CHEMICAL INVESTIGATIONS OF THE TOBACCO PLANT

IX. The Effect of Curing and of Fermentation on the Composition of the Leaves

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Part I

INTRODUCTION

The process whereby the fresh green leaf of the tobacco plant is converted into a form suitable for use in cigars consists of two main operations. The leaves, either picked singly from the plant ("primed") or harvested by cutting the stalk at ground level, are first allowed to cure by being hung in specially designed barns or sheds with maximal exposure to the air so that the greater part of the water can evaporate. A complex sequence of chemical reactions which affect the entire composition of the leaf accompanies the dehydration process, and it is with the details of these reactions as they are observed in Connecticut shade-grown tobacco used for cigar wrappers that the first part of the present bulletin is concerned. After the proper stage of dehydration and curing is attained, the leaves are packed tightly together in large rectangular piles called "bulks", which are roughly 6 x 12 x 5 feet and contain 3,000 to 4,000 pounds of leaf, and what is known as "fermentation" is allowed to take place. The second part of this bulletin deals with the chemical changes that then occur.

A widely different technique is used to prepare tobacco leaves of certain of the varieties used for the manufacture of cigarettes. These are usually cured in small square barns fitted with flues through which the hot gases from oil or wood fires are passed. The temperature at which the cure is carried out is carefully controlled and, at the point at which the leaves have become yellow, is rapidly raised so that the cells are killed. Nearly all of the remaining water in the leaves has been driven off when the proper stage is reached. Subsequently, the leaves are rehumidified and packed in hogsheads in which they are aged over a period of one to two years.

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There are many variants[1] of these two main methods for the curing of tobacco, and both the curing and the fermentation or aging steps involve broad experience and extensive practical knowledge if they are to be successfully conducted. Failure to control the conditions properly at any stage of the procedure is apt to diminish the value of the product and may result in its complete loss.

The variety of *Nicotiana tabacum* known as Connecticut shade-grown tobacco is produced on soils found mainly in the valley of the Connecticut River and is raised under shade-cloth tents. It is used almost exclusively for cigar wrappers. In the season of 1949, there were about 10,000 acres of land devoted to this crop, and its care and management represent a highly specialized form of agriculture.

The leaves are picked two or three at a time from each plant as they successively reach a stage of development recognizable by experts as technical ripeness. They are then taken to a curing barn and strung on cords attached at each end to wooden laths. About 40 leaves are suspended from each lath and the laths are arranged on racks of which there are usually seven tiers in the barn. A single barn provides room for about 20,000 laths or, roughly, 800,000 leaves.[2]

THE CURING PROCESS

The curing process consists essentially of the slow evaporation of the greater part of the water associated with the organic and inorganic solids of the living leaf. At the beginning, there are approximately 900 parts of water to 100 parts of solids; at the end, there are from 20 to 25 parts of water to 100 parts of solids and thus about 97 per cent of the initial water content of the leaves is ultimately removed. The proper management of this operation consists in establishing conditions in the curing barn such that evaporation proceeds at a favorable rate. During the early stages, it is customary to employ a moderate degree of artificial heat in the form of charcoal or gas fires on the floor of the barn; this is especially necessary in periods of humid weather. At the end of the process, which requires about two months, a day is selected when the humidity is high and the leaves are flexible and easily handled. Each lath is taken down, the leaves are bound into "hands", and the hands are packed in cases for transport to the warehouse where they are subsequently fermented in bulks, "mulled" in cases and graded. After being sorted for length, they are finally compressed into bales for marketing. The entire process usually requires more than six months in addition to the time required for curing.

Although the evaporation of water is by far the major change that takes place during the curing of tobacco leaves, at least in terms of the

[1] For example, Pennsylvania filler tobacco is stored for about a year after being cured ("storage fermentation"), and is then brought to a carefully determined moisture content and allowed to ferment for a number of successive periods in bulks or cases in a warm room. This last process is allowed to go much further than is the case with Connecticut shade-grown tobacco destined to be used as wrappers.

[2] A typical barn is 32 feet wide by 160 feet long and will accommodate the crop from about 5 acres of land. The fresh leaves will weigh about 36 tons and the cured leaves about 4,800 pounds.

quantity of material involved, it is only one of a complex sequence of events. Evaporation of water is essentially a physical process; accompanying it are other purely physical changes such as shrinkage and crumpling of the tissue, and the changes in color from the brilliant green of the fresh leaf to yellow and finally to a golden or light brown with occasional leaves showing greenish or reddish tones. The cured leaf reaches a stage at which it is remarkably responsive to variation in the humidity of the air to which it is exposed. At low humidity, water is given up and the tissue becomes crisp and easily broken so that it cannot be handled without damage. At a high humidity, water is quickly taken up and the leaf becomes soft and flexible, and is tough enough to be handled with ease.

These changes in physical properties are accompanied by a complex series of chemical changes that occur within the cells of the leaves. When the leaves are first hung up in the curing barn, they are still living organisms; respiration is evidenced by a substantial loss of organic solids which continues for a number of days, and the enzymes of the cells continue to play their part in the stimulation of innumerable reactions, although it is obvious that most of these reactions take place in the direction of decomposition of the normal components of the cell. The outstanding changes involve destruction of the pigments, losses of the carbohydrates, extensive digestion of the proteins to water-soluble nitrogenous substances and various transformations of these which result in the formation of amides and ammonia. Frankenburg (5) has recently reviewed the literature of tobacco curing and points out that the components of the green leaf and the changes they undergo during curing can be conveniently discussed under three broad headings. The *Static Group* includes components that change but little or not at all: included are the cellulose that forms the cell walls and which represents the main component of what is determined analytically as the so-called crude fiber; the pectins and pentosans, which are substances allied to the carbohydrates; a part of the ether-soluble components, which include a widely diversified group of substances such as hydrocarbons and waxes, higher alcohols, oils, resins, pigments, and phenols; and, finally, such other components as polyphenols, tannins, glucosides, and doubtless many other kinds of substances of as yet unrecognized chemical nature. Frankenburg includes oxalic acid in the static group because, although it belongs chemically with the other organic acids that can be extracted by ether from an acidified sample of tobacco leaf tissue, it exists in the leaf mainly as insoluble calcium oxalate and undergoes no detectable change during curing.

The *Nitrogen Group* of Frankenburg includes the proteins together with the amino acids, peptides, amides, and ammonia, the alkaloids, the nitrate which often makes up a substantial fraction of the soluble nitrogen in shade-grown tobacco, and a fairly small relative quantity of nitrogenous substances in which would be included such known components as adenine and choline together with doubtless a large number of substances of unknown nature. Most of the substances in the nitrogen group are extensively affected by the process of curing.

Finally, Frankenburg recognizes a *Dynamic Group* in which are included the most reactive of the non-nitrogenous substances found in the leaf. Perhaps the most important members of this group are the organic acids such as malic acid, citric acid, and succinic acid, the trans-

formations of which are today recognized as lying at the foundation of our understanding of respiration, and the carbohydrates such as starch, glucose, and sucrose. In addition, there is a substantial proportion of substances of unknown chemical nature which must be placed in the dynamic group because of the effect of curing upon them. According to Frankenburg's estimates based on a thorough survey of the literature and of data secured in his own laboratory, the static group makes up about 45 per cent of the solids of cigar-type green tobacco leaves, the nitrogen group about 24 per cent, and the dynamic group 31 per cent. The process of curing thus brings about chemical changes in more than one-half of the organic substances in the tissues.

Unfortunately, few of the kinds of chemical changes that take place in tobacco leaves during the process of curing can be detected and described in definite chemical terms. Analytical methods that are both specific and sensitive have been developed for only a few of the more important components of the leaf, such as nicotine, and for protein, asparagine amide, and ammonia nitrogen, for oxalic, citric, malic, and succinic acids, and for starch and the ash components. Reliance is placed, for the most part, on what may be called "group" methods or indirect methods. Such, for example, are the methods for total nitrogen, for amino and amide nitrogen, and for the simple carbohydrates that are reported as glucose and sucrose, but which in fact are merely determinations of reducing power under circumstances that make it probable that these specific sugars are being determined. Even less satisfactory, with respect to the possibility of precise chemical interpretation, are such determinations as those of total solids, ash, soluble solids, and soluble nitrogen, ether-soluble solids, and so forth. Although many important inferences can be drawn from the changes in such factors, exact statements that can be expressed in the form of chemical equations are for the most part impossible.

Nevertheless, in spite of the uncertainties involved, it has seemed desirable to study the chemical changes that occur during the processing of tobacco in as much detail as possible in the hope that light may be thrown on the nature of the events that take place. This is the more necessary since the problem was first investigated in this laboratory some 20 years ago (41, 42, 43) with interesting results, but the inadequacy of the methods available at that time was such that further work was suspended. Today, with better experimental methods at hand, a reconsideration of the matter is appropriate.

OUTLINE OF EXPERIMENT

It was desired to learn as much as possible about the chemical changes that occur when leaves of Connecticut shade-grown tobacco are cured in a barn under customary commercial conditions and are then fermented in a "bulk" along with a large quantity of similar tobacco, stored under the usual conditions and finally graded and prepared for market. To obtain this information effectively, three separate sets of samples collected from a uniform stand of tobacco plants in the field are necessary, one for the immediate study of curing, one to be cured along with the first and then used for the study of fermentation and

storage, and a third to be cured and fermented together with the first two and then graded and prepared for market and used for the study of the composition of the several grades.

The general technique employed for such experiments consists of the collection of a group of samples of green leaves in such a way that each sample resembles every other sample in the group as closely as possible in initial chemical composition. One of the samples is at once dried and prepared for analysis to furnish a base line or starting point and the others are strung on laths in the usual way for curing. Single samples are then removed from time to time, dried, and prepared for analysis. When analytical data for the entire set of samples have been obtained, it becomes possible to plot the composition of the successive samples on a scale of time in such a way as to show the behavior of each of the components for which analytical methods are available. Interpretation in chemical terms of some of the changes then becomes possible.

The analytical data for each separate sample are computed in terms of one kilo of the fresh weight of that sample at the time of collection. Only when expressed on such a common basis are the data for the individual samples strictly comparable, for it was only at the time of collection that the samples were identical in composition; and the only experimentally determined datum that can be conveniently obtained at the time of collection is the fresh weight. Other methods of expression are possible; for example, the data may be computed in terms of a unit area of the leaves. The disadvantage of this is that the determination of the area of leaves is a troublesome and inaccurate procedure and, moreover, has no special advantage over the determination of the weight. Expression of the data in terms of a percentage of the dry weight at each stage of the curing or fermentation is not a sound procedure unless corrections are applied to allow for the change in the quantity of solids that takes place during the process. Such corrections are difficult to estimate (with accuracy) and apply, and lead to no clear advantage over the method adopted in the understanding or presentation of the data. Expression of the data in terms of a definite number of leaves or plants is inappropriate in the present case.

Sampling Plan

The problem of collecting a group of samples of plant leaves in such a way that each sample is identical in composition at the start is manifestly a difficult one and considerable attention has been given in this laboratory to the development of methods whereby the ideal of precise identity in composition can be approached as closely as possible. An analysis of the problem carried out by Dr. C. I. Bliss (40) suggested that errors in the results of chemical analyses of separate samples that are presumed to be identical in composition arise from two main sources. One of these is the normal variation in the results of the analytical procedures in the laboratory. This can be estimated from the statistical analysis of analytical determinations carried out in duplicate. Attention must be paid to the technique of the analysis so that there shall be no subconscious bias in the mind of the chemist, when making a titration

for example, from the results of the titration of the previous duplicate sample. Thus, duplicate analyses should be obtained by making *single* determinations on each of a set of samples and then repeating the entire set of determinations, preferably taking the samples in a random order.

The second source of error arises from actual variation in the plant material itself. This may be studied by comparison of groups of samples collected by different sampling techniques. Analysis of the data, after segregation of the analytical error, should then lead to a judgment on the relative desirability of one or other of these sampling techniques.

In dealing with collections of leaves, it seems reasonable to assume that variability between any two samples has a component contributed by the individual plant and one contributed by the position of the individual leaf on the plant, since the age of the leaf is a function of position. In addition, there should be a random component dependent upon factors other than the variation of the individual plant or that due to leaf position. Accordingly, to collect samples of leaves that resemble each other as closely as possible in initial composition, steps should be taken so that each plant and each leaf position on the plant shall be equally represented in each sample. Samples secured in this way will not be exactly identical in composition because of the presence of the random component of variability, but they should approach identity more closely than samples collected on any other assumption of the sources of variation. The problem can be reduced, therefore, to the consideration of methods whereby leaves can be collected so that each plant and each leaf position are equally represented in each sample.

A simple example of how this can be done may help in following the more complex procedure employed in the present investigation. Suppose that the problem is to collect five samples of five leaves each from five plants. The five leaves to be taken are numbered in order up the stalk and the five plants are also numbered. Then leaf 1 from plant 1 is placed in sample A, leaf 2 in sample B, leaf 3 in sample C, and so on until all five leaves from the first plant have been collected. Then leaf 1 from plant 2 is placed in sample B, leaf 2 in sample C, leaf 3 in sample D, and so on, leaf 5 being placed in sample A. Leaf 1 from plant 3 goes into sample C, and so forth in sequence; leaf 1 from plant 4 into sample D, and so on, and, finally, leaf 1 from plant 5 goes into sample E, leaf 2 into sample A and so forth. At the end of the process, there are five leaves in each sample, one of which has come from each plant and one of these leaves was taken from each leaf position. If it is necessary to have larger samples, 10 or 15 or some other multiple of five plants can be used, or, for other types of experiments, 10 or 15 leaves would be taken from each plant according to a similar sequential process.

It is clear that the necessity of preserving an exact balance among several factors restricts the relative numbers of plants, leaf positions, and samples that can be used, for these must be numbers that are in general integral multiples of some selected small number. This is no great disadvantage, however, and the procedure is easy to design and apply in any specific case.

From the mathematical point of view, the plan of collection is based upon what may be called a systematized Latin square. It is systematized in order to facilitate the actual picking of the leaves. An ordinary randomized Latin square may also be used but is more difficult to apply

in practice. A diagram of the scheme is as follows, the letters within the rectangle, which is the actual Latin square, representing samples into which each of the designated leaves is placed.

	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5
Leaf 1	A	B	C	D	E
Leaf 2	B	C	D	E	A
Leaf 3	C	D	E	A	B
Leaf 4	D	E	A	B	C
Leaf 5	E	A	B	C	D

In the present case, the number of leaves that can be conveniently strung on a lath, namely 40, was the starting point in designing the method of sampling. This was also a convenient number of leaves to make up a single sample as it would yield approximately 125 gm. of dry tissue which was estimated to be sufficient for the analytical studies. It was desired to have the samples represent a typical "priming" of the crop and the contribution of each plant should probably have been three leaves. However, this number would not fit into the scheme of using 40-leaf samples and, accordingly, four leaves were taken from each plant. At least eight samples would be needed to give a fair distribution over the entire process of curing so that frequent samples could be taken in the early stages when the chemical changes were going on rapidly. A consideration of the possible numbers that could be selected for the construction of the necessary Latin square finally showed that 10 samples would be best, these to be taken from 100 plants arranged in a rectangular block of 10 rows, each row being 10 plants long.

The plan of collection shown in Table 1 was therefore drawn up. The symbols used for the designation of the samples, plants, leaf positions, and the order of the collection boxes in the rows are shown at the top of the table. The system as a whole is based upon a 10 by 10 Latin square[1] which is shown in the lower part of the table. This gives a completely randomized arrangement of the collection boxes used to hold the samples of leaves.

In preparation for the collection, sets of labels for the plants are prepared. These are conveniently made from shipping tags which can be attached to the plants with wire pipe-cleaners. For each block of 10 rows with 10 plants per row, labels are made with the numbers 1, 11, 21, ... 91, and also labels with the numbers 10, 20, 30, ... 100. These are used for the plants at the beginning and end of each of the 10 rows. In addition, a number of red labels are provided. At the time of collection, the carefully selected block of plants is defined by attaching label 1 to the plant at a corner of the block which is located well away from the wall of the tent. An adjacent plant in the next row is labeled 11, one in

[1] The characteristic property of a Latin square is that each of the letter symbols occurs once in each column and once in each row. With this restriction, the exact order in which the letters occur, except in row 1, is controlled entirely by chance. In the 5 by 5 systemized Latin square mentioned above, the order in which the the symbols occur in each row or column is the natural alphabetical order. That is to say, the order of the symbols is not randomized.

the third row 21, and so on across the rows of plants until the first plants in each of the 10 rows have been defined. Then the individual plants in each row are inspected, inferior plants or plants with damaged leaves being marked with red labels attached near the ground level so as to be readily noted when the actual collection begins. Finally, 10 perfect and uniform plants are counted off in each row and the end plant in each row of the block is labeled with the numbers 10, 20, and so forth across the rows. It is desirable that the marking of the plants should be at once checked by another worker to eliminate possibility of error.

TABLE 1. SAMPLING PLAN FOR COLLECTION OF LEAVES FROM SHADE TOBACCO

Plants	100	Samples indicated by capital letters
Rows	10	Plants indicated by numbers
Plants per row	10	Rows indicated by numbers
Samples	10	Leaf positions indicated by small letters
Leaves per sample	40	Order of boxes for each row of plants
Leaves per plant	4	indicated by Roman numbers
Plants per sample	40	

Samples	A	B	C	D	E	F	G	H	I	J
Order of boxes	I	II	III	IV	V	VI	VII	VIII	IX	X
Pick in order counting up	1a	1b	1c	1d	2a	2b	2c	2d	3a	3b
	3c	3d	4a	4b	4c	4d	5a	5b	5c	5d
Pick in order counting down	6d	6c	6b	6a	7d	7c	7b	7a	8d	8c
	8b	8a	9d	9c	9b	9a	10d	10c	10b	10a

Rows	Plants	Samples									
1	1-10	A	B	C	D	E	F	G	H	I	J
2	11-20	D	A	G	I	J	E	C	B	F	H
3	21-30	B	G	A	E	H	C	F	I	J	D
4	31-40	J	D	E	H	B	A	I	C	G	F
5	41-50	G	I	F	B	A	D	H	J	C	E
6	51-60	F	E	B	C	D	I	J	G	H	A
7	61-70	C	H	J	G	F	B	E	A	D	I
8	71-80	I	J	D	A	C	H	B	F	E	G
9	81-90	H	C	I	F	G	J	D	E	A	B
10	91-100	E	F	H	J	I	G	A	D	B	C

The provision of labels for the 10 collection boxes is somewhat more complex and the system employed was suggested by Dr. Bliss. It is designed to avoid the possibility of error in the field at the actual time of collection. The 10 collection boxes are conspicuously marked in order with the letters A to J; then for each box, a pack of 10 tags is provided, clipped together and held on a wire ring. The labels in the pack attached to box A are marked with the Roman numbers I, II, III, VI, V, X, VIII, IV, IX, and VII as shown in the vertical column under A in Table 2. The labels for box B have the numbers II, VIII, I, and so

forth as shown in Table 2 in the vertical column under B. Packs of labels marked in order as in the vertical columns in Table 2 are provided for each of the other boxes.

TABLE 2. POSITION OF SAMPLE BOXES DURING COLLECTION OF LEAVES FROM EACH ROW

Collection boxes to be placed in order of Roman numbers I to X in each successive row, the labels showing the set of Roman numbers for each row being exposed before the boxes are rearranged. The sample boxes A to J will then be in the order shown in each row as in Table 1. The pack of labels attached to each box is so made as to show Roman numbers in the order of the vertical columns.

Rows	Plants	A	B	C	D	E	F	G	H	I	J
1	1-10	I	II	III	IV	V	VI	VII	VIII	IX	X
2	11-20	II	VIII	VII	I	VI	IX	III	X	IV	V
3	21-30	III	I	VI	X	IV	VII	II	V	VIII	IX
4	31-40	VI	V	VIII	II	III	X	IX	IV	VII	I
5	41-50	V	IV	IX	VI	X	III	I	VII	II	VIII
6	51-60	X	III	IV	V	II	I	VIII	IX	VI	VII
7	61-70	VIII	VI	I	IX	VII	V	IV	II	X	III
8	71-80	IV	VII	V	III	IX	VIII	X	VI	I	II
9	81-90	IX	X	II	VII	VIII	IV	V	I	III	VI
10	91-100	VII	IX	X	VIII	I	II	VI	III	V	IV

At the time of collection, the boxes are placed, in the order of the Roman numbers on the top label in the pack, alongside the plants in the first row. This also places them in the alphabetical order of the letters A to J for this row. After the leaves from the 10 plants in row 1 have been picked, the second label in each pack of labels is exposed by turning the first label on the wire ring to the bottom of the pack. The boxes are then rearranged in the order of the Roman numbers from I to X that are thus exposed. The boxes as shown by the sample designations will then be in the order of the letters in the second row of Table 1, namely in the order, D, A, G, I, J, E, C, B, F, H. After the leaves from the second row of plants have been collected, the third label in each pack is exposed, the boxes are again rearranged and the leaves from the third row are collected. The process is repeated until the leaves from all 10 rows have been collected.

Errors can be avoided in the field if the labels in each pack show, in addition to the Roman number designation, the Arabic number that indicates the row at which the box should be placed when that number is exposed. The assistants, at the time of collection, can then quickly check all exposed labels to make sure that all box labels show the same row number.

The actual collection of the leaves requires the help of several assistants. The sampling for the present experiment was made after the first priming had been carried out so that the ground leaves had already been removed. The four leaves at the bottom of the stalk were designated a, b, c, and d as in Table 1. At the start of the operation, after the boxes were in position, one operator, using Table 1 as his guide, calls out the number "one" when the lowest leaf on plant 1 (leaf a) is picked and

this leaf is passed to box I. He calls out the number "two" when the second leaf (leaf b) is picked and this leaf is passed to box II, "three" when the third leaf (leaf c) is picked and "four" when the fourth leaf is picked, these being passed to the proper box. The assistant who is picking then moves to plant 2 and when he picks the lowest leaf (leaf a), the operator calls out "five" and the leaf is passed to box V, and so on. The second leaf (leaf b) from plant 3 goes to box X, the third leaf (leaf c) to box I, and the collection then proceeds in order for the first five plants. At this point, so as to improve the randomization of the collection, the leaves are picked from the last five plants in the row in order counting downwards from the fourth leaf remaining on the plant. The operator calls out "count down", and the assistant picks the fourth leaf from the bottom (leaf d). When he does this, the operator calls out "one" and the leaf is passed to box I, for the third leaf from the bottom (leaf c), the operator calls out "two" and this leaf is passed. The process then proceeds along the row of plants to plant 10.

At this point, the top labels in each pack are turned to the bottom of the pack and the boxes are rearranged in the order of the Roman numbers exposed. The process then continues along the second row exactly as in the first row. It is the responsibility of the person calling the numbers to see each leaf as it is picked and to call the box number clearly. One of the assistants passing the leaves makes it his responsibility to be sure that each leaf is placed in the box that has been called. As a rule, four or five other assistants are employed to pass the leaves to the sample boxes. This avoids the necessity for much moving about among the plants with consequent danger of damage to the leaves that are to be used.

As soon as the collection has been completed, the boxes are taken to the edge of the shade tent, the number of leaves in each is counted to make sure that no blunder has been committed, and the fresh weight of each sample is obtained on a balance furnished with accurate weights. The accuracy of this weight is most important, for it is used in the computation of all subsequent analytical results on that sample. With samples of 40 leaves, accuracy to four significant figures is easily attained.

Although this sampling process is undeniably complex, it has been found that, even with inexperienced assistants, the routine can be carried through quickly and without error. After a little experience has been gained, a group of workers can complete the collection from a single block of plants in about 45 minutes.

In order to illustrate the distribution, over the block of plants, of the leaves that are placed in any one sample, Figure 1 has been prepared. The symbols represent tobacco plants, the stalk being shown as a sloping line terminated with an arrow head and the four leaves used for the collection being represented by circles. There are 10 rows of 10 plants each. Capital letters to represent the sample into which each leaf is placed are entered in each of the circles. The letters are taken from the Latin square in Table 1 and, for the plants in the first row, follow the order of the first 10 letters of the alphabet, these 10 letters being repeated four times since 40 leaves are taken from the plants in the first row. In the second row of plants, the letters are in the order of the letters in the second row of the Latin square, the sequence again being repeated four times. Similarly, the sequence of the letters for each row of plants is taken from the appropriate row of the Latin square.

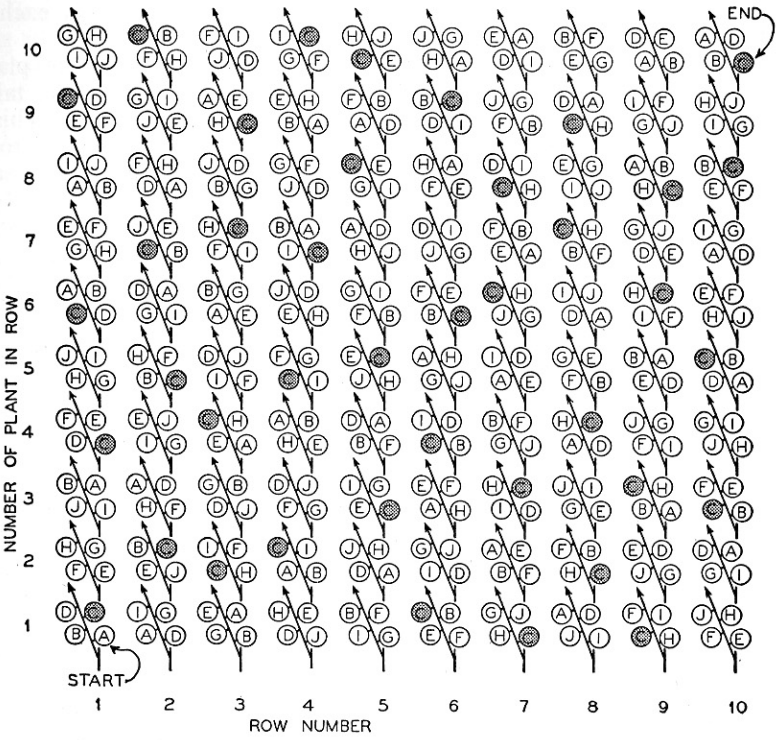

FIGURE 1

Diagram of a plot of tobacco plants 10 rows wide and 10 plants long as used for the collection of samples by the statistical method. Each symbol represents a single plant and the four leaves taken for the samples are represented by circles. The sample into which each leaf was placed is denoted by the letter entered in each circle. The order in which the sample collection boxes are arranged is taken for each row of plants from the appropriate row of letters in the Latin square shown in Table 1. All of the leaves that were placed in sample C are shaded in order to show the distribution over the entire plot of the leaves that found their way into this one sample. Examination of the diagram will show that an analogous randomized distribution of the leaves placed in each of the other nine samples was achieved.

All of the circles representing leaves that found their way into sample C have been shaded. It is clear that, although each row of plants contributed four leaves to sample C, the position of the shaded circles is scattered over the whole diagram. The scatter is random save for the restriction that no two leaves came from adjacent plants nor were there ever more than two plants intervening between two plants that contributed to sample C. Furthermore, within each row, one leaf was contributed from each leaf position. Examination of the diagram shows that similar statements are true for each of the samples. Thus, the 40 leaves in a single sample came from 40 different plants, these plants being distributed over

the entire block and there were 10 leaves in each sample from each of the four leaf positions, this distribution being also scattered.

Another feature of the diagram is of interest. If all of the plants numbered 1 within the rows are considered, only four leaves were taken for sample C, one coming from each of the four leaf positions. This is also true for the plants which bear the same number in all of the rows. It happens that plants numbered 2 and 7 in rows 2, 3, and 4 each contributed a leaf to sample C and a few cases have been noted (e.g. sample B from plant 4 in rows 4, 5, 6, and 7) where four plants opposite each other in adjacent rows contributed leaves to the same sample. Nevertheless, the randomization seems in general to be adequate, for, in the actual case in the field, the plants which bore the same numbers in adjoining rows were rarely exactly opposite to each other owing to irregularities in the spacing within the rows or to the presence of occasional inferior plants arbitrarily omitted from the collection.

Precision of Sampling

Data for the fresh weight of five collections of leaves made by this sampling method are shown in Table 3. The spread in the coefficients of variation from 1.3 per cent to 2.7 per cent is somewhat disappointing but it seems clear that, in general, samples can be obtained by this method, the variation of which is less than 2 per cent. The exaggerated variation of the second set of samples in the table arises almost entirely from two samples which were respectively unusually high and unusually low in fresh weight. If these are eliminated, the coefficient of variation of the remaining eight samples drops to 1.9 per cent with only a negligible change in the mean fresh weight of the set. If analytical results on such a set of samples showed marked irregularities referable to the two samples of discrepant fresh weight, it would probably be justifiable to assign less significance to them in the interpretation of the data as a whole.

TABLE 3. MEAN FRESH WEIGHT WITH STANDARD DEVIATION AND COEFFICIENT OF VARIATION OF SETS OF SAMPLES OF TOBACCO LEAVES COLLECTED BY THE STATISTICAL METHOD

Samples	Mean fresh weight gm.	Standard deviation gm.	Coefficient of variation per cent
1949 (A1 — J1)	1371.9	± 19.7	1.44
1949 (A2 — J2)	1349.6	± 36.5	2.71[1]
1949 (FA — FJ)	1287.0	± 16.9	1.32
1950 (A — J)	1147.6	± 18.0	1.57
1950 (FA — FJ)	1156.8	± 28.0	2.42

[1] The large coefficient of variation in this set of samples arises mostly from two discrepant samples, one of which was high, the other low. Omitting these two, the mean of the remaining eight samples was 1347.6 ± 25.5, the coefficient of variation being only 1.9 per cent.

As an illustration of the improvement in precision of sampling introduced by the use of the statistical method, results obtained from samples of 60 leaves collected in 1935 may be mentioned. The individual samples

for these sets were counted out from a large quantity of leaves, reliance being placed upon random selection of the individual leaves from a large mass to obtain uniformity. As computed from the data for seven samples in each set, the coefficients of variation of the fresh weights were, respectively, 4.6, 4.9, and 4.7 per cent. That this variability extended to the composition of the samples is evident from the fact that the coefficients of variation of the total nitrogen were also greater than 4 per cent and in one of the sets was 7.5 per cent, largely because of one sample of widely discrepant nitrogen content.

For the present experiment, three sets of samples were taken by the statistical method on the same morning from adjacent blocks of 100 plants in the same field. Two of these sets were taken so that the data of the curing experiment could be secured in duplicate if the variability of the samples appeared to be excessive. The third sample was to be used for the fermentation experiment.

The data for the mean fresh weight of these sets are shown in the first three lines of Table 3. The differences in the mean fresh weight furnish a striking illustration of the variability to be found in tobacco plants in the field even when the samples are taken from a stand of plants selected for the present purpose because of their apparent uniformity of development. Even greater attention was paid in 1950 to the selection of a uniform stand for the sampling and this time with a fair measure of success. Nevertheless, the leaves collected in 1950 were considerably lighter in fresh weight than were any of the samples taken in 1949.

In addition to the three sets of samples collected for the present experiment by the statistical method, a single sample of 400 leaves was taken from the same leaf positions from an adjacent block of 100 plants. This sample, which was designated FER, was subsequently cured and fermented in the same manner as the other samples and was ultimately employed for the study of the grades (see Part III).

Management of Samples

The samples were collected July 22, 1949 from a field at Windsor, Conn., the property of the Imperial Agricultural Corporation.[1] The plants were of the kind known as "Regular Cuban Strain" of the variety Connecticut shade-grown, and were well developed and uniform. The ground leaves had been primed a few days before the collection so that there was ample room to work among the plants. The weather was cloudy but bright, humid, and hot. Picking was begun at 10:20 A.M. and all samples had been obtained and weighed by 1:45 P.M. The leaves were at once taken to Shed 1 of Farm 4 of the Imperial Agricultural Corporation. Samples A1 and A2 were dispatched to the New Haven laboratory where they were dried for analysis, being placed in the oven at 3:45 P.M. The remaining samples were strung on laths and the laths were placed

[1] It is a pleasure to acknowledge our indebtedness to the Imperial Agricultural Corporation of Hartford for their courteous assistance in carrying out the present experiment, in particular to Mr. E. Temple, Dr. P. J. Wijga, and Mr. Allen H. Green. Throughout the entire process of collecting the samples, curing and fermenting them, and grading and storing the final product, we enjoyed the closest cooperation from these gentlemen. No small share of the success of the experiments was due to their generous help.

in the second tier of leaves in the barn and were marked with labels so as to be easily located later, this operation being completed by 3:00 P.M.

On the days shown in Table 4, successive samples from the A1 — J1 and the A2 — J2 series were taken from the barn to the laboratory and dried for analysis. Descriptions of the appearance of the samples are collected in Table 5. These descriptions are approximate only, but they suffice to show that the individual leaves pass through the early stages of the curing process at somewhat different rates, notwithstanding the fact that these samples were collected in such a way as to provide for the utmost uniformity in initial composition. To anticipate some of the results of the chemical analysis of these samples, it may be pointed out that respiration ceased to be significant after eight days of curing. It is at this point that most of the leaves within the samples had become more

TABLE 4. INITIAL AND FINAL EQUILIBRATED DRY WEIGHTS OF SAMPLES OF 40 TOBACCO LEAVES SUBJECTED TO CURING

The dried samples were equilibrated with air at 50 per cent relative humidity and 75° F. (24° C.). Figures in italics refer to the duplicate A2 — J2 series of samples.

Sample	Initial fresh weight	Time in barn	Weight on removal from barn	Equilibrated dry weight	Factor A[1]
	gm.	days	gm.	gm.	
A1	1387	0	1387	129.30	0.9322
A2	*1317*		*1317*	*119.15*	*0.9047*
B1	1376	2	1056	124.95	0.9081
B2	*1297*		*988.5*	*114.15*	*0.8801*
C1	1354	4	741.9	115.25	0.8512
C2	*1377*		*780.0*	*117.40*	*0.8526*
D1	1358	6	509.4	113.10	0.8328
D2	*1341*		*544.3*	*110.05*	*0.8207*
E1	1337	8	331.9	109.90	0.8220
E2	*1389*		*323.0*	*112.45*	*0.8096*
F1	1376	12	182.2	112.85	0.8201
F2	*1358*		*145.6*	*108.45*	*0.7986*
G1	1366	16	126.1	112.35	0.8225
G2	*1336*		*118.9*	*106.30*	*0.7957*
H1	1385	20	122.0	112.75	0.8141
H2	*1418*		*123.3*	*115.25*	*0.8128*
I1	1408	32	119.7	116.45	0.8271
I2	*1342*		*110.8*	*108.15*	*0.8059*
J1	1372	63	129.5	112.90	0.8229
J2	*1321*		*120.3*	*104.80*	*0.7933*

[1] Factor A is the factor by which the result of an analytical determination, expressed as a percentage of the equilibrated dry weight, is multiplied in order to give the quantity of the component in terms of grams per kilo of initial fresh weight of the leaves (see p. 21).

TABLE 5. GENERAL APPEARANCE OF 40 LEAF SAMPLES AT EACH STAGE OF THE CURING PROCESS

Figures are numbers of leaves to which the description applies approximately. Figures in italics represent the A2 - J2 series.

Sample	B	C	D	E	F	G	H	I	J
Days cured	2	4	6	8	12	16	20	32	63
Green: wilted: faintly mottled	35								
	35								
Green: wilted: mottled: few yellow spots	5	9							
	5	*13*							
Green: patches of yellow		27	8						
		12	*5*						
More yellow than green: trace of brown		4							
		15							
Yellow and green: brown spots				8					
				7					
Yellow and green: brown patches 10% of area: main vein turgid			10						
			15						
No green: yellow with 20 to 30% of area brown: vein mostly turgid			10	8	9				
			11	*13*	*5*				
Yellow with 50 to 60% of area brown: main vein collapsed			12	11	7	1			
			9	*9*	*3*	*1*			
Brown 90%: yellow spots: mid vein dry				13	12		7	7	
				11	*11*		*8*	*8*	
Brown: faint greenish tinge					6	17	10	10	
					11	*15*	*11*	*11*	
Strong yellowish brown					6	22	23	23	
					10	*24*	*21*	*21*	
Uniform brown both sides									35
									35
Greenish brown									5
									5

or less brown in color, and the onset of this change in color may therefore be taken as an index of the death of the cells. The leaves had lost 75 per cent of their initial weight at this point, mainly through the evaporation of water, and thus the concentration of the solutes in the cell sap must have been increased by a factor of between 3 and 4. This situation was manifestly incompatible with continuance of the life of the cells.

It cannot be assumed that the rate of evaporation of the water from each of the leaves in an individual sample strung on cord and hung in the curing shed was exactly the same in all cases. Some leaves may have been so crowded against others, especially for the first day or two, that an even flow of water vapor out of the tissue was impossible. Accordingly, the obvious wide spread in the rate of curing through the yellow phase may in part arise from differences in the physical situation in which

certain of the leaves or certain areas of some of the leaves were placed. Clearly, it would seem to be desirable to study the exact rates at which presumably identical leaves pass through this phase when the individual leaves are handled in such a manner that uniform rates of evaporation of the water may be assumed to have occurred. The information obtained from such experiments would help materially in the interpretation of the present observations. It is clear that the results of chemical analysis of the present samples will reveal only the average rates at which many of the changes occurred.

ANALYTICAL METHODS

Preparation of Samples

Inasmuch as the weights of the individual samples of fresh leaves and the equilibrated dry weight of the material obtained from each of them are quantities that are used in the computation of the analytical results for each sample, conditions are established that permit the measurement of these weights to four significant figures. The individual samples of fresh leaves are weighed at the field using a beam balance of a capacity of about 3,000 gm. and sensitive to 0.2 gm. The balance is set up and leveled in a position completely sheltered from currents of air, and accurate brass weights are used. The weighing is done immediately after the collection of each set of samples is completed. Since samples of 40 leaves weigh from 1,200 to 1,400 gm., adequate accuracy is obtained.

After the samples have been subjected to the experimental treatment, in the present case curing and subsequent fermentation, the treated samples successively removed from the curing barn or from the warehouse are taken to the Experiment Station laboratory in New Haven and weighed on a torsion balance sensitive to about 0.1 gm. The sample is then dried until the midribs are crisp in a Proctor and Schwartz tray drier provided with forced circulation of air and maintained at 80° C. by thermostatic control. The dry leaves are broken up and transferred to an accurately weighed new one-gallon paint can and a rough weight is obtained. The can is then tightly closed and is stored until the entire set of samples has been collected and dried. The set of cans is then opened in an air-conditioned room at 24° C. and 50 per cent relative humidity and rough weighings (to 0.1 gm.) are made from time to time until no further change in weight is noted. At this time, a final weighing is obtained on a beam balance of 3,000 gm. capacity sensitive to 13 mg., on which the weights of the empty cans had previously been obtained. Since the dry samples weigh somewhat more than 100 gm., accuracy to four significant figures for the so-called equilibrated dry weight is obtained.

After equilibration, the samples including the midribs[1] are ground in the air-conditioned room in a small Wiley mill and are stored in screw-

[1] The decision to include the midrib in the samples was made in order to avoid subsequent complexities in interpretation of the results. Each sample represents an isolated biochemical system that can be compared with each other sample. Many of the advantages of the method adopted for the presentation of the data would be lost if the lamina and midribs had been separated before preparing the samples for analysis.

capped glass bottles which are protected from dust by being enclosed in one-quart ice-cream cartons. The samples are removed from the room only when taken to the analytical balance for the purpose of weighing out subsamples for analysis. They are returned as soon as possible. By careful attention to these precautions, it is possible to maintain the moisture content of the equilibrated samples unchanged indefinitely.

There is one exception to this routine. The samples which represent the grades (derived from the 400 leaf FER sample) were separated into lamina and midrib fractions before being dried and equilibrated. Accordingly, the treatment of the analytical data from these samples is different. Details of this treatment are given in a later section.

All analytical results on the cured or cured and fermented samples are calculated as percentages of the equilibrated dry weight or, in the case of the organic acids, in terms of milliequivalents (m.eq.) per 100 gm. In order to convert such data to the common basis of 1 kilo of initial fresh weight of the leaves of the individual sample, it is necessary to know what the equilibrated dry weight would have been if the initial fresh weight had been exactly 1,000 gm. This is computed from the proportion:

$$\text{Initial fresh weight} : \text{equilibrated dry weight} = 1{,}000 : X$$

$$\text{Whence } X = \frac{\text{Equilibrated dry weight} \times 1{,}000}{\text{Initial fresh weight}}$$

An analytical result expressed in percentage (say B per cent) or as milliequivalents per 100 gm. of equilibrated dry weight is then computed from the proportion:

$$B : 100 = Y : X \text{ whence } Y = \frac{B \times X}{100}$$

where Y is the quantity of the component in gm. (or m.eq.) in the dry material derived from 1 kilo of fresh leaves.

Substituting the value for X

$$Y = B\frac{\text{Equilibrated dry weight}}{\text{Initial fresh weight}} \cdot \frac{1{,}000}{100}$$

Thus, the value for any analytical result in terms of grams per kilo of initial fresh weight of the leaves that form the sample is calculated by multiplying the percentage of the component by 10 times the ratio of the equilibrated dry weight of that sample to its initial fresh weight. This quantity is designated "Factor A" and is computed once for all for each sample and tabulated as soon as the equilibrated dry weights of the sets of samples have been obtained.

Moisture[1]

Experience has shown that rigid adherence to the following practices is essential if reproducible and trustworthy results are to be obtained.

Samples of approximately 500 mg. are weighed to 0.1 gm. and dried for exactly 4 hours at 110° C. The vessel used is a low form porcelain crucible (45 mm. diameter x 25 mm. deep). All crucibles are

[1] Most of the details of the following methods were developed by the late Dr. G. W. Pucher and the late Mr. C. S. Leavenworth of this laboratory. The present description includes changes that have been made since an earlier description (46).

throroughly cleaned by being boiled for a few minutes with 0.25 *N* hydrochloric acid and are then rinsed and dried in the oven before being weighed, the weight of a lid being included in the tare. The same lid is used with the whole series of crucibles, and is kept in the desiccator in which the dried samples are cooled until transferred to the balance pan. With determinations carried out in humid summer weather, the crucible containing the sample is enclosed in a large weighing bottle to prevent moisture absorption. These weighing bottles are 48 mm. inside diameter x 28 mm. deep, with a 55/12 standard taper, hood-style cap. The heaviest of a group of the bottles is used as a counter-balance in weighing and is kept in the desiccator. The 4-hour drying period is timed carefully. About 1 hour is allowed for cooling the samples in the desiccator before they are weighed, but the weighing is done as soon as possible thereafter; overnight storage in the desiccator before weighing is avoided. Determinations are usually run in sets of 12, including two pairs of duplicates chosen at random out of a set of 10 samples.

Ash

After the determination of moisture, the sample is charred by heating the crucible over a low flame until all volatile material has been driven off. The tissue is then heated in the muffle for exactly 2 hours at 580 ± 20° C. If any visible carbon remains, the sample is cooled and weighed, but is then heated for 1 additional hour, and reweighed. The ashed samples are allowed to cool in a desiccator for at least an hour, but should not be allowed to stand overnight before being weighed. Samples of tobacco leaf midrib tissue often ignite explosively even when heated over a very low flame and must therefore be charred by careful application of the flame of a micro burner to the top surface of the sample. For precise work, it is desirable to allow about 2 hours for the muffle to become stabilized at 580° before the crucibles are placed in it. Only six crucibles are heated at one time, and are placed well back in the chamber. One pair of duplicates is usually run in each set of six samples.

Alkalinity of Ash

A suitable quantity, e.g. 10 ml., of standardized 0.25 *N* HCl is added to the crucible containing the ash, which is then warmed for 10 minutes on the steam bath, being covered with a watch glass if necessary. A small glass rod is provided for each crucible and the ash is stirred two or three times during the digestion. The contents of the crucible are then washed into a 150 ml. beaker, bringing the total volume to about 25 ml. The solution is boiled 2 to 3 minutes to expel CO_2 and titrated, while still hot, to the phenolphthalein end-point with standardized 0.10 *N* NaOH. If Tashiro indicator is used, the solution must be cooled to room temperature before titration. Calculation:

$$\text{m. eq. per 100 gm. Eq.D.W.} = \frac{(A-B)\ 100}{\text{Sample wt. in gm.}}$$

where A = 10.00 × normality factor HCl

B = ml. NaOH × normality factor NaOH

Nitrogen

Total Nitrogen

Reagents: Concentrated H_2SO_4 (A.R. grade)
Iron powder (by hydrogen, low nitrogen)
Sodium sulfate solution, approximately 20 per cent by weight of the anhydrous salt
Sodium selenate solution, 2.5 per cent of the anhydrous salt
Concentrated NaOH solution (18 to 19 *N*)
Standardized 0.02 *N* HCl
Standardized 0.02 *N* NaOH
Tashiro indicator solution

The apparatus employed is modified from that described by Folin and Wright (4). The distillation tube has a spherical bulb trap (Kjeldahl connecting tube) at the top. The receiving flask is set into a pan of water through which cold water is circulated by suitable inlet and overflow tubes during the distillation.

A sample of about 100 mg. of nitrate-containing tissue powder, weighed to 0.2 mg., is placed in a 300 ml. Kjeldahl flask: 12 ml. of distilled water, 1.5 ml. of concentrated H_2SO_4, and a measured volume of iron powder (approximating 300 mg.) are added and mixed thoroughly with the tissue powder and the mixture is boiled gently for 5 to 10 minutes. Then 5 ml. of 20 per cent sodium sulfate, 5 ml. of concentrated H_2SO_4, and 2 ml. of the sodium selenate solution are added and the sample is digested on the Kjeldahl rack, continuing the heating for 2 hours after the digest is colorless. The flask is turned occasionally during the digestion. A small glass bead has been found to be effective in controlling bumping. After cooling the flask and contents, 50 ml. of water are added and the flask is cooled and clamped at an angle of about 45° on a ring stand; 15 ml. of concentrated NaOH solution are added so as to form a layer under the diluted digest, and the neck of the flask is carefully washed down with water, the upper portion being then wiped dry with a piece of filter paper. The flask is turned to the vertical position and, after the addition of a small quantity (roughly 50 to 100 mg.) of granulated zinc metal (to control bumping), a short loose plug of glass wool or non-absorbent cotton is placed in the neck of the flask and the distilling tube and receiver are attached. The rubber stopper is roughened with sandpaper before each series of distillations. A 250 ml. Erlenmeyer flask, charged with exactly 25 ml. of the 0.02 *N* HCl, is used as the receiver.

The contents of the Kjeldahl flask are thoroughly mixed by rotation of the flask before starting the distillation, which is continued for 8 minutes after the first drop comes over. With practice, one is able to control the distillation rate so that the contents of the flask are just beginning to bump at the end of 8 minutes. During the last half-minute of the distillation, the receiver is removed from the cooling bath and lowered so that the delivery tube is well above the liquid level. The end of the delivery tube is then washed carefully before removing the burner. The contents of the receiver are made to an arbitrary standard volume of 80 ml., cooled

to room temperature, and titrated with 0.02 *N* NaOH, using 10 to 12 drops of Tashiro indicator.

Blank determinations on the reagents are run frequently.

Calculation:

$$\text{Total N as per cent Eq.D.W.} = \frac{100\ (A-B)\ 0.28-\text{blank}}{\text{Sample wt. in mg.}}$$

where A = ml. 0.02 *N* HCl × normality factor
B = ml. 0.02 *N* NaOH × normality factor
blank = mean of several determinations of nitrogen in reagents calculated as mg. of N

Protein Nitrogen

Accurately weighed samples of approximately 200 mg. are wrapped securely in 2-inch squares of fine cotton cloth (previously extracted with alcohol) so as to form flat packets about ¾ inch square. Each packet with its accompanying small cardboard label is held together with a wire paper clip. Groups of samples so prepared are placed in a stainless steel wire-mesh basket and extracted for 16 hours (overnight) with 70 per cent alcohol in the siphon beaker of the Nolan extraction apparatus (17, 39). The 70 per cent alcohol is a mixture of 70 parts of 95 per cent ethanol and 30 parts of distilled water; the hot vapor phase and condensate contain between 85 and 90 per cent of alcohol. The alcohol-extracted samples are dried for 1 hour at 110° C. and then transferred quantitatively from the cloth to a 25 x 200 mm. test tube, using a wide-mouth funnel and washing the particles from the cloth with water. The transfer is best made by moistening the packet with water before unfolding it; it is then spread out on the inside surface of the funnel and a spatula is used to loosen the bulk of the tissue from the cloth and to remove any particles that do not come off easily in the stream of wash water.

The volume of water in the test tube is diluted to 20 ml., a stirring rod is introduced into each tube, and the sample is extracted in the steam bath for 15 to 20 minutes, being occasionally stirred. The extracted tissue is centrifuged and the wash water is decanted through a paper filter. The residue is then transferred quantitatively to a 300 ml. Kjeldahl flask along with any particles caught on the filter paper. The test tube must be scrubbed thoroughly with a policeman and a considerable quantity of water may be required to complete the transfer to the Kjeldahl flask. The nitrogen is determined as for total nitrogen, omitting the nitrate reduction step. The excess water should be boiled off carefully at the beginning of the digestion, since there is some initial tendency toward foaming.

Blank values obtained for this determination should correspond to those found for the total nitrogen determination.

Careful tests of this method have shown it to be reliable. The coefficient of variation of determinations on sets of samples of tobacco of presumably constant protein content is, as a rule, more satisfactory than the coefficient of variation of parallel determinations of total nitrogen, presumably because of the removal of nicotine in the alcohol extract.

Soluble Nitrogen Fractions

It is advisable to prepare the extract by a vacuum infiltration procedure to insure rapid and complete wetting of the sample. Samples of 2.000 gm. are placed in 100 ml. beakers and about 15 ml. of distilled water are added. The beakers are then placed in a vacuum desiccator (without drying agent), which is carefully exhausted and maintained under vacuum for a few minutes after air has ceased to be evolved from the suspension of the sample in water. Air is allowed to flow back into the desiccator slowly. Thorough wetting of the sample is indicated if all of the solids have settled, leaving only tiny particles of lyophobic materials on the surface. If the wetting seems to be incomplete, a second evacuation is carried out after the addition of about 10 ml. more of water. Hot water (80 to 85° C.) is then added to the wetted samples, bringing the total volume to about 80 ml., and the beakers are held in a water bath at 80 to 85° C. for 10 minutes with frequent stirring. The beakers are cooled in a pan of cold water, each mixture is transferred quantitatively to a 100 ml. calibrated test tube, made to volume, centrifuged, and the extract is collected by decanting the solution through a dry paper filter. Aliquots of this solution are used for all of the soluble nitrogen fraction determinations with the exception of the nitrate determination, which is done on the organic acid extract (see below).

It is advantageous to preserve the aliquots of the water extract by freezing rather than by adding toluene to the entire solution. A convenient technique is to transfer a sufficient number of 5 ml. and 10 ml. aliquots immediately after the extract is prepared to cylindrical shell vials of 8 ml. and 14 ml. capacity respectively. The vials are labeled and capped with pliofilm and are stored in racks in the freezing compartment of the refrigerator. The vials needed for a single day's work are removed and the contents allowed to thaw before use. In making the transfers from the vials, variations in the technique described in the following pages are employed: For free ammonia nitrogen, the 10 ml. of phosphate-borate buffer followed by 2 to 3 ml. of water are used as a rinse, a pipette being used in delivering the buffer solution. For glutamine amide nitrogen, the 10 ml. of buffer are used to rinse the contents of the vial into the hydrolysis tube. For asparagine amide nitrogen, 5 ml. of water are used as a rinse and 2 ml. of 6 *N* sulfuric acid are used for hydrolysis instead of 1 ml. For peptide hydrolysis, the 5 ml. of 12 *N* sulfuric acid are used as a rinse in transferring to the hydrolysis tube. For total nitrogen, 10 ml. of water are used in the transfer to the Kjeldahl flask, reduction is carried out as described, and 3 ml. of concentrated sulfuric acid are employed for the digestion instead of 1.5 ml.

Acid-Base Titration of Ammonia

The ammonia distillation apparatus is that described by Pucher, Vickery and Leavenworth (24). All of the distillations are carried out under reduced pressure (*ca.* 20 mm.) at 40 to 45° C. for a 15 minute period. In assembling the apparatus, the receiver is charged with 3.00 ml. of 0.1 *N* HCl which has been carefully standardized against the standard 0.02 *N* NaOH. The measurement of the 3 ml. of HCl must be done with the greatest possible accuracy, and the same pipette should

be used for standardization and for all determinations and reserved for this measurement only. The apparatus is assembled ready to start the distillation before the addition of alkali to the solution in the distilling flask. After addition of the appropriate quantity of sodium hydroxide or alkaline buffer solution, the distilling flask is closed, the contents are mixed thoroughly, and the aspirator is turned on full. As soon as the maximal vacuum is obtained, with air introduced at the rate of one to two bubbles per second, the warm water bath is raised around the lower half of the distilling flask, and the distillation is continued for 15 minutes from that time. The alkaline solutions are introduced through the side opening which accommodates the air inlet. The air admitted during the distillation should be passed through a trap containing dilute sulfuric acid.

At the end of the distillation period, the stopcock between the receiver and aspirator is closed, the water bath is lowered, and air is carefully admitted to the apparatus at the rubber tube connection between stillhead and delivery tube. In bringing the apparatus back to atmospheric pressure, the clamp on the air inlet tube should be opened sufficiently to prevent the mixture in the distilling flask from being sucked back into the air inlet trap. If the residue in the distilling flask is to be saved, the stillhead and air inlet tube are washed with distilled water, draining the washings back into the flask.

The delivery tube is washed down with three small portions of water, and the parts of the joint between delivery tube and receiver that have been exposed to possible spattering are also washed. The washings are caught in the receiver, the contents of which are transferred to a 250 ml. Erlenmeyer flask. The receiver is then washed out with three small portions of water which are added to the flask. After being diluted to a standard volume (arbitrarily chosen) of 80 ml., the distillate is titrated with standardized 0.02 *N* sodium hydroxide, using 10 to 12 drops of Tashiro indicator.

Blanks on the apparatus and reagents should be run frequently. They should be total blanks, including hydrolysis or whatever other treatment is given the extract in the course of the determination. Furthermore, it is necessary to steam out the apparatus at the beginning of each day's work by running a blank distillation that is discarded.

Since 3 ml. of 0.1 *N* hydrochloric acid are equivalent to 4.2 mg. of nitrogen, quantities as high as 3.5 mg. can be determined by this method. When the quantities are at the 0.1 mg. level, however, the precision is only about 10 per cent.

Hypobromite Oxidation Method to Determine Ammonia

For the determination of small quantities of ammonia, i.e., less than 0.3 mg., the hypobromite method developed by Reifer (27) is much to be preferred to acid-base titration.

The distillation is carried out exactly as described by Pucher, Vickery and Leavenworth (24). However, the distillate and washings from the delivery tube are not transferred to a flask, but the titration of the ammonia is made in the receiver from the still.

Reagents:

Hypobromite solution: For the stock bromine solution, 20 gm. of KBr are dissolved in 50 to 100 ml. of water and 2.5 ml. of bromine

are added. The solution is shaken until the bromine is dissolved and then diluted to 1 liter. The hypobromite reagent solution is made by diluting 10 ml. of stock bromine solution to 100 ml. with 0.05 *N* NaOH and should be prepared on the day of use.

Sulfuric acid 5 *N*.

Potassium iodide solution, 20 per cent. This should be stabilized with about 0.1 gm. of sodium carbonate per 100 ml., and prepared fresh every few days.

Starch indicator solution (approximately 0.5 per cent).

Sodium thiosulfate 0.01 *N* (chloroform used as a preservative) standardized frequently against standard potassium iodate.

Procedure: The distillate is received in 3 ml. of 0.1 *N* sulfuric acid.[1] After the addition of exactly 10 ml. of the hypobromite reagent solution, the mixture is stirred thoroughly, with care to avoid the introduction of air bubbles, and is allowed to stand for 2 minutes to complete the oxidation of the ammonia. The timing is not critical, but an effort should be made to keep within the limits of 2 and 2.5 minutes; 1 ml. of 20 per cent potassium iodide solution and 5 ml. of 5 *N* sulfuric acid are then added. This mixture is stirred and titrated with 0.01 *N* thiosulfate, adding 10 to 12 drops of starch indicator solution when approaching the completion of the titration.

This is a differential titration procedure. The blank on the hypobromite solution is obtained by adding exactly 10 ml. to a mixture of 40 ml. of distilled water and 3 ml. of 0.1 *N* sulfuric acid, using tubes of the same dimensions as the distillation receivers.[2] Blanks on the distillation apparatus and on the various procedures for the forms of ammonia nitrogen have generally been negligible, but should be checked occasionally. The differential titration measures the loss of hypobromite incidental to the oxidation of ammonia to gaseous nitrogen, but the total of hypobromite and free bromine is included in the blank titration, which therefore does not indicate the stability of the hypobromite. When there is any doubt as to whether there is sufficient excess of hypobromite to oxidize the desired quantity of ammonia, a titration should be run on standard ammonium sulfate solution. Ordinarily, the hypobromite solution is stable for 2 or 3 days, and standardization with standard ammonium sulfate solutions need be done only infrequently if fresh reagent is made up each day.[3]

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[1] The accuracy of this measurement is not critical, as it is in the acid-base titration procedure.

[2] Since the tube in which the titration is run is about three-quarters full, mixing during the titration cannot be done by shaking or swirling. A thin glass rod, with a loop formed at the end at right angles to the shaft, is used for vertical stirring. Stirring should be rather vigorous, but with care taken to avoid breaking the surface with the loop on the rod, since experience has shown that erratic results are obtained if air is thus introduced during the titration.

[3] Interference from nicotine in the hypobromite method for ammonia has been briefly investigated.

When 0.25 mg. of ammonia nitrogen was recovered from mixtures with 1.17 mg. of nitrogen as nicotine, it was found that between 1 and 2 per cent of the nicotine was oxidized in the distillate. The error thus introduced into the ammonia

Since 1 NH_3 is equivalent to 3 $Na_2S_2O_3$,

1 ml. 0.01 *N* $Na_2S_2O_3 = 0.1401/3$, or 0.0467 mg. N_2:

10 ml. of 0.01 *N* thiosulfate would then be equivalent to 0.467 mg. of nitrogen, and the method is useful for quantities up to about 0.3 mg. of nitrogen. The sensitivity is about six times that of the acid-base titration procedure in which 1 ml. of 0.02 *N* sodium hydroxide is equivalent to 0.28 mg. of nitrogen.

Free Ammonia Nitrogen

A 10 ml. aliquot of the tissue extract (containing a maximum of about 2 mg. of nitrogen) is transferred to the ammonia distillation apparatus; 10 ml. of phosphate-borate buffer,[1] pH 6.5 (used in the transfer if a frozen aliquot is taken), and 5 ml. of sodium hydroxide borate mixture are then added, the flask is shaken and the distillation is carried out as described. The ammonia is determined by acid-base titration, or by oxidation by hypobromite, depending on the quantity present and the accuracy desired. The residual solution in the distilling flask is saved for the determination of free α-amino nitrogen.

Glutamine Amide Nitrogen

A 5 ml. aliquot of the tissue extract is pipetted into a 25 x 200 mm. test tube together with 10 ml. of the phosphate-borate buffer. Special test tubes, fitted with a 24/40 standard taper ground glass joint, are used; the inner part has a 30 cm. length of 1 mm. bore capillary tubing fused to it to serve as an air condenser. Before closing the tube, a drop or two of water is run into the lower end of the capillary. For the hydrolysis, the tube is placed in a constant-level boiling water bath at 100° C. for exactly 2 hours and is then removed and cooled in cold water, a few drops of water at the same time being allowed to be drawn down through the capillary. The contents of the tube are washed into the ammonia distillation apparatus with 10 ml. of water and the ammonia is distilled and determined as described under free ammonia nitrogen.

Glutamine amide nitrogen is the difference between the ammonia nitrogen found after a 2-hour hydrolysis at pH 6.5 and the free ammonia nitrogen.

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recovery was between +5 and +10 per cent. Since the ratio of ammonia to nicotine nitrogen is rarely found to be as high as this, interference from nicotine will ordinarily be negligible. If a situation should develop in which the relative proportions of ammonia and nicotine would lead to a significant error in the ammonia determination, it should be possible to make a correction after determination of nicotine in the extract.

[1] Phosphate-borate buffer, pH 6.5: A mixture of 750 ml. of 0.1 molar potassium dihydrogen phosphate (13.6 gm. per liter) and 250 ml. of 0.05 molar borax (19.1 gm. per liter).

Sodium hydroxide-borate mixture: 100 gm. of borax are dissolved in a mixture of 1,000 ml. of 1.0 *N* sodium hydroxide and 1,000 ml. of water. The solution is boiled for 15 minutes, cooled, and diluted to 2,000 ml. with water.

Asparagine Amide Nitrogen

A 5 ml. aliquot of the tissue extract is transferred into one of the hydrolysis tubes (as used in the glutamine amide nitrogen determination), together with 1.00 ml. of exactly 6.0 *N* sulfuric acid (low nitrogen), (or 2.00 ml. if a frozen aliquot is used and 5 ml. of water have been added as a rinse). The tube with the capillary air condenser attached is heated for 3 hours in the boiling water bath, being agitated at the end of the first hour. It is then cooled and the contents are transferred to the distillation apparatus with 20 ml. of water; the ammonia is distilled after the addition of 7 ml. of 1.50 *N* sodium hydroxide and titrated as described.

Asparagine amide nitrogen is the difference between the ammonia obtained in this determination and the ammonia after a 2-hour hydrolysis at pH 6.5.

Free Amino Nitrogen

The solution remaining after the free ammonia distillation is acidified with 2 ml. of glacial acetic acid and transferred to a 50 ml. volumetric flask. The distilling flask is then washed with two or three portions of water, which are added to the volumetric flask, and the volume is adjusted; 5 ml. portions of this solution are analyzed for amino nitrogen in the Van Slyke manometric apparatus. For details of the procedure see Peters and Van Slyke (20).

Ammonia Nitrogen after Peptide Hydrolysis

A 5 ml. aliquot of the tissue extract together with 5 ml. of 12 *N* sulfuric acid is pipetted into one of the 25 x 200 mm. hydrolysis tubes which is closed as in the other determinations and heated in a boiling water bath for 6 hours with occasional agitation. The tube is then cooled, the contents are transferred to the distillation apparatus with 15 ml. of water[1] and the ammonia is distilled off after the addition of 8 ml. of 10 *N* sodium hydroxide. The ammonia is received in 3 ml. of 0.1 *N* hydrochloric acid, and titrated as described.

Total Amino Nitrogen (After Acid Hydrolysis)

The solution remaining after distilling off the ammonia from the hydrolysate is acidified with 3 ml. of glacial acetic acid, and transferred, with two or three washings, to a 50 ml. volumetric flask; 5 ml. aliquots are analyzed for amino nitrogen as above.

Total Nitrogen of Water Extract

A 10 ml. aliquot of the extract is transferred to a 300 ml. Kjeldahl flask. To this are added 1.5 ml. of concentrated sulfuric acid and a measured volume of iron powder (approximately 300 mg.). Reduction and digestion are carried out as described.

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[1] In making this and other transfers it is convenient to use a wash bottle made from a 100 ml. graduated cylinder.

Nitrate Nitrogen

A 5 ml. aliquot of the *Organic Acid Fraction* (see below) is pipetted into a 300 ml. Kjeldahl flask, 2.5 ml. of 18 *N* sulfuric acid and approximately 300 mg. of reduced iron powder are added and the mixture is boiled gently over a micro burner for 5 minutes. After being cooled, 50 ml. of water and 10 ml. of Kjeldahl sodium hydroxide (18 to 19 *N*) are added and the solution is distilled for 8 minutes, the apparatus and technique being the same as used in the total nitrogen determination. To control frothing, 10 to 15 drops of white mineral oil are added to the flask before inserting the glass wool plug. The distillate is received in an appropriate quantity of standardized 0.02 *N* hydrochloric acid, and after cooling the flask to room temperature, the excess acid is titrated with 0.02 *N* sodium hydroxide, using Tashiro indicator.

Blanks, substituting 5 ml. of water for the organic acid fraction, are usually found to be in the vicinity of 0.1 ml. of 0.02 *N* sodium hydroxide.

Calculations for Soluble Nitrogen Fractions

Computations are simplified if the number of milligrams of original tissue powder equivalent to the final aliquot (used for the ammonia distillation or the amino nitrogen determination) is recorded along with the titration or gas measurement data.

For the acid-base titration method,

$$\text{Nitrogen as per cent Eq.D.W.} = (A-B) \times 0.02/n \times 0.28 \times 100/W,$$

where A = ml. standard NaOH equivalent to 3 ml. 0.1 *N* HCl
B = titration, ml. of standard NaOH
n = normality of standard NaOH
0.28 = mg. of N equivalent to 1 ml. of 0.02 *N* NaOH
W = mg. of original tissue represented by final aliquot.

Before multiplying by 100/W, the quantity $(A-B) \times 0.02/n \times 0.28$ should be corrected by subtracting the blank, computed as milligrams of nitrogen. Alternatively, the blank, as milliliters of standard sodium hydroxide, may be subtracted from A at the start of the computation.

For the hypobromite oxidation method,

$$\text{Nitrogen as per cent Eq.D.W.} =$$

$$(\text{Blank}-\text{Titration}) \times 0.0467 \times n/100 \times 100/W =$$

$$\frac{(\text{Blank}-\text{Titration}) \times n \times 4.67}{W},$$

where Blank = ml. thiosulfate required to titrate 10 ml. of the hypobromite
Titration = ml. thiosulfate required to titrate the unknown after oxidation of the ammonia
n = normality of the thiosulfate
0.0467 = mg. N equivalent to 1 ml. of 0.01 *N* thiosulfate
W = mg. of tissue represented by final aliquot.

The blank on apparatus and reagents in this determination is negligible.

The method of calculating the amino nitrogen is given in Peters and Van Slyke (20). The difference between total amino nitrogen and free amino nitrogen is arbitrarily taken to represent the peptide nitrogen of the extract. The quantity measured is obviously only the peptide nitrogen of the less stable peptides and does not include peptides of proline if such are present.

Petroleum Ether Extract

A 1 gm. sample is placed in a paper thimble (Whatman, ether extracted, 19 x 50 mm.) and covered with a loose pad of glass wool. The thimble is set in the siphon cup of the extraction apparatus (Fisher Scientific Co., catalogue No. 9-624), using, if necessary, a few quartz pebbles in the bottom of the cup to adjust the level of the top of the thimble to about 1 mm. above the top of the siphon.

The sample is extracted for 3 hours, with 100 ml. of petroleum ether in a wide-mouth 500 ml. Erlenmeyer flask. The siphon cup is hung from the tin coil condenser by a wire stirrup, and the heating is done in a water bath on an electric hot plate at low heat. The cycling time is from 2 to 2.5 minutes, giving a total of about 75 to 90 extractions during the 3-hour period.

The siphon cup with thimble is emptied of solvent and placed in a vacuum desiccator for one-half hour to remove the remaining petroleum ether. The extract is filtered if necessary through sintered glass (medium porosity) or a pad of asbestos in a Gooch crucible, evaporated to small volume in a beaker or flask, and transferred to a tared weighing bottle, from which the solvent is carefully evaporated. The residue of oil is weighed after being dried for one-half hour in the steam oven at 95 to 100° C.

Carbohydrates

Preparation of Alcohol Extract

After completing the petroleum ether extraction, the apparatus is reassembled, the flask is charged with 100 ml. of 70 per cent (v/v) ethyl alcohol and is boiled at low heat on the hot plate, two or three tiny quartz pebbles being used to prevent bumping. The extraction is continued for a total of 15 to 16 hours. The alcohol is evaporated on the hot plate at low heat, using a fan to disperse the vapors, with the addition of two or three small portions of water to prevent the extract from going to dryness while the last traces of alcohol are being driven off. The water solution of the material from the alcohol extract is then transferred to a volumetric flask[1] and made to volume. Sugar determinations are run on this water solution.

For details of the titration method see Pucher, Leavenworth and Vickery (22). The Somogyi phosphate sugar reagent is standardized against Bureau of Standards glucose.

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[1] 100 ml. flasks were used for the A through H series of samples, and 50 ml. for the set containing I, J, and the six fermentation samples.

Free Reducing Substances

A 5 ml. aliquot of the sugar solution is transferred to a test tube calibrated at 25 ml. After the addition of 3 ml. of 0.05 *M* lead acetate solution (18.95 gm. $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ to 1,000 ml.), the mixture is diluted to the mark, mixed, and allowed to stand for 15 minutes. After being centrifuged, all of the supernatant fluid is poured into a 50 ml. Erlenmeyer flask, 0.1 ml. of 3.072 *M* dibasic potassium phosphate (53.5 gm. anhydrous K_2HPO_4 to 100 ml.) solution is added and the solution is mixed and allowed to stand for 10 minutes. One gm. of Lloyd's reagent is added, the mixture is shaken for 2 minutes and the suspension is filtered on a dry Whatman No. 4 filter paper; 5 ml. aliquots of the filtrate are analyzed for sugar. Two or more blanks, each having 5 ml. of distilled water substituted for the sugar solution, are run in parallel with each series of determinations.

Total Sugars

Three drops (about 0.15 ml.) of invertase solution[1] are added to 5 ml. of the sugar solution in a 25 ml. calibrated test tube. The solution is mixed and incubated for one hour at room temperature. At the end of the incubation period, 3 ml. of lead acetate solution are added and the procedure outlined under *Free Reducing Substances* is followed from that point. Blanks, containing 5 ml. of distilled water and 3 drops of the invertase solution, are run in parallel with each series of determinations.

Reducing Substances after 0.18 N Acid Hydrolysis

A 5 ml. aliquot is incubated with invertase as above, then 5 ml. of 0.35 *N* sulfuric acid are added and the mixture is heated for 30 minutes in a water bath at 100° C. After being cooled, 2 drops of 0.04 per cent phenol red are added, and the solution is neutralized with a cold saturated solution of barium hydroxide (about 1.37 *N*) delivered from a burette. The pink color is discharged with a few drops of 0.1 *N* oxalic acid and, after the addition of 3 ml. of lead acetate solution, the determination is completed as above. Blanks, starting with 5 ml. of water in place of the sugar solution, are run in parallel with each series of determinations.

Reducing Substances after 0.8 N Acid Hydrolysis

One ml. of 5 *N* sulfuric acid is added to 5 ml. of the sugar solution in a calibrated 25 ml. tube, which is then shaken, capped with a glass bulb and heated at 100° C. in the water bath for 2 hours. After being cooled, the solution is neutralized with barium hydroxide and the determination is completed as above, blanks being run in parallel.

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[1] Invertase solution: A 1 per cent solution of the flakes of "Invertase, Analytical (Mellibiase free)", Nutritional Biochemicals Corp., Cleveland, Ohio, is made on the day of use. Generally, 3 to 5 ml. of this solution will suffice for a day's work. A test of the activity of the material should be made.

Unfermentable Reducing Substances

A 10 ml. aliquot of the sugar solution is placed in a 50 ml. conical bottom centrifuge tube (the tubes are equipped with lips which serve as vents during the incubation), and 3 ml. of a standard yeast suspension are added. The tube is stoppered and shaken for 30 minutes by machine. The tube is supported at an angle (with the lip up) to provide for continuous mixing of the suspension. At the end of this period, the tube is centrifuged at high speed for at least 5 minutes. A 5 ml. aliquot is then withdrawn carefully, so as not to stir up the packed yeast cells, transferred to a calibrated 25 ml. test tube, and the determination completed as for *Free Reducing Substances.* Blanks, starting with 10 ml. of distilled water and 3 ml. of yeast suspension, are run along with each series of determinations.

Preparation of Standard Yeast Suspension

A 6 gm. portion of active dry yeast is washed thoroughly with four portions of distilled water in a 500 ml. centrifuge bottle. Each washing is completed by centrifuging and pouring off the wash water. The yeast cells should be suspended and washed without the use of a stirring rod. After the fourth washing, the yeast cells are suspended in a small amount of water and the suspension is made to a volume of 50 ml. For use, the suspension is transferred to a 125 ml. Erlenmeyer flask, which is swirled during the pipetting so as to maintain a uniform concentration of yeast cells. Occasionally, the activity of yeast suspensions so prepared should be tested against glucose or sucrose. Ordinarily, 3 ml. of the suspension will absorb at least 10 mg. of sugar during a 30-minute period of incubation on the shaking machine. It is desirable to prepare the yeast suspension on the day of use, rather than to store it under toluene. The suspension can be prepared the day before if necessary, but should then be washed once and resuspended immediately before use. For convenience, the standard yeast suspension may be made from the contents of a single envelope of Fleischmann's active dried yeast, which contains approximately 8 gm. After being washed, this is made up to a volume of 65 ml. in a graduated cylinder, and provides enough material for 20 determinations.

This procedure is a modification of an earlier one for the determination of unfermentable carbohydrates. In computing the results it is assumed that the unfermentable reducing substances are distributed uniformly through the cells and solution, and the 5 ml. aliquot of the sugar solution after fermentation is assumed to have been diluted by a factor of 10/13 with respect to the concentration of the original 10 ml. of solution taken for fermentation.

Calculations of Carbohydrate Fractions

Computations are simplified if the number of milligrams of original tissue equivalent to the final aliquot taken for sugar titration is recorded along with the titration data.

The *normality factor* converts the actual normality of the thiosulfate to 0.005 *N*, of which 10.00 ml. is equal to 5.00 ml. of the sugar reagent, which is 0.01 *N* potassium iodate.

NF = normality factor
GE = glucose equivalent of the sugar reagent, as mg. per ml. of 0.005 *N* thiosulfate
T = net titration, the appropriate blank titration minus the unknown
W = mg. of original tissue equivalent to the final aliquot taken for sugar titration

Free Reducing Substances, as glucose, as per cent of Eq.D.W.

$$= NF \times GE \times T \times \frac{100}{W}$$

The other determinations are computed in the same way, except for the unfermentable carbohydrates, where the factor of 10/13 must be introduced into the above formula to allow for the dilution by the yeast suspension.

BEHAVIOR OF TOBACCO LEAVES DURING CURING

Changes in Water Content

Data are shown in Table 6 for the water content of the two sets of samples examined during the curing process. The fourth column shows the water as calculated by subtracting the equilibrated dry weight of each sample from its weight as removed from the curing barn. The figures thus refer to the water in each sample in excess of the quantity which is held by the tissue when in equilibrium with air at 50 per cent relative humidity and 25° C. In order to permit a closer comparison between the sets of samples, the data are expressed in the fifth column in terms of 1 kilo of initial fresh weight. The mean chemically determined moisture content of the samples of the A1 — J1 series after equilibration was 6.39 ± 0.22 gm. per kilo so that a constant amount of water remained after this treatment. The agreement between the two sets of samples is remarkably close and indicates that the conditions in the curing barn were satisfactorily constant from day to day at least over the region in which the samples were hung. Water evaporated at approximately the same rate from both sets.

The rate of evaporation was such that approximately one-half of the water in the tissues had disappeared after four days of curing. Reference to Table 5 shows that at this time most of the leaves were beginning to show patches of yellow color owing to the decomposition of the chlorophyll. At the eight-day point, only 18 per cent of the original water remained and more than one-half of the leaves were brown in color over the greater part of their area. The solutes in the cell sap must therefore have attained a concentration roughly five times greater than normal. Table 5 indicates that only a few leaves still retained small areas of green color and even these had brown spots when this stage of dehydration had been reached.

TABLE 6. CHANGES IN WATER CONTENT DURING CURING

Figures in italics refer to the duplicate A2 — J2 series of samples.

Sample	Time	Weight of sample as removed from barn	Water content[1] of sample	Water content[2]	Moist weight as percentage of fresh weight
	days	gm.	gm.	gm./kg.	
A1	0	1387	1258	907	100
A2		*1317*	*1198*	*910*	*100*
B1	2	1056	931	677	76.7
B2		*988*	*874*	*674*	*76.2*
C1	4	742	627	463	54.8
C2		*780*	*663*	*481*	*56.6*
D1	6	509	396	292	37.5
D2		*544*	*434*	*324*	*40.6*
E1	8	332	222	166	24.8
E2		*323*	*211*	*152*	*23.7*
F1	12	182	69	50	13.2
F2		*146*	*38*	*28*	*10.7*
G1	16	126	14	10	9.2
G2		*119*	*13*	*10*	*8.9*
H1	20	122	9	6.5	8.8
H2		*123*	*8*	*5.6*	*8.7*
I1	32	120	4	2.8	8.5
I2		*111*	*3*	*2.2*	*8.3*
J1	63	130	17	12	9.5
J2		*120*	*15*	*11*	*9.1*

[1] These figures represent the water content in excess of that held when the tissues are equilibrated with air at 50 per cent relative humidity and 24° C.

[2] The actual water content of the samples is obtained by adding 6.39 $\pm$ 0.22 gm. per kilo to each of the figures in this column, this being the mean value for chemically determined water in the equilibrated samples.

Dehydration became extreme as the brown areas enlarged more and more. The sample taken at 32 days was, in fact, so dried out that it was impossible to handle the leaves sufficiently to permit an accurate classification with respect to the extent of the areas of brown and yellow color, and the description in Table 5 depends on the general observation that this sample did not seem to have changed much in comparison with the previous one. The actual water content when collected was only 10.8 per cent. The last sample was taken during a period of damp weather when the leaves had become hydrated to a point such that they could be easily sorted.

The data in the right hand column of Table 6 show the weights of the samples at the time of collection as percentages of the initial fresh weights of the samples. Although the greater part of the change indicated

arises from the loss of water, these figures are also affected by the loss of organic solids which occurred mainly during the first eight days. The data are plotted as a broken line in Figure 2 and furnish what is perhaps the most easily appreciated general view of the effect of the curing process. The major events were manifestly completed during the first 16 days.

Ash and Moisture

The data in Table 7 for the water content and ash of the equilibrated samples illustrate the desirability of placing analytical results obtained upon leaf tissue on a common basis. The actual water content of the samples ranged from 6.98 to 8.10 per cent of the dry weight, a moderately narrow range. However, when the figures are recomputed in terms of a kilo of initial fresh weight of the leaves, the range becomes less and the mean water content of the entire set was 6.39 ± 0.22 gm. per kilo, the coefficient of variation (C.V.) being only 3.4 per cent. This coefficient expresses the variation due to the combined errors of sampling, of the analytical method employed, and of the equilibration process. It is clear that this process was satisfactorily controlled, for each sample behaved in essentially the same way with respect to its capacity to absorb and hold moisture.

TABLE 7. WATER, ASH AND ORGANIC SOLIDS CONTENT OF SAMPLES EQUILIBRATED AT 24° C. AND 75 PER CENT RELATIVE HUMIDITY

Data in grams per kilo of fresh leaf.

Sample	Days cured	Water	Ash	Organic solids
A1	0	6.51	16.5	70.2
B1	2	6.43	16.6	67.8
C1	4	5.94	16.4	62.8
D1	6	6.14	16.7	60.4
E1	8	6.28	16.8	59.1
F1	12	6.40	17.0	58.6
G1	16	6.66	16.7	58.9
H1	20	6.43	17.3	57.7
I1	32	6.55	16.8	59.4
J1	63	6.57	16.8	58.9
Mean		6.39	16.76	
S.D.		± 0.22	± 0.25	
C.V. (%)		3.4	1.5	

The values for ash in terms of the fresh weight of the leaves are in even closer agreement with each other than those for the moisture content. After the individual leaves are detached from the plant and hung in the curing barn, there is no opportunity for the ash content of the samples to change as there is, for example, when tobacco leaves are

cured on the stalk and migration of ash components into the stalk can occur. The coefficient of variation of 1.5 per cent is very little greater than that of the fresh weight (Table 3) and is evidence of the close similarity in composition of the samples within the set. The constancy of the data supports the view that close agreement in fresh weight of samples collected by the statistical method is presumptive evidence of agreement in fundamental chemical composition. The plot of the data for ash in Figure 2 is, as would be anticipated, a horizontal straight line, the slight irregularities in it being wholly negligible.

Through the courtesy of the Department of Analytical Chemistry, determinations were made of the most important components of the ash of these samples by the spectrographic method. The upper part of Table 8 shows the range of the values together with the means. The potassium, calcium, magnesium, and phosphorus account for the greater part of the ash, these together making up 7.66 gm. per kilo, the trace elements accounting only for another 0.23 gm. per kilo if the mean values are used. However, in accounting for the composition of the ash, the sum of the corresponding oxides should be taken; this amounts to 10.28 gm. and, accordingly, 6.48 gm. of the mean ash weight of 16.76 gm. is left unaccounted for. This quantity presumably is mostly carbon dioxide combined as carbonate in the ash, although sulfate and possibly chloride may also be present. That the order of magnitude is correct is clear if this quantity is converted to milliequivalents by means of the relation 1 m.eq. of carbon dioxide = 0.022 gm. The deficit so calculated accounts for 295 m.eq. of carbon dioxide which may be assumed to be present in combination with the alkali of the ash. As will be shown in detail later, the actual mean alkalinity of the ash was 297 m.eq. So close an agreement as this is doubtless fortuitous; in contrast, the data for the FA — FJ series when computed in the same way gave 238 m.eq. of carbon dioxide to be compared with 292 m.eq. of alkalinity of ash. Nevertheless, there is little doubt that carbonate accounts for most of the deficit in the weight of the ash.

TABLE 8. SPECTROGRAPHIC ANALYSIS OF ASH

Data in grams per kilo of fresh leaf except for copper and boron which are in milligrams per kilo. Data for the samples subjected to fermentation in lower half of table.

Sample	K	Ca	Mg	P	Mn	Fe	Al	Zn	Na	Cu	B
A — J											
Highest	4.61	2.47	0.84	0.25	0.012	0.068	0.086	0.034	0.066	3.1	3.8
Lowest	4.12	2.22	0.72	0.22	0.009	0.056	0.070	0.016	0.045	1.9	3.3
Mean	4.31	2.34	0.78	0.23	0.010	0.062	0.075	0.025	0.056	2.2	3.5
FA — FJ											
Highest	4.82	2.46	0.79	0.25	0.010	0.050	0.044	0.041	0.064	2.4	4.1
Lowest	4.21	2.04	0.64	0.18	0.007	0.042	0.035	0.016	0.038	1.6	1.6
Mean	4.47	2.23	0.69	0.20	0.008	0.045	0.040	0.023	0.046	2.0	2.3

Organic Solids

The organic solids of the samples, unlike the ash, did not remain constant. The plot in Figure 2 shows that, during the first eight days of the curing process, organic solids disappeared at a moderately constant rate, but then reached a level which was maintained with little change for the rest of the curing period. The loss of organic solids is evidence that chemical reactions of a fundamental nature were taking place. The average value for the organic solids of the last six samples in the series is 58.8 gm. per kilo. The leaves at the start contained 70.2 gm.; thus 11.4 gm. of organic solids or 16.2 per cent of the solids present initially disappeared, presumably as volatile products of chemical reactions that took place within the cells. Nearly the whole of this change had been completed at the end of eight days, for no further significant change had taken place after 12 days. Referring back to Table 5, it is clear that the leaves at eight days were about at the end of the yellow phase of curing; only eight of the 40 leaves are recorded as retaining any green color and more than half of them were brown or mostly brown. At 12 days only a few of the leaves retained much yellow color. Thus, at this point in the curing process there was a striking change in leaf color. If it is assumed that the formation of the brown color represents a stage at which substances of essentially phenolic nature are subjected to oxidation processes with the formation of products of a dark color perhaps allied with the vaguely understood substances classified as "humins" or "melanins," it seems clear that at a certain point of time some event occurred within the cells which initiated this reaction. Browning did not occur suddenly over all of the leaves; on the contrary, traces of brown color were observed on some of the leaves at the end of four days. But

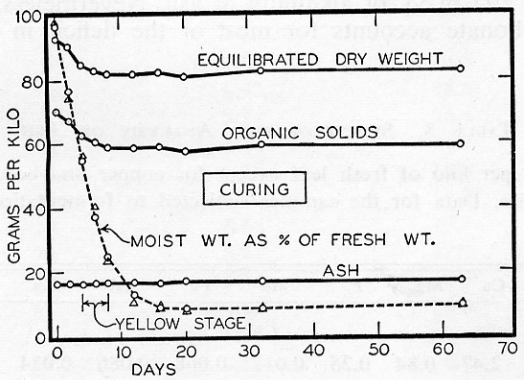

FIGURE 2

Ash, organic solids, and equilibrated dry weight of the samples (Series A1 — J1) subjected to curing, expressed in grams per kilo. The broken line shows the rate of change of the moist weight of the samples as collected, expressed as a percentage of the initial fresh weight (ordinate to be read as per cent) and thus gives an over-all picture of the change in weight that occurred. Data for both A1 — J1 and A2 — J2 series are included on this curve, the points for the latter series being shown by triangles. The extent of the yellow stage of curing is also indicated.

within a given area of a single leaf, the onset of browning is a moderately rapid event; brown spots form and extend in size and, within a space of a day or two, become confluent and extend over more and more of the surface. The significant point, with respect to the present data, is that when the brown color became dominant over all of the leaves of the sample, loss of organic solids ceased.

The most logical interpretation of the loss of organic solids from the green leaves, which were undeniably living organisms at the time they were placed in the curing barn, is that it represents the effect of uncompensated respiration of the cells. It is an effect that is invariably observed when records are made of the changes in organic solids of detached leaves whether subjected to the curing process in dim light or cultured in water in darkness. The effect can be stimulated in magnitude by culture of tobacco leaves in solutions of salts of organic acids in darkness if allowance is made for the uptake of solute from the solution. However, if the detached leaves are illuminated during culture with light of an intensity sufficient to give rise to photosynthesis, gain rather than loss of organic solids may take place (44).

At the point in the curing process at which the brown coloration had spread over the greater part of the leaf area, the concentration of the solutes in the leaves had increased by a factor of approximately 5 owing to the evaporation of water. Loss of organic solids then ceased. This is equivalent to the statement that respiration ceased and this, in turn, to the statement that death of the cells had occurred, if one accepts the view that the presence of the respiratory process is an essential criterion of the presence of life in the cells of the leaf.

This does not mean that all capacity for chemical reactions within the leaf tissues ceases when the leaves have become brown. As will become evident, many kinds of chemical changes can go on, although at a slow rate, and some of these, particularly those that take place during the fermentation process, are of the greatest importance in the technology of tobacco manufacture. What we are dealing with at the point where a definite respiratory process can no longer be observed is a change such that the ordered enzymatically controlled reactions characteristic of the living cell can no longer continue. The causes for the change are doubtless complex, but dehydration with the accompanying abnormal concentration of the cell sap is probably the main precipitating cause. Other factors, such as exhaustion of the substrates for some of the essential reactions, may also play a rôle.

Consideration must be given to the presence of living microorganisms such as bacteria, molds, and other fungi on the surfaces of the leaf. Leaves usually support a large population of such organisms, and it is well known that, under improper conditions of humidity in the curing barn, these organisms may multiply freely and cause serious losses. The microflora on the leaf surfaces doubtless continue to respire, and sufficiently sensitive methods would reveal their presence; but this phenomenon is of a much smaller order of magnitude than the respiration of the leaf cells while they are still alive. The essential point in the technique of the curing process is to conduct the dehydration of the leaves in such a manner that the effects of the life processes of the microflora are minimized, although scope may be given to these processes during the fermentation operations later on.

Notwithstanding the fact that the cells ceased to respire after 8 to 12 days, enzymes and enzyme systems remained which were still capable of bringing about chemical changes. Although when the leaves turn wholly brown the organization of these enzyme reactions into the mutually interdependent system characteristic of the living cell has been broken down, some of the enzymes retain the capacity for activity, although probably at reduced rates as compared with the conditions in the living leaf. Thus, the sudden cessation of all chemical change is not to be expected unless steps are taken to inactivate the enzyme systems in some irreversible manner such as by exposure of the tissue to a high temperature as is customary in the flue-curing process applied to cigarette tobaccos.

Still other possibilities of chemical changes in the partially cured leaves must be considered. The browning reaction itself is probably an evidence of the possibility of reactions of a kind that may not be exclusively enzymatic. Considered as an over-all process, it is probably an oxidation reaction in which molecular oxygen from the air is concerned followed by polymerization, the substrate being phenolic substances that are made available for oxidation only upon the general disintegration which accompanies the extensive dehydration of the leaves. Although enzymes of the tyrosinase or catacholase type are probably concerned, it does not follow that all stages of the change are controlled by catalysts. And the browning reaction is clearly one that continues at a slow rate throughout the greater part of the curing operation; during the second month, the outstanding visible change is an evening of the color over the whole surface of the leaves.

At the top of Figure 2 is shown the curve for the equilibrated dry weight of the series of samples. Inasmuch as the weight of the ash is approximately constant as well as the weight of the moisture associated with the samples, the curve is essentially parallel with that for the organic solids although it is slightly less regular. It is included in the figure to give a measure of the actual weight of the samples as removed from the curing barn, expressed in terms of grams per kilo of the initial fresh weight of the leaves, with the additional proviso that the samples had been brought to a stabilized moisture content by equilibration under fixed conditions.

An Uncertainty in the Measurement of the Organic Solids

Consideration must be given to the chemical meaning of the data recorded as organic solids in Table 7 and plotted in Figure 2. The data are computed by subtracting the combined weights of the determined moisture and ash from the equilibrated dry weight of the samples. This is sound chemical practice, but it ignores the fact that a certain part of the weight of the ash consists of carbonate, the carbon of which may be assumed to have had its origin from the carboxyl groups of organic acids and the oxygen either from the carboxyl groups or the air in the muffle furnace or both. If neutral potassium malate, for example, which is one of the salts to be expected as a component of the curing leaves, is heated in the muffle, a residue of potassium carbonate weighing 44.54 per cent of the weight of the salt should remain. Of this, 25.2 per cent represents the potassium ion of the salt, the remainder of 19.34 per cent is carbonate ion. In reckoning what is "organic" and what "inorganic" in a salt such

as this, should one consider that only the potassium in the ash is "inorganic"? The answer in this simple case is clearly in the affirmative, but application of the principle involved to the correction of the weights of the ash obtained from leaves is fraught with complexities. To begin with, a solution of dipotassium malate is approximately at pH 7 or a little higher; the pH of the samples of leaves ranged from 5.4 to 5.7 and a solution of malic acid neutralized to this reaction consists of a mixture of about 60 per cent of divalent malate ions and 40 per cent of monovalent acid malate ions. The relative proportions of these ions change rapidly in this region of the pH scale. Thus, if such a solution were evaporated to dryness and ashed, exact knowledge of the pH and of the dissociation constants of malic acid would be required in order to compute the quantity of malic acid present from the amount of potassium carbonate found. The situation in the case of salts of the trivalent citric acid is even more complex. When the mixture of acids in the leaves is ashed, interpretation of the results becomes subject to many assumptions.

Even more difficult is the problem raised by the presence in the leaf of several cations. Potassium, calcium, and magnesium are the chief ones. Potassium carbonate is stable at the temperature of the muffle, magnesium carbonate is decomposed completely, and calcium carbonate at 600° C. is at a point where a certain amount of decomposition may be anticipated if the heating period is prolonged. It is probably for this reason that the time of heating in the muffle must be closely controlled if reproducible results for the ash of tobacco leaves are to be obtained. The determination of the carbonate in the ash might be assumed to give an approximate correction to be added to the weight of the organic solids and subtracted from that of the ash. This is not entirely sound, however. In the ash of potassium malate, only one of the two carbons of the carboxyl groups and only three of the four oxygen atoms of these groups are represented. Although an accurate factor can be computed in this simple case to convert the weight of the ash to the weight of the malate ion in the salt, the situation becomes more complicated when a mixture of malate and citrate is present, and in the actual case of the leaves, with unknown proportions of several other acids, not all of which have been identified, and several possibilities with respect to the cations, to say nothing of the uncertainties arising from the pH of the leaf, it seems wiser to make no attempt to compute a correction. In considering the data, it is necessary to understand that the ash as a measure of the "inorganic" components is somewhat overestimated and that the true "organic" solids are underestimated by the same amount. With leaves of the same initial composition, the error is a constant one and applies to all samples equally but cancels out when attention is paid only to the changes in composition from one sample to the next. Accurate correction of the data should not change the shape of the plots but should merely lead to identical lines located somewhat differently on the scale of ordinates.

Total Nitrogen

Table 9 shows the data for the total nitrogen of both the A1 — J1 and the A2 — J2 series. The data for the first of these sets are plotted in Figure 3. Examination at once suggests that a loss of nitrogen took

place during the period of curing and an analysis of variance kindly carried out by Dr. C. I. Bliss leads to the conclusion that the slope of the line in which all of the data were plotted is highly significant, that is to say, there is no reasonable doubt that an actual loss of nitrogen took place. In addition, there is no significant difference between the two sets of data, for the plots of the two sets do not differ significantly in either position or slope.

TABLE 9. NITROGENOUS COMPONENTS OF LEAVES DURING CURING

Figures in italics refer to the duplicate A2 — J2 series of samples. Data in grams per kilo of fresh weight.

Sample	Days cured	Total nitrogen	Protein nitrogen	Soluble nitrogen	Nitrate nitrogen	Ammonia nitrogen	Glutamine amide nitrogen	Asparagine amide nitrogen	Amino nitrogen Free	Amino nitrogen Corrected
A1	0	3.80	2.27	1.53	0.82	0.022	0.013	0.019	0.112	0.080
A2		*3.76*								
B1	2	3.76	1.88	1.88	0.81	0.036	0.040	0.078	0.344	0.227
B2		*3.63*								
C1	4	3.73	1.33	2.40	0.84	0.037	0.050	0.292	0.570	0.228
C2		*3.80*								
D1	6	3.66	1.07	2.59	...	0.10	0.041	0.435	0.680	0.204
D2		*3.67*								
E1	8	3.62	1.01	2.61	0.79	0.17	0.042	0.467	0.643	0.135
E2		*3.74*								
F1	12	3.69	1.02	2.67	0.81	0.227	0.026	0.465	0.610	0.120
F2		*3.61*								
G1	16	3.65	0.98	2.67	0.79	0.230	0.035	0.449	0.579	0.095
G2		*3.61*								
H1	20	3.65	0.97	2.68	0.81	0.226	0.035	0.449	0.573	0.089
H2		*3.59*								
I1	32	3.59	0.99	2.60	0.78	0.237	0.036	0.454	0.592	0.102
I2		*3.52*								
J1	63	3.60	0.97	2.63	0.77	0.206	0.018	0.426	0.537	0.093
J2		*3.55*								
Mean		3.675			0.80					
S.D.		±0.069			±0.02					
C.V. (%)		1.9			2.7					

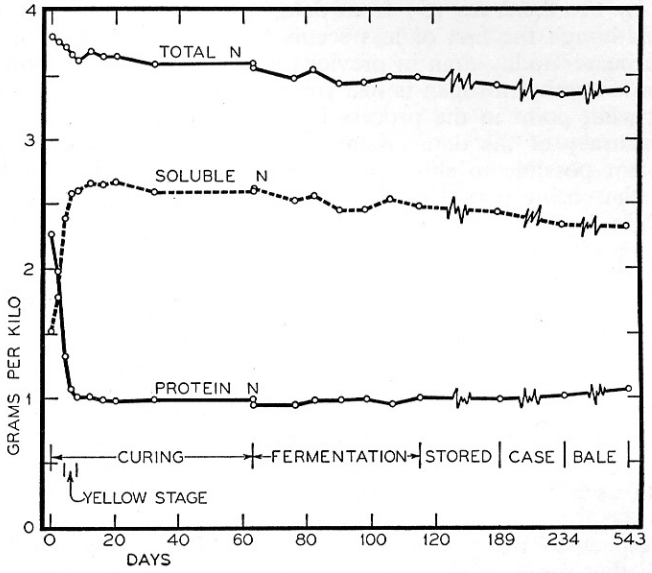

FIGURE 3

Protein nitrogen, soluble nitrogen, and total nitrogen of the samples subjected to curing (Series A1 — J1) and also of those subjected to both curing and fermentation with subsequent storage (Series FA — FJ). The small discontinuity in the curves at 63 days arises from the fact that sample FA was not precisely identical in composition with sample J1 although both were collected on the day the curing operation was completed. The time scale has been greatly compressed for the successive samples collected after storage, after the mulling operation in the case, and after 309 days of storage in a bale. This is indicated on the curves by the zigzag lines.

The data as a whole (20 observations, each in duplicate) conform to the equation

$$\text{T. N.} = 3.662 - 0.1273\ (t - 0.997)$$

where t = log (time in days + 1).[1] From this equation, the loss of nitrogen at the end of 63 days was 0.23 gm. per kilo or 6 per cent of the nitrogen present at the start. That a loss of nitrogen occurs during the normal curing period was pointed out in 1931 in an earlier bulletin (41) and the magnitude of the loss in the present case is similar to that

[1] The use of "time in days +1" in this equation is merely a mathematical device so that the observations at zero time can be included in the calculation.

Dr. Frankenburg has analyzed the data for total nitrogen on the assumption that a large part, represented by N_o, is stable while a small part, represented by B, disappears completely according to an exponential law. These assumptions lead to the equation

$$N_o + B = 3.55 + 0.21 \times 10^{-0.31t}$$

where t = time in days.

This equation fits the data at least as accurately as the least squares equation given in the text and may well be a more realistic expression of the behavior of the nitrogen.

estimated by Frankenburg (5) from data in the literature. Unfortunately, however, although the fact of loss seems to be established unequivocally, we are no nearer today than in previous work to an understanding of the mechanism whereby nitrogen is lost from the leaves, nor to a demonstration of at what point in the process it occurs. In spite of the increase in general accuracy of the data arising from the sampling methods now in use, it is not possible to show whether the loss takes place either early or late in the curing period or that it is continuous throughout. Data for the first eight days do indeed follow a straight line which slopes downwards in a manner that suggests that much of the loss occurred in this period. However, the remainder of the data are less regular, and when it is remembered that the coefficient of variation of the entire set of figures is 1.9 per cent, it is clear that no great significance may properly be attached to the apparent regularity of the results for the first few days. In any case, loss of nitrogen from leaves that are still green is not a probable event. No such loss has ever been detected from tobacco leaves subjected to culture in water for protracted periods. It seems more conservative, therefore, to accept the equation which represents the behavior of the data as a whole as the best general picture of the progress of the reaction.

With respect to the mechanism of the loss, the most probable hypothesis is that some of the ammonia that was formed by the oxidative deamination of the amino acids together with a little nicotine may have escaped from the tissues. Although the vapor pressure of ammonia from solutions at the pH of the leaves must be minute, there is no reason to believe that it is zero and, in consideration of the large area of the leaves and of the protracted time involved, loss by this mechanism does not seem impossible. The presence of ammonia in the air of the curing shed has been detected by Garner, Bacon and Foubert (8) and ammonia is easily recognized in the air surrounding bulks of fermenting tobacco. The pH of fermenting tobacco is only a little more alkaline than that of curing tobacco.

Protein Nitrogen

The behavior of the protein of the leaves is shown in Table 9 and is plotted in Figure 3. The measurement consists of the determination of the nitrogen that remains insoluble when a sample of the dry finely ground tissue is exhaustively extracted with 70 per cent alcohol at boiling temperature and then with hot water. It is assumed that, under these circumstances, all of the protein of the leaf is denatured and rendered insoluble, and that no insoluble form of nitrogen other than protein nitrogen remains.

The data indicate that rather more than one-half of the proteins of the cells disappears during the first six days of curing, the greater part of the change having occurred, in fact, within four days; subsequently, the protein nitrogen remains substantially constant. Reference to Table 5 shows that the cessation of the digestion of the proteins corresponds in time with the rapid extension of the brown color over the surface of the leaves, which has in turn been correlated with the death of the cells. This would suggest that the conditions essential for the activity of the proteolytic enzymes no longer prevailed when the water content of the tissues had been reduced to about one-fifth of that present at the start.

It is of considerable interest to note that the extent of the digestion of the protein in the present case closely resembles that observed when tobacco leaves are subjected to culture in darkness in water or in culture solutions that contain glucose or an ammonium salt. Under these circumstances, the leaves pass through a series of changes in color that are quite similar to those that occur in the curing process. After six days, they have become almost entirely yellow through the extension outward of the color change that begins along the main veins. In Table 10 are quoted results obtained with leaves from the crop of 1934 (44). The DW series of samples which were cultured in water in darkness gave results that are almost identical with those from the present experiment with respect to the quantity of protein found in the leaves at the start and after six days of treatment; 56 per cent of the protein disappeared in this interval. The leaves from a higher position on the plant used for the culture experiments in glucose solution (DG samples) and in ammonium salt (DN samples) were higher in protein at the start and smaller proportions (respectively 49 and 44 per cent) of the protein had been digested in the same interval. It was necessary to terminate two of these solution culture experiments at the end of six days because of the invasion of the base of the midveins by bacteria; the culture in glucose solution was, however, extended for eight days and digestion of the protein continued, although at a diminished rate. Further extension of the culture period would probably lead to additional loss of protein (see data in (43)), but the results become increasingly difficult to interpret owing to complications arising from bacterial decomposition at the base of the leaves in contact with the culture solution.

TABLE 10. CHANGES OF PROTEIN NITROGEN IN TOBACCO LEAVES SUBJECTED TO CULTURE IN WATER IN DARKNESS

Units are grams per kilo of fresh weight.

Samples	Date picked	Position of leaves on plant	Protein nitrogen		
			Initial	After 6 days	After 8 days
DW	July 17, 1934	1 to 3	2.27	1.00	...
DG	July 24, 1934	4 to 6	2.35	1.20	1.11
DN	July 31, 1934	7 to 9	2.61	1.45	...

The comparison of the present results with those obtained from leaves cultured under conditions such that a serious degree of dehydration does not take place indicates that decomposition of the protein is a normal process and that its rate and extent are not critically affected by dehydration until this has become extreme. The important point is that digestion then ceases and no further significant change in protein content takes place either during the remainder of the curing operation or, as will be seen later, during fermentation in spite of the fact that the leaves during the fermentation process contain about 18 per cent of water.

This observation suggests that irreversible changes in the proteolytic enzymes may have occurred at the time that the leaves first became wholly brown in color. The desirability of the examination of partially and fully

cured leaves for the presence of proteolytic enzymes in a still active condition is clearly indicated. Such tests were not possible with the present material because the samples had been dried at a temperature that would destroy all enzymes.

Soluble Nitrogen

The differences between the data for total nitrogen and for protein nitrogen are shown in Table 9 and are plotted in Figure 3 as "soluble nitrogen." A comparison is shown in Table 11 between data for the FA — FJ series of samples computed in this way and the results of direct determinations of nitrogen in extracts prepared by heating the tissue with water in the steam bath. The agreement between the two results is moderately close, the mean difference of 0.13 gm. being 5 per cent of the soluble nitrogen. The computed value is obviously sufficiently accurate for the present purposes. The difference is presumably due to the presence of a small proportion of nitrogenous substances (e.g. chlorophyll) which are extracted by hot alcohol while preparing the samples for the determination of protein nitrogen but which are insoluble in hot water.

TABLE 11. COMPARISON OF VALUES FOR SOLUBLE NITROGEN COMPUTED FROM THE DIFFERENCE BETWEEN TOTAL AND PROTEIN NITROGEN WITH DIRECT DETERMINATIONS OF WATER-SOLUBLE NITROGEN

Data in grams per kilo of fresh weight.

Sample	Total nitrogen minus protein nitrogen	Soluble nitrogen	Difference
FA	2.61	2.35	0.26
FB	2.53	2.33	0.20
FC	2.57	2.44	0.13
FD	2.46	2.38	0.08
FE	2.46	2.39	0.07
FF	2.54	2.38	0.16
FG	2.49	2.47	0.02
FH	2.45	2.33	0.12
FI	2.34	2.20	0.14
FJ	2.32	2.24	0.08
Mean			0.13

Inasmuch as the total nitrogen is essentially constant, the curve for soluble nitrogen is very nearly symmetrical with that for protein nitrogen, and illustrates the rapid rate of conversion of insoluble protein into soluble components during the first six days. The soluble nitrogen reached a maximum at 12 days which was maintained for some time, but the last two observations indicate that a small loss subsequently occurred, possibly due to the evaporation of a little ammonia.

The soluble nitrogen fraction doubtless represents an extremely complex mixture of substances. In view of the rapid decomposition of the protein during the first six days, nearly half of the soluble nitrogen then present would be expected to consist of amino acids and their metabolic products. Regarding the nature of the soluble nitrogen present at the beginning, which amounts to about 1.5 gm. per kilo, about 0.1 gm. consists of amino nitrogen and represents the amino acids of the green leaves, 0.77 gm. is nitrate nitrogen and 0.17 gm. the nitrogen of nicotine, these accounting together for two-thirds of the whole. The remaining 0.5 gm. of soluble nitrogen is distributed among a large group of components present in small amounts such as adenine and choline, and includes the non-amino nitrogen of peptides and of basic and cyclic amino acids. An appreciable part of this residual nitrogen consists of substances that Frankenburg has found are strongly adsorbed on magnesium oxide when this reagent is used to render the extract alkaline for the distillation of ammonia. He has also found that some of this nitrogen can be precipitated by silicotungstic acid and presumably therefore is combined in essentially basic substances.

Ammonia, Amide and Amino Nitrogen

Table 9 shows the data for asparagine amide nitrogen, glutamine amide nitrogen, ammonia nitrogen, and the amino nitrogen of amino acids. In an additional column are shown the data for amino nitrogen after subtraction of the contributions from asparagine and glutamine. These figures therefore represent the quantities of amino acids other than the two amides. The data are plotted in Figure 4.

The most striking chemical event is the formation of asparagine, which became evident within two days after the leaves were picked and continued at a high rate for six days. The maximum was reached at about eight days. Synthesis of asparagine then rather suddenly ceased and the quantity of asparagine amide nitrogen subsequently diminished slightly. There was a little asparagine present in the leaves at the start, but the increase of 0.448 gm. of asparagine amide nitrogen in the first eight days represents the formation of 4.23 gm. of anhydrous asparagine per kilo. This amounts to 7.2 per cent of the organic solids present in the leaves at the end of the curing process.

The question of the origin of this asparagine is at once raised. If it is assumed that asparagine was derived from the protein as a direct product of enzymatic hydrolysis, one may calculate, since the protein nitrogen diminished by 1.26 gm. in this period and, accordingly, approximately 7.9 gm. of protein underwent digestion, that the part of the proteins of the leaves that was decomposed by the proteolytic enzymes must have contained about 53 per cent of aspartic acid. This is a highly improbable result: the highest aspartic acid content recorded by Lugg (15) for the proteins of healthy spermatophyte leaves is the equivalent of 8.8 per cent, although tobacco mosaic virus protein has been found by Knight (10) to yield as much as 13.5 per cent. Only a few of the seed globulins are recorded as yielding proportions of aspartic acid as high as this and no proteins of higher aspartic acid content have been reported. Thus it is extremely unlikely that the asparagine found in these

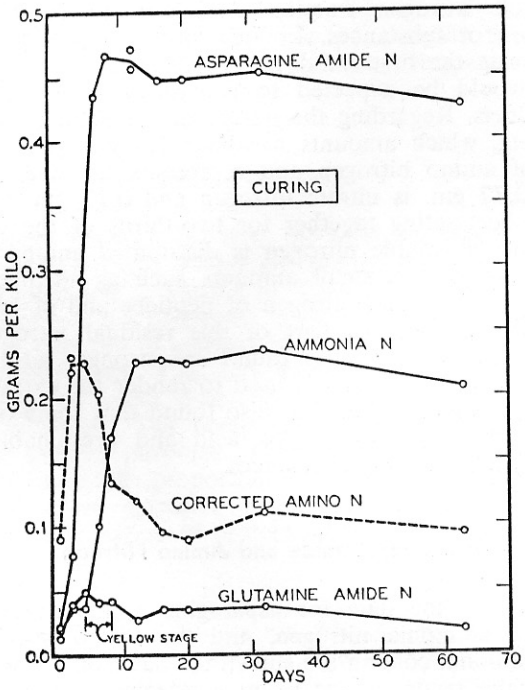

FIGURE 4

Amide, ammonia, and corrected amino nitrogen of the samples subjected to curing. The curve for corrected amino nitrogen represents amino nitrogen of amino acids other than asparagine and glutamine.

leaves after eight days of curing could have been a direct product of the digestion of the protein.

The alternative view, that the asparagine arose by a secondary synthetic process, is in conformity with the general theory of amide metabolism developed in 1898 by Schulze (30) with special reference to the metabolism of sprouting seeds and later extended by Prianischnikow (21) (see (44) for a full discussion). According to this view, the protein of the plant tissue undergoes decomposition to amino acids under the influence of proteolytic enzymes, and these substances are then oxidatively deaminized with the production of ammonia. The ammonia combines with a non-nitrogenous substance, which is today generally supposed to be oxaloacetic acid, to form aspartic acid and this substance finally combines with an additional mole of ammonia to give asparagine. Enzyme systems present in the cells catalyze each step in this process.

In view of the results of recent research (e.g. Elliott (3)) on the mechanism of the formation of glutamine in animal tissues, the suggestion may be advanced that the final step possibly involves a transfer of a phosphate radical to the β-carboxyl group of the aspartic acid to form β-aspartyl phosphate. The interaction of this with ammonia then leads to the synthesis of asparagine and the liberation of a phosphate ion.

Elliott holds that the over-all reaction of glutamate involves interaction with adenosine triphosphate according to the equation

$$\text{L-glutamate} + \text{ATP} + NH_3 \rightarrow \text{L-glutamine} + \text{ADP} + \text{phosphate ion}$$

Although the over-all reaction appears to have been established, the detailed mechanism via the formation of a mixed anhydride of phosphoric acid is still speculative since the occurrence of this reaction has not been demonstrated, the experimental observations being mainly confined to the demonstration of the formation of phosphate ion. The extension of this hypothesis to the case of asparagine formation in leaves is justified only by the possibility that the suggestion may give rise to experimental investigation.

Little or nothing is known of the ultimate fate of the residues of the amino acids which have undergone oxidative deamination, but evidence has been obtained in the case of rhubarb leaves subjected to culture in water in darkness (45) that such residues may form a part of the substrate of respiration. Meiss (16) has obtained evidence, for the case of etiolated lupine seedlings, that gives rise to the suggestion that amino acid residues contribute to the formation of the oxaloacetic acid required for the synthesis of asparagine.

It is not impossible that some similar type of reaction occurs in the tobacco leaf. The data in Table 9 show that 4.23 gm. of asparagine were synthesized during the first eight days. Since the molecular weights of oxaloacetic acid and asparagine are both 132, 4.23 gm. of oxaloacetic acid would therefore be required and it is justifiable to search through the analytical data for a possible source of this much material.

It is known that carbohydrates can be metabolized in certain animal tissues by the reactions of the Krebs cycle into oxaloacetic acid, one mole of glucose yielding at the maximum one mole of this substance. The data for the changes in the quantities of carbohydrates in the present samples of tobacco leaves are shown on later pages in Table 14 and for starch in Table 15. A rough estimate of the total quantity of simple carbohydrate that disappeared during eight days of curing is 2.3 gm. and of starch 0.48 gm. If it is assumed, for the sake of the argument, that all of this was transformed into oxaloacetic acid to support the synthesis of asparagine, the maximal amount that could have been provided would be 2.03 gm. Accordingly, some other source for the remaining 2.2 gm. of oxaloacetic acid required must be found.

On general grounds, the most likely other source would be the residues of the amino acids that remain after the deamination process. It has already been shown that 7.9 gm. of protein underwent digestion during the first eight days of curing. This should yield 6.6 gm. of nonnitrogenous residues as an outside estimate. It is now necessary to assume that metabolic transformations of these residues can be brought about such that at least 2.2 gm. of oxaloacetic acid would be made available. This is not an unreasonable assumption. The part of the decomposed protein that consisted of aspartic acid, possibly 10 per cent of the whole, would already be present as oxaloacetic acid and many enzyme reactions have been detected in recent years whereby other amino acids can be converted into substances which are well-known substrates of the respiratory cycle as it is known to operate in certain animal tissues. If analogous enzyme systems are present in tobacco leaf, a reasonable hypothesis of the

source of the non-nitrogenous precursor of the asparagine can be advanced. It should be pointed out, however, that if the carbohydrate and a part of the protein residues were employed for this purpose, equivalent quantities of other components of the tissue must have been used in respiration and converted into volatile products in order to supply the energy and account for the loss of more than 10 gm. of organic solids from the system.

Consideration of the curves shown in Figure 4 shows that the data conform in every particular with what would be expected if the asparagine were synthesized in accordance with the sequence of reactions that has been outlined. The prompt formation of asparagine is accompanied during the first two days by a parallel formation of amino nitrogen in excess of the amino nitrogen of the asparagine itself as is shown by the curve for "corrected amino nitrogen." This implies that more than sufficient amino nitrogen was produced in this interval of time to supply the required nitrogen. Synthesis of asparagine continued as the corrected amino nitrogen reached a maximum that was held constant for the next two days. The corrected amino nitrogen then diminished, slowly at first as asparagine synthesis continued, and it had dropped almost to its initial level at the time that the asparagine amide nitrogen reached its maximum. The relationships between the two curves are such as to indicate an initial production of amino nitrogen at a rate greater than that at which it was used. During the third and fourth days of curing, synthesis of asparagine became so rapid that the level of corrected amino nitrogen remained constant and, subsequently, the rate of production of amino nitrogen was unable to keep up with the rate at which it was used; the curve for corrected amino nitrogen accordingly fell. At the end of eight days, the formation of asparagine amide nitrogen abruptly ceased but the amino nitrogen continued to decrease although more slowly.

The explanation of the fate of the amino nitrogen which disappeared between the 8th and the 12th day is to be found in the behavior of the ammonia nitrogen. Ammonia in the form of ammonium ion accumulated sluggishly during the first four days; the shape of the curve suggests that it was being utilized almost as soon as it was liberated. It then began to rise rapidly although not nearly so fast as the curve for asparagine amide nitrogen. When asparagine synthesis ceased at the expiration of eight days, ammonia still continued to increase although more slowly. This was accompanied by a slow diminution of the corrected amino nitrogen. It would therefore appear that the reactions that led to oxidative deamination of the amino acids were able to continue for a few days longer than the reactions that led to the synthesis of asparagine. However, perhaps the most striking observation is that nearly all chemical reaction ceased at the end of 12 days; save for some small irregularities in the curve for corrected amino nitrogen, the composition of the tissues with respect to asparagine, ammonia, amino acids, and glutamine subsequently remained constant for several weeks. Only toward the end of the curing process is there evidence for small additional losses of these components.

It seems extremely likely that the continuation of the complex series of rapid reactions that occurred in the first eight days of curing is dependent upon the maintenance of the organization of the cells and is associated with the presence of the respiratory process. When respiration ceased, the processes which led to amide metabolism also ceased. It may therefore be inferred that the support of the amide metabolism is dependent

upon respiration for the necessary supply of either the substrate or of the energy required for the synthesis of asparagine. Both requirements probably need to be satisfied.

The chemical conditions under which these reactions occurred must have been extremely complex. The system as a whole was undergoing dehydration at a rate that increased the concentration of the reactants by a factor of 2 at the end of four days and by a factor of 5 at the end of eight days. Although this situation may well have influenced the velocity and extent of some of the reactions, it is one that makes extremely difficult any inferences based upon concepts of equilibrium between reactants. It is clear that the integrity of the cells as living units was largely destroyed at the end of eight days; after 12 days, the system had degenerated into a disorganized mixture of organic and inorganic substances of the utmost complexity but with too little water remaining to permit enzymatically catalyzed reactions to proceed at more than minimal velocity if at all.

The curve for the corrected amino nitrogen shows that only small quantities of amino acids, other than asparagine and glutamine, were present at the termination of the reactions that led to the formation of asparagine. The 0.095 gm. of amino nitrogen that remained after 16 days represents only a little over 7 per cent of the 1.29 gm. of protein nitrogen that had been decomposed in the tissues during this period. Inasmuch as the leaf protein could be expected to yield between 60 and 70 per cent of its nitrogen as amino nitrogen after complete hydrolysis, the intensity of the oxidative attack upon these amino acids as soon as they were formed is evident. The specificity of the oxidative deamination reactions is also interesting. Why, if amino acids were being oxidized as extensively and rapidly as this, do asparagine and to a lesser extent glutamine survive? Such evidence as is available suggests that asparagine is dissolved as such in the sap of the cells so that no hypothesis of a specific protective mechanism owing to some obscure form of combination can readily be invoked. Accordingly, one is inclined to suspect that the hydrolytic and oxidative reactions are in some way closely associated with each other both in time and space; the product of the reactions, ammonia, then becomes available for the subsequent synthesis of asparagine under the influence of another enzyme system possibly located elsewhere in the cell.

The formation of a small proportion of glutamine during the first few days of the curing process may but probably does not represent a secondary process of synthesis. The maximal amount of glutamine amide nitrogen formed, about 0.040 gm. after eight days, represents 3.2 per cent of the 1.26 gm. of protein nitrogen that had been hydrolyzed in this interval. If it is assumed that glutamine is a direct product of hydrolysis, the quantity actually formed would account for 6.4 per cent of the protein nitrogen; that is, twice the increase in the quantity of glutamine amide nitrogen observed since glutamine contains two atoms of nitrogen. Although there are no data available on the quantity of glutamic acid yielded by tobacco leaf protein, Lugg (15) has given estimates for a group of proteins derived from leaves of higher plants that range from 6 to 7 per cent of the protein nitrogen as glutamic acid nitrogen. Accordingly, if all of the glutamic acid these proteins contain were combined as glutamine, they would thus have from 12 to 14 per cent of their

nitrogen in this form. Clearly, therefore, it is quite possible that the glutamine that was produced during the hydrolysis of the proteins of the curing tobacco leaves may have arisen as a primary product of hydrolysis. If these assumed figures are of approximately the correct magnitude, they suggest that about one-half of the glutamine liberated from the protein survived the processes of hydrolysis and oxidative deamination. If this is so, the unusual position occupied by the amides of the dicarboxylic amino acids is again emphasized. It is by no means clear why a substantial fraction of the glutamine survived when more than 85 per cent of the rest of the amino acids produced by hydrolysis were destroyed.

In Figure 5 the several forms of soluble nitrogen are plotted in a somewhat different manner and data for nitrate nitrogen and for amino nitrogen after acid hydrolysis are also given. Furthermore the results of the examination of the leaves subjected to fermentation are included. These data will be discussed in detail in a later section.

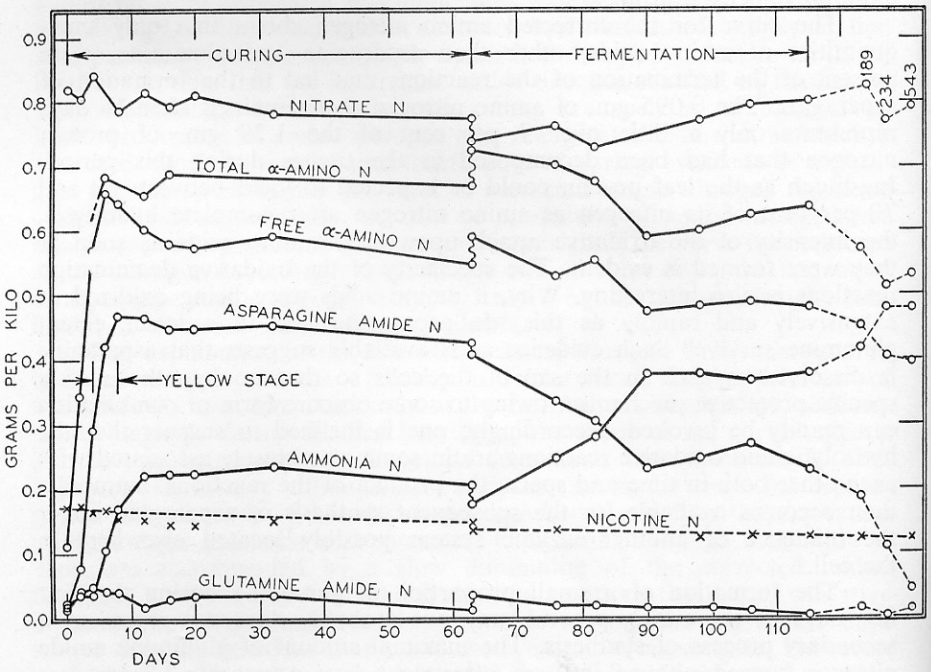

FIGURE 5

Forms of soluble nitrogen in cured samples and also cured and fermented samples. For explanation of discontinuity of the curves at 63 days, see legend of Figure 3 and text. The discontinuity of the time scale due to the periods of storage, mulling, and storage in the bale is indicated by the broken lines at the right of the drawing. The elapsed time at each of these operations is indicated on the uppermost curve.

Nitrate Nitrogen

Nitrate is clearly the major form of soluble nitrogen present in the samples of tobacco leaves; it makes up 53.6 per cent of the soluble nitrogen of the fresh leaves and the presence of so large a proportion is

evidence of the liberal application of nitrogenous fertilizer characteristic of the system of agriculture used for the production of this type of tobacco. The plants are obviously grown with a luxury supply of nitrogen.

Disregarding the minor irregularities which arise mainly from the inaccuracies of the analytical method, the curve (Figure 5; data in Table 9) suggests that a small proportion (of the order of 3 per cent) of the nitrate present at the start may have disappeared during the curing process. There is no indication whatever that synthesis of a substantial proportion of nitrate nitrogen occurred during the first few days as was observed with the crop grown in 1931 (43). The great improvement in sampling methods since that early work suggests that the observation of an increase in nitrate, although this was noted both during curing and during culture of similar leaves in water in darkness, may have been in error. The present experiments are the first that have been carried out in which adequate sampling methods were used and the result to which they point seems unequivocal: the nitrate changes little if at all during the process of curing tobacco leaves under the usual conditions. The data that are plotted are averages of values obtained at least in triplicate and, although the precision leaves something to be desired, the results fall into a consistent pattern; there is at most only a slight loss of nitrate nitrogen.

Uncorrected Free Amino Nitrogen

The curve in Figure 5 for free amino nitrogen shows the quantities of amino nitrogen of the amino acids together with the amino nitrogen of the asparagine (equal to the asparagine amide nitrogen) and that of the glutamine. Because of the small quantity of glutamine amide nitrogen present, no correction has been applied to the data to allow for the fact that most of the glutamine amide nitrogen is decomposed in the nitrous acid procedure used for the determination of amino nitrogen and thus appears as amino nitrogen. The sharp rise of the curve in the first six days of curing corresponds with the rapid decomposition of the protein in this interval. The maximum was approximately maintained for two days and the amino nitrogen then slowly decreased for a few days, presumably owing to oxidative deamination with the production of ammonia. After the 12th day, there was little further change in amino nitrogen, and both the ammonia and the asparagine amide nitrogen likewise remained essentially constant.

Peptide Nitrogen

The curve for total amino nitrogen represents the quantity of amino nitrogen present after hydrolysis of the extract with 6 *N* sulfuric acid. The difference between this curve and that for free amino nitrogen presumably represents nitrogen present as the peptide groups of peptides. Disregarding minor irregularities in the data, it seems probable that a quantity of the order of magnitude of 0.1 gm. of nitrogen was present in this form at the time that hydrolysis of the protein ceased and that the substances represented by this quantity remained essentially unchanged in amount throughout the rest of the period of curing. In fact, as is obvious from the curves in Figure 5, a closely similar quantity of material with

the same chemical properties was present in the samples subjected to fermentation and changed little if at all in amount during this process. If this interpretation is correct, it would suggest that enzymes capable of hydrolyzing peptide groups became completely inactivated at the end of 12 days of curing.

Nicotine Nitrogen

Data for the quantities of nicotine nitrogen in the samples are plotted in Figure 5, the points being shown by crosses distributed about a straight broken line. We are indebted to Dr. W. G. Frankenburg for these figures (see p. 86). The general slope of the curve suggests that about one-seventh of the nicotine present in the green leaves had disappeared at the end of the curing period and about one-quarter at the time that the fermentation process had been completed. Tobacco leaves of the type used in these experiments are low in nicotine content. The fresh leaves contained 0.17 gm. per kilo of nicotine nitrogen or the equivalent of 1.14 per cent of the dry weight as nicotine itself. The disappearance of a part of the nicotine in the course of the processing of the tobacco leaf is a well-known characteristic. Some of the loss is possibly due to evaporation from the surfaces of the leaves but chemical change of nicotine into other substances, especially during fermentation, has been demonstrated to occur in other types of tobacco. With some types the destruction is nearly complete. Study of the details of the process has not yet been attempted with the present material.

Changes in Acidity during Curing

Table 12 shows the pH values obtained when 100 mg. of the dry tissue are suspended in 2.5 ml. of water and tested with the glass electrode. The data are averages of duplicate or triplicate determinations which agreed within a few hundredths of a pH unit. Precautions such as subjecting the suspension to a vacuum in a desiccator to remove all traces of air and to ensure complete wetting of the sample made no significant difference in the results. The observations are plotted in Figure 6.

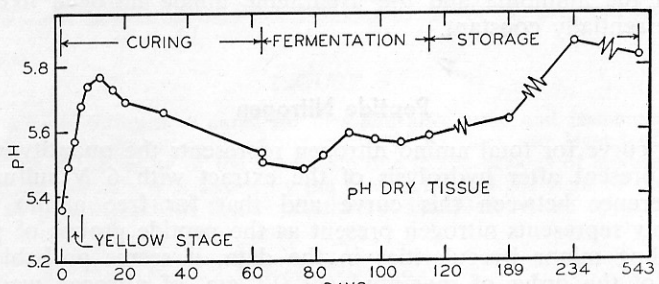

FIGURE 6

Changes in pH during curing, fermentation, and storage. The discontinuity in time scale for the last operations is indicated by zigzag lines.

The outstanding change is the prompt increase of about 0.4 units in pH during the first phase of the curing operation. The change suggests a partial neutralization of the acidity of the tissue, and examination of the data that might bear on this point shows that approximately 0.2 gm. of ammonia nitrogen were set free in the first 12 days; this is 14 m.eq. A titration with 0.1 *N* sodium hydroxide of a suspension of tissue from the fresh leaf sample showed that 12.4 m.eq. of alkali were required to bring the pH from an initial 5.36 to 5.76, a change identical with the one that occurred during the first 12 days. Accordingly, it seems possible that the initial sharp rise in pH may have been the result of a simple neutralization process owing to the liberation of ammonia in the course of the metabolic reactions that occurred in this interval.

However, this interpretation overlooks the possibility that there was a contemporaneous change in the total ether-soluble organic acids. Table 12 indicates that the acids apparently decreased by approximately 22 m.eq. in the first 12 days, mostly during the first four. The alkalinity of the ash remained constant so that such a change might be expected to have an effect upon the pH of the extract of the tissue. Whether or not this played a role remains uncertain.

The gradual drop in pH from the 12th to the last day of curing is difficult to account for. Metabolic reactions which liberate ammonia had ceased and both ammonia and the organic acids remained essentially constant throughout this period. The reaction which brought about the change is apparently a slow liberation of a significant quantity of acid and one possibility is that the demethylation of esters of pectic acid may have occurred. Further investigation will be required to test the validity of this view.

Alkalinity of Ash

The data for the alkalinity of the ash (Table 12) are plotted in Figure 7. Within certain limitations, this quantity is a measure of the organic acids present in the leaves at the time of collection, for it represents the sum of the carbonates and oxides that remain when the salts of the organic acids in the tissue are burned. The mean value for the set of 10 samples was 296.8 m.eq. and the coefficient of variation was only 2.7 per cent. This figure includes both the sampling error and the analytical errors involved in preparing the ash and titrating it. Because of the large scale adopted for plotting the data, the irregularities are somewhat unduly emphasized in the figure. It will be recalled that the data for the weight of the ash in Figure 2 when plotted gave a horizontal straight line. The coefficient of variation of the ash weights was 1.5 per cent (see Table 7) which is unusually small for data on samples such as these.

It is clear that none of the metabolic changes that occur during the curing operation can affect the distribution of the inorganic components of the ash. These changes may and obviously do alter the acid-base relationships in the cells so that the pH of extracts may change. However, when all of the organic material has been burned away, the remaining inorganic basic components are constant in quantity and the inorganic anions such as phosphate, sulfate, and carbonate are likewise fixed. The titration value should thus also be constant and the experiment shows that this is so within the limits of the accuracy of the measurement; a

TABLE 12. ACIDITY AND ORGANIC ACIDS

Data expressed in milliequivalents per kilo of fresh leaf.

Sample	Days cured	pH	Alkalinity of ash	Total organic acids[1]	Citric acid	Malic acid	Oxalic acid	Undetermined acid
A1	0	5.36	304	309	26.7	159	36.2	88
B1	2	5.49	301	307	38.6	142	37.9	88
C1	4	5.57	281	284	64.0	95.4	42.3	82
D1	6	5.68	300	283	84.3	83.1	41.7	74
E1	8	5.74	298	273	92.1	74.4	41.1	66
F1	12	5.77	303	286	98.8	65.0	43.5	79
G1	16	5.73	290	278	98.9	67.9	40.7	70
H1	20	5.69	287	282	96.0	65.5	43.8	77
I1	32	5.66	304	291	103.0	72.0	44.0	72
J1	63	5.53	300	281	100.8	65.3	43.2	72
Mean			296.8					
S.D.			± 8.0					
C.V. (%)			2.7					

[1] The term "total organic acids" as used in this bulletin refers to the total ether-extractable organic acids.

better picture of the events would perhaps be provided if the alkalinity of the ash were represented by a horizontal straight line placed at the average value of 297 m.eq.

Total Ether-Soluble Organic Acids

The data in the fourth column of Table 12 give the titration values of the total ether-extractable acidity of the tissues; they are plotted in Figure 7. This quantity is one that is difficult to measure accurately and the scale employed emphasizes the irregularities. Nevertheless, examination of the figures indicates that the total organic acidity, which was about 309 m.eq. in the leaves at the time of collection, dropped to about 280 m.eq. in the first four days of the curing period, and then remained substantially constant. The mean value of the acidity in the eight samples collected from the fourth day to the end of curing was 282 ± 5.3 m.eq., the coefficient of variation being only 1.9 per cent. The precision of this particular set of determinations is considerably higher than is usually attained in titrations of total organic acidity and it seems clear that reactions took place during the first few days that led to the disappearance of roughly 9 per cent of the ether-soluble organic acids. The situation then apparently became stabilized and no further change occurred in the *total quantity* of organic acids present. This interpretation of the observations is not unreasonable. It was during the early period that metabolic reactions were proceeding with high velocity, and dehydration of the tissues had not become excessive by the time the apparent destruction of organic acids ceased. Accordingly, comparison may be made with observations upon tobacco leaves subjected to culture in water in darkness. In experiments carried out with the crop of 1934 (44), leaves cultured in

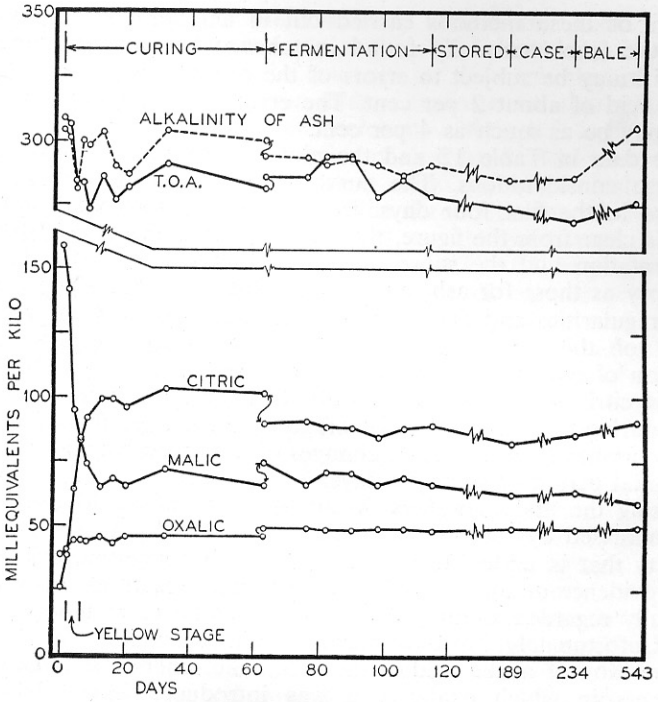

FIGURE 7

Alkalinity of ash (broken line) and organic acids of the cured and the cured, fermented, and stored samples. Discontinuities in scales are indicated by zigzag lines.

water or in glucose solution in darkness underwent only moderate change in the total organic acidity for the first two days but subsequently showed evidence for losses of the order of 10 to 15 per cent. Because of the inadequate sampling method used at that time, the accuracy of these early observations is not high and comparison in detail with the present data is scarcely justified. However, it seems probable that tobacco leaves may lose an appreciable fraction of their organic acids if cultured in darkness for periods substantially in excess of two days. It is important to note, nevertheless, that the change in the first two days is usually small and is often negligible. This is true of the present data as well.

Oxalic, Malic, and Citric Acids

In considering the data for the individual organic acids and the changes that they underwent during curing, it is necessary to have in mind the accuracy with which the several determinations can be made. The method for oxalic acid is simple and highly accurate, that for citric acid is complex although moderately accurate, but that for malic acid is complex and least accurate of the three. An examination of the analyti-

cal errors of these methods carried out in another connection suggests that, at the levels normally found in tobacco leaves, determinations of malic acid may be subject to errors of the order of 5 per cent and those of citric acid of about 2 per cent. The error of the titration of the total acidity may be as much as 4 per cent.

The data in Table 12 and the plots in Figure 7 are in conformity with these considerations. The curve for oxalic acid, disregarding the small rise in the first four days, is practically a horizontal straight line and, as is clear from the figure, there is no change throughout the process of fermentation and the subsequent period of storage. The data are as satisfactory as those for ash in Figure 2. The curve for citric acid shows minor irregularities and that for malic acid shows somewhat greater ones. In spite of this, however, the data as a whole suggest that, after completion of the major changes that occurred in the first 12 days, both malic and citric acids remained essentially constant and changed but little if at all during fermentation and storage of the leaves. It is clear that the more important of the metabolic changes of the organic acids are restricted to the initial period of some 12 days.

During the first four days of curing, the oxalic acid content of the tissues increased by some 6 m.eq. (0.27 gm.) or by nearly 16 per cent, a quantity that is undoubtedly greater than the experimental error. The effect is evidence of somewhat intense oxidative reactions, for oxalic acid is generally regarded as an end product of oxidative metabolism in plant tissues. Unfortunately, however, there is little information in the literature on the position of oxalic acid in the metabolic scheme. It is known, from experiments in which oxalic acid was introduced into tobacco leaves in culture solution (36), that this acid is not connected by reversible enzymatic reactions with the system in which malic and citric acids are normally involved. Nevertheless, that an enzyme is present in tobacco leaves which oxidizes glyoxylic acid to oxalic acid has recently been demonstrated by Kenten and Mann (9). Hydrogen peroxide is produced at the same time and the enzyme therefore has properties analogous to those of an enzyme known to be present in mammalian liver. In the present case, simple carbohydrates were undergoing rapid decomposition during the first few days of the curing process as will be seen from data to be presented on a later page. That glyoxylic and, subsequently, oxalic acid may have been produced in small proportion in the course of these reactions is quite possible but no direct evidence in favor of this view is at present available.

The apparent indifference of oxalic acid to chemical reaction during the curing process is probably related to the form in which it is present in the leaves. Experiments in which both fresh and cured tobacco leaves were extracted with boiling water (42) have shown that from 80 to more than 92 per cent of the oxalic acid remained insoluble, the higher values being observed with cured leaf. Although indirect, the evidence points strongly to the probability that oxalic acid is normally combined in the leaf as insoluble calcium oxalate and it is for this reason that Frankenburg places oxalic acid among the "static" components of the tissue. In the present case, the analysis of the ash has shown that there were on the average 2.34 gm. of calcium per kilo of fresh weight of these samples of leaves. This is 117 m.eq. or nearly three times as much as is required to combine with all of the oxalic acid the leaves

contained. Accordingly, it would seem that little of the oxalic acid would be present in a form soluble in water at the pH of the tissues. Nevertheless, it should be pointed out that the evidence on the extraction of the oxalic acid was obtained on killed tissue under circumstances that would permit interaction between oxalic acid and calcium. Although it is likely that the cells do contain a substantial proportion of their oxalic acid content in combination with calcium, this proportion may not be as high while the cells are still alive as is indicated by the determinations of the solubility of the oxalic acid at the pH of the dried and ground tissues.

The data for citric and malic acid in Table 12 and the plot in Figure 7 illustrate one of the most interesting reactions of organic acids that has been observed in tobacco leaves. During the first 12 days of curing, the quantity of malic acid dropped from 159 m.eq. to 65 m.eq. or by 94 m.eq. This is equivalent to the disappearance of 6.3 gm. of malic acid, a quantity which amounts to almost 9 per cent of the organic solids present in the tissues at the start. The change involves 30 per cent of the total ether-soluble organic acids present. In the same period, citric acid increased from 26.7 m.eq. to 98.8 m.eq. or by 72.1 m.eq. or the equivalent of 4.61 gm. This is also a substantial proportion of the organic solids of the tissues and of the total organic acidity. Even more striking is the obvious chemical relationship between these figures. Malic acid diminished by 47 millimoles (one-half of 94) while citric acid increased by 24 millimoles (one-third of 72). The ratio between these quantities is almost exactly 2 and the evidence points to the possibility that for every two moles of malic acid that disappeared one mole of citric acid made its appearance; in other words, that malic acid underwent transformations such that two moles were used up in the formation of one mole of citric acid. It is important to note that during the entire course of the curing process the total acidity due to ether-soluble organic acids changed to only a negligible extent, in comparison to the changes in malic and citric acids.

The occurrence of this reaction in tobacco leaves subjected to culture in water in darkness was first demonstrated in 1937 (25) and it has since been studied in considerable detail. Although nothing definite is yet known regarding the enzyme mechanisms whereby the transformation is brought about, the reaction has been shown to involve an equilibrium or series of equilibrium reactions, for the change can be reversed by culturing tobacco leaves in 0.2 *M* solutions of potassium citrate in darkness (37). Furthermore, the formation of citric acid can be stimulated by culture of the leaves under similar conditions in L-malate or succinate (38) and also by culture in fumarate, pyruvate, or L-lactate (23). Even when *d*-isocitrate, an acid that has not hitherto been demonstrated to be present in leaves of this species, is administered to tobacco leaves (35) an increase in citric acid occurs and the loss of malic acid is diminished. In general, culture of tobacco leaves in solutions of alkali salts (either potassium or sodium) of any acid that is a member of the Krebs tricarboxylic acid cycle leads to a stimulation of the formation of citric acid; furthermore, culture in solutions of certain other related substances has the same effect. However, there are strict limitations to the scope of the reaction; culture in tartrate or malonate brought about no increase in citric acid greater than that in the control (23) nor did culture in oxalate (36). None of these substances appears to be able to enter into the

series of enzymatic equilibrium reactions which affect the relative proportions of citric and malic acids in this tissue.

That an increase of citric acid occurs in tobacco leaves subjected to the curing process under normal conditions is not a new observation. In experiments carried out with leaves from the crop of 1929 (42), it was shown that citric acid may increase as much as six-fold although the concomitant loss of malic acid was not detected at that time, probably owing to the inadequacy of the methods then available. The increase of citric acid has also been observed by Pyatnitskii (26). The present data are, however, unequivocal; during the early phases of curing, a transformation occurs that seems to be in every way analogous to the transformation that takes place when tobacco leaves are subjected to culture in water in darkness. The enzyme systems that bring about this reaction or series of reactions are clearly active for the first 12 days, that is to say during and perhaps even a little beyond the period when respiration as evidenced by the loss of organic solids was still detectable.

Discussion of the possible enzyme systems of tobacco leaf tissue that may bring about this change is premature at the present time. However, the nature of the chemical reactions that occur can perhaps be made clearer if consideration is given to the structure of the substances involved and to the enzyme systems in other tissues to which the formation of citric acid is attributed. The fundamental reaction is the mechanism whereby malic acid, a substance that contains four carbon atoms, is converted into citric acid, a substance that contains six carbon atoms. Accordingly, attention must be given in the first place to reactions whereby malic acid can be converted into a four-carbon substance which can undergo such a reaction. Secondly, the nature of the two-carbon fragment which combines with the four-carbon substance must be sought.

The four-carbon substance which undergoes condensation in such a way as to lead to the formation of citric acid has been held to be oxaloacetic acid ever since the fundamental work of Knoop and Martius (11) in 1936. An enzyme mechanism whereby malic acid can be converted into oxaloacetic acid is today well known. It is a typical dehydrogenation reaction and is set forth by Stern and Ochoa (32) as follows:

$$\underset{\text{L-Malic acid}}{COOH\text{-}CH_2\text{-}CH(OH)\text{-}COOH} + \underset{\substack{\text{Diphosphopyridine nucleotide}\\ \text{(oxidized)}}}{DPN_{ox}} \rightleftharpoons \underset{\text{Oxaloacetic acid}}{COOH\text{-}CH_2\text{-}CO\text{-}COOH} + \underset{\substack{\text{Diphosphopyridine nucleotide}\\ \text{(reduced)}}}{DPN_{red}}$$

The reaction proceeds in the presence of the enzyme malic dehydrogenase which is found in many tissues (e.g. pig heart muscle). Ordinarily, the equilibrium position of the reaction is far in the direction of the reduction of oxaloacetic acid to malic acid, but, if another mechanism is present in the system which uses oxaloacetic acid, the reaction can proceed to the right.

The nature of the substance which provides the two-carbon fragment which combines with oxaloacetic acid to form citric acid was for a number of years a puzzle. It was early supposed to be pyruvic acid (CH_3-CO-COOH) (13, 14) and the product of the condensation of pyruvic acid

with oxaloacetic acid was supposed to be subsequently oxidized to citric acid somewhat as follows:

$$\underset{\text{Oxaloacetic acid}}{COOH\text{-}CO\text{-}CH_2\text{-}COOH} + \underset{\text{Pyruvic acid}}{CH_3\text{-}CO\text{-}COOH}$$

$$\rightarrow COOH\text{-}C(OH)(CH_2\text{-}CO\text{-}COOH)\text{-}CH_2\text{-}COOH \xrightarrow{O} \underset{\text{Citric acid}}{COOH\text{-}C(OH)(CH_2\text{-}COOH)\text{-}CH_2\text{-}COOH} + CO_2$$

This particular reaction is the one observed by Knoop and Martius (11) and takes place at room temperature in alkaline solution without the intervention of a catalyst. The oxidation step was carried out with hydrogen peroxide.

The present-day view of the sequence of reactions holds that the oxidation step takes place before the condensation and that the two-carbon fragment is accordingly a form of acetic acid so activated that reaction takes place at the methyl group. This activated form is, according to Novelli and Lipmann (18), acetyl phosphate which can be produced from acetate and adenosinetriphosphate (ATP) by an enzyme (acetate-ATP enzyme) present in extracts of *Escherichia coli,* adenosinediphosphate (ADP) being the by-product of the reaction

$$\underset{\text{Acetic acid}}{CH_3\text{-}COOH} + ATP \rightarrow \underset{\text{Acetyl phosphate}}{CH_3\text{-}CO\text{-}O\text{-}PO(OH)_2} + ADP$$

Acetyl phosphate then reacts with a sulfhydryl group present in the widely distributed substance coenzyme-A to produce acetylcoenzyme-A, the enzyme which catalyzes this reaction being a transacetylase which is known to be present in an extract of *Clostridium kluyveri* (31).

$$CH_3\text{-}CO\text{-}O\text{-}PO(OH)_2 + CoA \rightarrow \underset{\text{Acetylcoenzyme-A}}{CH_3\text{-}CO\text{-}S\text{-}CoA} + H_3PO_4$$

The final step of condensation of acetylcoenzyme-A with oxaloacetic acid takes place under the influence of the recently described condensing enzyme of Stern and Ochoa (32), an enzyme that has been isolated in crystalline form by Ochoa, Stern and Schneider (19) from pig heart muscle and which has been shown to be present in a number of other animal tissues as well as in yeast.

$$CH_3\text{-}CO\text{-}S\text{-}CoA + COOH\text{-}CO\text{-}CH_2\text{-}COOH \rightarrow COOH\text{-}C(OH)(CH_2\text{-}COOH)\text{-}CH_2\text{-}COOH + CoA$$

This series of enzyme reactions has been extensively studied by Ochoa and his collaborators in the past few years, and artificial systems have been devised which will catalyze the formation of citric acid from oxaloacetic acid or from malic acid. For example, a system buffered with phosphate at pH 7 and containing manganese chloride, cysteine, ATP,

coenzyme-A, an extract of *E. coli* and an extract that contains the condensing enzyme will rapidly convert oxaloacetate to citrate or will convert a mixture of pyruvate and L-malate to citrate. The system is thus able to convert L-malate into oxaloacetate as well as to bring about the condensation to citrate. Proof that the sequence of reactions in the systems studied is essentially that described has been provided by Stern, Shapiro, Stadtman and Ochoa (33) and the equilibrium position has been shown to be markedly in favor of the formation of citric acid.

In order that these considerations may have a bearing on the problem of the formation of citric acid from malic acid in tobacco leaves, it is also necessary to develop a mechanism whereby the necessary acetyl group may be derived from L-malic acid. Since L-malic acid can be oxidized to oxaloacetic acid, one possibility is the decarboxylation of oxaloacetic acid to yield pyruvic acid:

$$COOH\text{-}CO\text{-}CH_2\text{-}COOH \rightleftharpoons CH_3\text{-}CO\text{-}COOH + CO_2$$

This reaction is catalyzed by oxaloacetic carboxylase, an enzyme found in bacteria and in animal liver. Pyruvic acid can then act as the acetyl group donor by a reaction that is known to take place in the presence of extracts from *E. coli* if diphosphopyridine nucleotide (DPN) is added. The reaction has been studied by Korkes, del Campillo, Gunsalus and Ochoa (12). It proceeds in two steps

$$\text{Pyruvate} + \text{phosphate} + \text{DPN} \rightarrow \text{Acetyl phosphate} + CO_2 + \text{DPNH}$$

$$\text{Pyruvate} + \text{DPNH} \rightarrow \text{Lactate} + \text{DPN (lactic dehydrogenase)}$$

which add to give the reaction

$$2\ \text{Pyruvate} + \text{phosphate} \rightarrow \text{Acetyl phosphate} + CO_2 + \text{lactate}$$

If oxaloacetate and condensing enzyme are also present, this reaction would lead to the formation of citrate.

It is clear that enzyme reactions are well known today whereby malic acid can be converted into citric acid. However, these reactions have been studied mainly by employing extracts or concentrates of a variety of tissues. A demonstration has yet to be obtained in which all of the essential enzymes and co-factors have been derived from a single tissue, whether of animal or bacterial origin, and in no case has the demonstration been made in an experiment in which an artificial system has been set up the components of which were all derived from leaf tissue. Nevertheless, the analytical evidence that this reaction takes place smoothly and rapidly in tobacco leaves is most impressive, and final proof that the citric acid produced is in fact derived from malic acid has been obtained by Zbinovsky and Burris (47). These authors have infiltrated L-malic acid labeled with C^{14} into tobacco leaves and have examined the labeling of the acids isolated from the leaf after four hours in darkness. Citric acid was found to be strongly labeled leaving "no question that carbon from malic acid can be used in the synthesis of citric acid in the darkened tobacco leaf." Obviously, however, the exact nature of the enzyme systems involved, and especially the explanation of the observation that two moles of malic acid appear to be used for the synthesis of one

mole of citric acid, *with no significant change in total acidity,* remain for future research. When these matters have been worked out, there is little doubt that a great deal of light will be shed upon the mechanism of respiration in green leaves.

Lipides

Table 13 and Figure 8 show the data for the lipides of the samples subjected to curing. Two different methods were employed. The A.O.A.C. method (1)[1] involves extraction with ethyl ether after the analytical sample (2 gm.) has been thoroughly dried in a vacuum; the extract is evaporated and the residue of oil is weighed. The apparatus and technique used for the analysis of feeds were employed. As an additional control, samples were extracted with petroleum ether by a slightly different technique, the extracted oil being also weighed. The results of the two methods were closely similar. The data obtained by the A.O.A.C. method are plotted as a broken line in Figure 7, the data from the extraction with petroleum ether as a solid line.

TABLE 13. LIPIDES AND CRUDE FIBER

Data in grams per kilo of fresh weight.

Sample	Days cured	Lipides		Crude fiber
		A.O.A.C. method	Petroleum ether-soluble	
A1	0	5.16	5.12	7.36
B1	2	4.26	4.77	7.31
C1	4	3.41	3.60	7.02
D1	6	3.19	3.04	7.25
E1	8	...	2.75	6.91
F1	12	2.38	2.71	7.22
G1	16	2.57	2.80	7.28
H1	20	2.74	2.64	7.10
I1	32	2.80	2.58	7.44
J1	63	2.58	2.49	7.43
Mean				7.23
S.D.				±0.17
C.V. (%)				2.4

The oils obtained from samples A and B were somewhat acrid and unpleasant in odor and were dark green in color owing to the presence of chlorophyll. The odor changed progressively through the series, becoming sweet and pleasantly aromatic, and the color also changed; the oil from sample D was yellow with a slight green tinge and the subsequent samples were clear orange yellow.

[1] We are indebted to the Department of Analytical Chemistry for the determinations by the A.O.A.C. method.

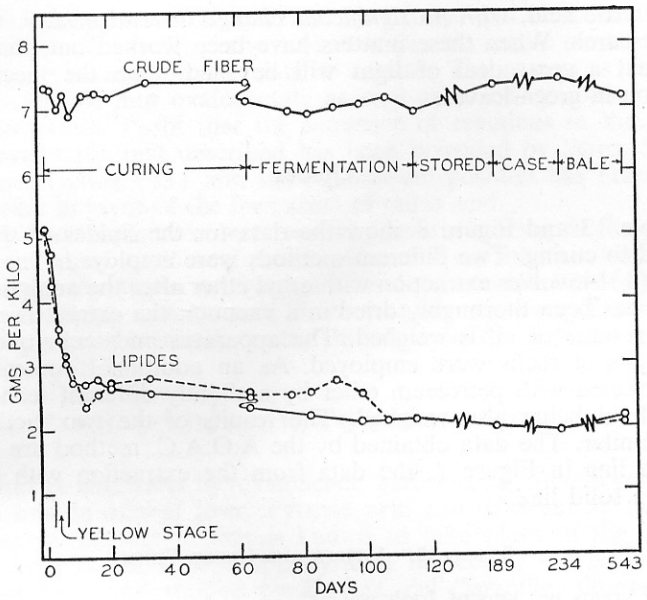

FIGURE 8

Crude fiber and lipides of the cured and the cured, fermented, and stored samples. Discontinuity in time scale is indicated by zigzag lines.

The curves show that about one-half of the ether-soluble material present in the green leaves underwent chemical change during the first eight days of curing such that the products were no longer soluble in the fat solvent. The changes in color both of the leaves and of the extracts indicate that a part of this effect can be attributed to the destruction of chlorophyll with the production of ether-insoluble substances.

The content of ether-soluble extractives in this lot of samples of tobacco leaf was considerably lower than that found in the samples from the crop of 1929 studied earlier in this laboratory (41). That lot of samples consisted of the 8th to the 11th fully developed leaf counting from the bottom of the plant and contained 8.5 gm. per kilo of ethyl ether-soluble material of which about one-third disappeared in the early stages of curing. Similarly, samples from the crop of 1931 (13th to 16th leaf) (43) contained 11.6 gm. per kilo of ether extractives and also lost about one-third of these by the time the brown stage of curing had been reached. These observations are in conformity with Frankenburg's statement (5) that the lower leaves of the tobacco plant contain less ether-extractable material than the upper leaves. Nevertheless, the leaves in each of these three sets of samples were collected at the point of "technical ripeness" and were thus presumably of somewhat similar actual age.

Although the total ether extractives of different lots of tobacco leaves may differ somewhat widely in amount, the quantities of ether-soluble components that disappeared from these three sets of samples were strikingly similar. The 1929 leaves lost approximately 2.8 gm. per kilo, the 1931 leaves lost 3.9 gm., and the present samples (1949) lost 2.8 gm.

It would appear that the more labile components together with chlorophyll make up a substantial fraction of the ether extractives and are present in Connecticut shade tobacco leaves in moderately constant amount at the stage of technical ripeness regardless of the position of the leaf on the plant or of the crop year.

The ether extractives of tobacco leaves comprise an extremely complex mixture of substances. Frankenburg (5) has reviewed the literature of the subject and has pointed out that even qualitative knowledge of the components of the mixture is still far from complete. Important components are hydrocarbons, including both paraffins and terpenes, alcohols, esters, true fats, and so-called resin esters as well as polymerization products of high molecular weight but of only vaguely known chemical relationship. Chibnall, Piper, Pollard, Williams and Sahai (2) have made a brief study of the paraffin hydrocarbon fractions obtained from both cured and fresh tobacco leaves and were able to show the probable presence of hydrocarbons with 33 and 31 carbon atoms and the possible presence of the next three lower homologues with odd numbers of carbon atoms. These components accounted, however, for only about 5 per cent of the total petroleum ether extract of fresh leaves.

The ether extractives of the tobacco leaf provide one of the most difficult and challenging problems in tobacco chemistry. Since the components of the leaf that give rise to the characteristic odor of tobacco before it is smoked and to the aroma of the smoke itself may be due, at least in part, to components included in this fraction, the importance of an understanding of the chemistry of the substances involved can hardly be overestimated.

"Crude Fiber"

The conventional A.O.A.C. method (1)[1] to determine crude fiber was employed. The residue which remains after extraction of tobacco leaf tissue with acid and with alkali under the conditions of the method consists of cellulose together with some contaminating insoluble material, chiefly lignin. Inasmuch as these components of the samples would not be expected to be affected to any significant extent during the curing process, the quantity present in the set of samples should, when plotted, give a horizontal straight line. The plot in Figure 8 shows that this is approximately true. The mean value for the crude fiber of the cured samples is 7.23 $\pm$ 0.23 gm. per kilo, the coefficient of variation being 2.4 per cent. The analytical method is thus a thoroughly reliable one and earlier observations that crude fiber does not change during curing (41) are confirmed. Nevertheless, the mean value for the crude fiber of the samples from the 1929 crop was 10.9 gm. per kilo which is appreciably higher than that of the present samples. The higher position on the plant of the leaves used in the earlier study may have a bearing on this difference.

Reducing Carbohydrates

Tobacco leaves of the type used in the present investigation are characterized by the small proportion of the organic solids they contain in the form of simple carbohydrates and starch. The actual quantities found

[1] We are indebted to the Department of Analytical Chemistry for these determinations.

in any specific sample will depend upon such factors as the age of the leaf, its position on the plant, the weather conditions at the time of sampling and presumably also the time of day at which the leaves were picked, to say nothing of the exact strain of the tobacco type and the conditions under which the plants were grown. Connecticut shade tobacco usually contains from 2 to 4 gm. per kilo of reducing substances calculated as glucose at the time of technical ripeness and, when the leaves are subjected to the curing process, this reducing substance disappears somewhat rapidly. The behavior of the reducing substances in the present set of samples is shown in Table 14 and the data are plotted in Figure 9 on a time scale that has been greatly extended in comparison with the other figures so that an adequate separation of the curves could be obtained. The figure shows the results of the analytical determinations on the samples collected during the first 20 days.

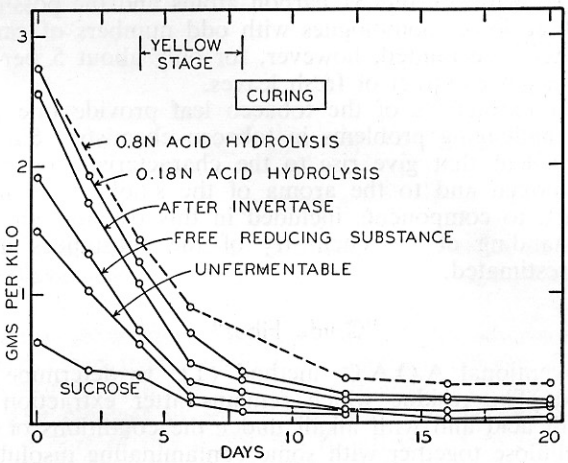

FIGURE 9

Changes in soluble carbohydrates of samples during first 20 days of the curing process.

The analytical methods employed are not absolutely specific for sugars. The samples are extracted with hot dilute alcohol and the reducing power of the substances in the extract is determined with the copper reagent of Somogyi after various treatments designed to indicate the chemical nature of the simple carbohydrates the extracts contain. Nevertheless, there is no assurance that all of the reducing power found and calculated in terms of glucose originates from simple sugars. It is quite possible that small quantities of other reducing substances may be present as contaminants. The curves in Figure 9, then, represent the net changes in reducing power as determined with a sugar reagent. The data are calculated as glucose equivalents and the statements that are made with respect to the changes indicated by these curves are subject to the reservation that a part of the effects observed may be due to the behavior of substances other than sugars. In spite of the deficiencies

TABLE 14. SOLUBLE CARBOHYDRATES

Data expressed as glucose equivalents in grams per kilo.

Sample	Days cured	Total sugar after inversion	Total reducing substance after hydrolysis with dilute acid	Free reducing substance	Total reducing substance after 1 *N* acid hydrolysis	Unfermentable reducing substance	Sucrose	"Glucoside" sugar	Glucose
A1	0	2.56	2.76	1.92	2.76	1.50	0.64	0.20	0.42
B1	2	1.74	1.94	1.32	. .	1.03	0.42	0.20	0.29
C1	4	1.09	1.25	0.72	1.43	0.61	0.37	0.16	0.11
D1	6	0.46	0.70	0.31	0.93	0.20	0.15	0.24	0.11
E1	8	0.33	0.40	0.24	. . .	0.16	0.09	0.07	0.08
F1	12	0.16	0.21	0.10	0.34	. . .	0.06	0.05	. . .
G1	16	0.16	0.19	0.08	0.28	0.02	0.08	0.03	0.06
H1	20	0.13	0.16	0.11	0.28	0.03	0.03	0.03	0.08
I1	32	0.14	0.18	0.15	. . .	. . .	0.00	0.04	. . .
J1	63	0.13	0.19	0.14	. . .	. . .	0.00	0.06	. . .

of the analytical methods, however, there is every reason to assume that most of the chemical effects observed do indeed represent changes in carbohydrate components of the leaves.

The outstanding observation is that all of the several forms of reducing substances present in the fresh leaves rapidly diminish in amount so that, at the end of 12 days, only negligibly small quantities remain. It is doubtless significant also that these small residual quantities of reducing substances undergo little or no detectable further change even during the fermentation process. This observation suggests that the rapid changes that take place in the first few days of curing actually do represent decomposition of simple carbohydrate components and that these reactions run to completion. The small residual amount of more stable reducing material may thus furnish a rough estimate of the reducing substances other than true carbohydrates that were present in the alcohol extracts of the tissues.

With these qualifications of the validity of the analytical determinations in mind, the data may be interpreted as follows. The fresh leaves contained at the start 1.92 gm. per kilo of sugar, some of which was glucose, in the free form. In addition, a carbohydrate, which is presumably sucrose, is present in sufficient amount to provide 2.56 gm. of glucose equivalents after treatment of the extract with invertase. The difference, 0.64 gm., thus represents the quantity of sucrose present and is so recorded in Table 14 and in the figure.

There is also present a substance or substances which yield reducing power after treatment of the extract with dilute acid at the boiling point. The total reducing power after acid hydrolysis is recorded in the table and was 2.76 gm. per kilo for the fresh leaves. Sucrose would be hydrolyzed under the conditions chosen so that 2.56 gm. of this quantity would arise from the initial sugar present together with the sucrose. The small additional reducing power, 0.20 gm. per kilo, if analytically significant, furnishes an estimate of substances which yield glucose on acid hydrolysis under the arbitrary conditions adopted and which may possibly represent glucosides.

Finally, only a part of the reducing substances present in the extract is removed by yeast, the remainder being reported as "unfermentable reducing substance." The part which is absorbed by yeast is reported in the last column of Table 14 as glucose. The four columns at the right in Table 14 thus represent the composition of the alcohol extract of the samples with respect to simple carbohydrates. The major component of the fresh leaves is a form of reducing sugar that is not fermented by yeast, the other components are probably sucrose and glucose together with a small quantity of what is presumably glucoside glucose.

Indications of the presence of still another form of carbohydrate were obtained by determining the reducing power after hydrolysis with 0.8 *N* sulfuric acid. A small quantity of reducing power in addition to that liberated by dilute acid hydrolysis was revealed; this possibly represents additional glucoside-like material.

The behavior of these presumptive forms of simple carbohydrates during curing is shown in Figure 9. The unfermentable material diminished rapidly along a straight line and only 0.2 gm. remained after six days. Sucrose likewise dropped to a low level in six days. Curves for glucose and for the hypothetical glucoside sugar have not been plotted, but the

behavior can be readily seen from the table. Glucose dropped to a low level in six days, but the so-called glucoside sugar appears to have been relatively stable during this period. Nevertheless, with the onset of the brown color of the leaves after the sixth day, this component likewise rapidly diminished. The quantities of all of these forms of carbohydrate that are tabulated after eight days of curing are of doubtful analytical significance. It is clear that the intense respiratory activity of the first few days of curing drew heavily upon the small amounts of simple carbohydrates that were present in the leaves at the time of collection.

Starch

The starch content of field grown tobacco of the type used in the present investigation is usually low. Samples from the crop of 1934 studied in connection with an investigation of the effect of culture of detached leaves in darkness and in light (44) contained from 0.25 to 1.4 gm. per kilo and, when the leaves were kept in darkness, the starch dropped to negligible values in from two to four days. Nevertheless, a mechanism for the synthesis of starch is active in such leaves, for when cultured under otherwise similar conditions but in light, the starch content increased materially.

The results of the determinations of starch in the present samples are shown in Table 15. The initial starch content of 0.58 gm. per kilo had disappeared for all practical purposes within two days and the subsequent small variations in the apparent quantity of starch are merely a reflection of the difficulty of the analytical determination at this extremely low level. The actual titrations involved were of the order of 0.1 ml. of reagent. It may be assumed, therefore, that starch promptly becomes involved in the metabolic processes that take place after the leaves are detached from the plant. Whether or not the somewhat more sluggish early drop in the content of glucose that is evident in the data in the right hand column of Table 14 is associated with the disappearance of the starch is not clear, but it seems certain that starch is immediately called upon to provide energy for the various metabolic processes that take place in the detached leaves and that all of it is soon exhausted.

TABLE 15. STARCH

Sample	Days cured	Starch gm./kg.
A1	0	0.58
B1	2	0.05
C1	4	0.03
D1	6	0.07
E1	8	0.10
F1	12	0.09
G1	16	0.17
H1	20	0.18

Part II

BEHAVIOR OF LEAVES DURING FERMENTATION

The samples that were to be used for the study of the effect of fermentation were cured, along with the others, in a position in the barn adjacent to those used in the curing study. At the end of the curing operation on September 23, 1949, these samples, namely the series designated FA — FJ together with the 400-leaf sample designated FER, were taken to the warehouse of the Imperial Agricultural Corporation in Hartford and a few days later were placed in a bulk along with a large quantity of tobacco of similar type. Sample FA was taken for analysis in order to provide the zero point for the study of the changes during fermentation. At each subsequent "turning" of the bulk, a sample was removed and prepared for analysis. The details of the weights of the samples and the approximate location within the bulk are shown in Table 16. The samples beginning with FF were transferred to an adjoining bulk since the tobacco in the first one used had completed its fermentation at the time Sample FF was removed. The next time this second bulk was turned, the experimental samples were judged to be fully fermented and sample FG was taken. Subsequently, for a period of 74 days, the remaining samples were stored in a cool room along with a large quantity of other experimental material. Sample FH was then taken and samples FI and FJ were packed into a case together with other tobacco which was stored at a moderately high temperature (*ca.* 80° F.) for 45 days for the process of "mulling." Finally, sample FJ was compressed into a bale with other similar tobacco; 309 days later, it was recovered from the bale for analysis.

Each of these samples, when removed from the processing, was taken to the New Haven laboratory and prepared for analysis exactly as were the samples from the curing process. Details of the data, including the equilibrated dry weight and the conversion factor for the analytical results, are shown in Table 16.

Ash and Moisture

The data for the moisture and ash content of the samples subjected to fermentation are collected in Table 17 and are plotted in Figure 10. The six successive turnings of the bulk are indicated on the figure as well as the subsequent period of cool storage for 74 days, of "mulling" in the case for 45 days, and of storage in the bale for 309 days. The scale of time of the figure is arbitrarily compressed for these last three intervals, and this fact is expressed on the curves by the zigzag lines.

Like the samples for the curing experiment (see Table 7 and Figure 2), this set also contained a remarkably uniform quantity of water after equilibration at 24° C. and 75 per cent relative humidity. The mean moisture content was 6.08 $\pm$ 0.13 gm. per kilo of initial fresh weight of the leaves, the coefficient of variation being only 2.1 per cent. The plot of the equilibrated dry weight is essentially a horizontal straight line.

TABLE 16. FUNDAMENTAL DATA ON SAMPLES SUBJECTED TO FERMENTATION AND SUBSEQUENTLY MULLED AND BALED

Sample	Initial fresh weight gm.	Time from start of fermentation days	Interval between samples days	Weight at end gm.	Equilibrated dry weight gm.	Factor A	Maximum temperature of bulk °F.	Location in bulk, etc.
FA	1286	0	0	118.8	102.65	0.7982		
FB	1298	13	13	120.9	103.25	0.7954	114	near top: outside
FC	1296	19	6	119.8	103.50	0.7986	114	center: outside
FD	1281	27	8	115.95	101.10	0.7892	111	inside
FE	1262	35	8	112.25	98.90	0.7837	110	outside
FF	1316	43	8	116.4	102.50	0.7789	109	inside
FG	1288	52	9	113.9	101.15	0.7853	108	outside
FH	1278	126	74	116.3	98.50	0.7707		stored cool
FI	1263	171	45	107.95	96.60	0.7648		mulled in case
FJ	1302	480	309	106.50	101.59	0.7803		baled
Mean						0.7854		
S.D.						± 0.0121		
C.V. (%)						1.54		

It may be inferred that, whatever the chemical changes that occur during fermentation may be, they do not affect the water-holding capacity of the tissue to any significant extent.

Emphasis should be placed on the fact that the moisture content of these samples after equilibration is no indication of their water content during the actual fermentation of the tobacco. The data for the water content at the time the samples were taken are shown in Table 17. The leaves as removed from the curing barn contained 17.7 per cent

TABLE 17. WATER, ASH, AND ORGANIC SOLIDS CONTENT OF SAMPLES EQUILIBRATED AT 24° C. AND 75 PER CENT RELATIVE HUMIDITY

Data in grams per kilo of fresh weight.

Sample	Days from start of fermentation	Percentage moisture during processing	After equilibration		
			Water	Ash	Organic solids
FA	0	17.7	5.89	15.26	58.67
FB	13	18.8	6.05	15.46	58.02
FC	19	17.3	6.24	15.61	58.01
FD	27	17.8	6.24	15.15	57.52
FE	35	16.4	6.07	15.17	57.13
FF	43	16.3	6.11	15.27	56.51
FG	52	16.1	6.08	15.20	57.25
FH	126	20.2	5.92	15.23	55.92
FI	171	16.0	5.99	15.11	55.38
FJ	480	11.0	6.23	15.82	55.98
Mean			6.08	15.33	57.04
S.D.			±0.13	±0.22	±1.05
C.V. (%)			2.1	1.4	1.8

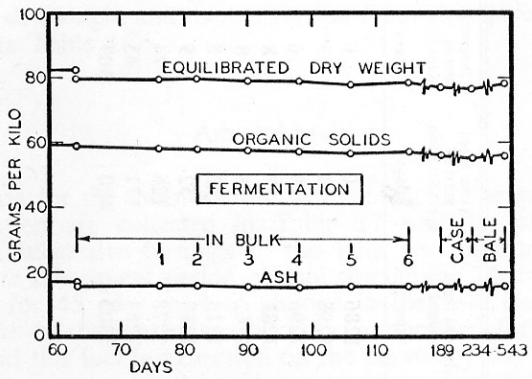

FIGURE 10

Ash, organic solids, and equilibrated dry weight of samples subjected to curing, fermentation, and storage (samples FA — FJ). The termination of the curves that show the data for the cured samples is indicated at the left. Discontinuity in time scale is indicated by zigzag lines.

of water and, save for an initial rise to 18.8 per cent due to the "conditioning" of the leaves before the first bulk was built, this proportion changed very little as fermentation progressed. After the leaves had been stored for 74 days (sample FH), the moisture content increased, but it diminished again during the mulling process in the warm room, and the leaves recovered from the bale (sample FJ) in which they had been packed for 309 days were quite dry. It is obvious that analytical determinations cannot conveniently be based upon the weight of material of such variable water content and it is for this reason that the samples were brought into equilibrium with air at a controlled temperature and humidity before analysis.

The data for the ash, like those for the samples that were examined after the curing process, are constant and the plot is approximately a horizontal straight line. The coefficient of variation of only 1.4 per cent indicates the high accuracy of the sampling and of the analytical results. This set of samples was manifestly satisfactorily uniform in initial composition. Reference may be made to Table 8 (page 37) for the detailed analysis of the ash of these samples, and comparison of the results for the two sets of data there shown indicates that, with the possible exception of a difference in the trace quantity of aluminum they contained, the samples derived from adjacent plots in the same field were closely alike in mineral constituents.

Organic Solids

The data for the organic solids plotted in Figure 10 clearly show that a small loss of organic material occurs during fermentation. The effect appears to be continuous although slow. During the actual fermentation in the successive bulks, a loss of about 1.4 gm. per kilo took place. This is 2.4 per cent of the organic solids present at the start of fermentation and 2.0 per cent of the organic solids present in the fresh leaves. During the long period of storage, an additional loss of about 1.3 gm. occurred.[1] Thus approximately 4 per cent of the organic solids of the original green leaves are removed from the system as volatile products in the course of the second stage of processing the tobacco. It has already been pointed out that the loss during curing is about 16 per cent. The net result, accordingly, is that approximately one-fifth of the organic matter of the green leaf disappears during the curing and fermentation of wrapper tobacco.

The fermentation process has long been known to be accompanied by losses of a significant proportion of the organic substances of the tissue. Data for many types of tobacco have been collected by Frankenburg (6) and his figures suggest that the losses found in the present case are of about the magnitude that would be anticipated although they are at the lower limit. Wrapper tobaccos are reported to lose from 4 to 7 per cent of their dry weight in the course of the entire fermentation, mulling and aging processes; other types of cigar tobacco, especially filler, may lose upwards of 20 per cent. However, the technical methods that

[1] A regression line fitted to these data indicates that the loss at the end of fermentation in the bulk was 0.25 gm. and at the end of storage in the bale was 2.24 gm.

are used differ widely for different types of tobacco and in most cases appear to involve far more severe treatment than is customary for high grade wrapper tobacco. With this material, the preservation of the strength and elasticity of the final product are of the utmost importance, and, as a consequence, only what may be termed a mild degree of fermentation is allowed to take place.

The explanation of the fundamental nature of the fermentation process has been a subject for debate for many years. The literature of the subject has been reviewed in detail by Frankenburg (6) and only an outline of the main views need be given here. The observations that fermenting tobacco increases in temperature and that carbon dioxide is liberated early suggested that fermentation is essentially an oxidative process, and three hypotheses have been advanced in an effort to explain the mechanism. According to the first of these, the changes that are observed are the result of the metabolism of microorganisms, mainly bacteria, which are present on the surfaces of the leaves and also presumably embedded within the tissue. The growth of these organisms is stimulated by the conditions that obtain when the tissue is moistened and packed into a firm mass, and the rise of temperature and the chemical effects are supposed to be the result of this growth. The second hypothesis holds that the metabolism of the microorganisms is a subordinate phenomenon, the main reactions that take place being stimulated by the intrinsic enzymes of the leaves which are activated under the conditions that are established. The third view maintains that the oxidations are stimulated by inorganic catalysts and that the intervention of enzymes, whether derived from microorganisms or already present as original components of the tissues, is not an essential part of the process.

Frankenburg has summarized the conflicting evidence and claims in the literature by pointing out "that it is futile to strive for one single and general theory of tobacco fermentation." As he has said, it seems clear that the reactions are initiated and sustained by catalysts and that catalysts of all types, including enzymes, are to be found in the tissues. Present day knowledge of enzyme chemistry would suggest that much of what happens is the result of establishing conditions favorable for the activity of these substances; no definite line can be drawn, however, between the activity of enzymes that were present in the leaves from the start and of those that are elaborated by contaminating microorganisms.

Notwithstanding this, however, attention should be drawn to the fact that the rapid chemical changes that occur in the green leaves during the early stages of the curing process come to an end shortly after the leaves have become brown; the subsequent changes in the curing leaves are extremely slow. These early changes seem obviously to be associated with the activities of the intrinsic enzymes of the living tissues and they involve chemical processes some of which are fairly well understood today. These reactions practically cease, however, at the time of death of the leaf cells. Nevertheless, there is a moderate resurgence of chemical activity when the leaves are exposed to the conditions that obtain in the fermenting bulk. New factors must therefore have been introduced into the chemical system. One of the new factors may be the addition of sufficient water to provide a more favorable medium for chemical reactions however catalyzed.

Total Nitrogen

The data for the total nitrogen content of the samples subjected to fermentation are shown in Table 18 and are plotted in Figure 3. Considered as a whole, the figures suggest that the slow loss of nitrogen that was observed to take place during curing continued during fermentation. However, the compression of the scale of time in the plot gives a somewhat distorted idea of the rate of the loss. A regression line fitted to the data for the total nitrogen of the fermented samples suggests that the values observed at the start and after 19 days are somewhat too high and that the loss during the actual period of fermentation in the successive bulks was in fact quite small. Nevertheless, the loss over the entire period of fermentation and storage amounted to 0.15 gm. per kilo, a quantity that is appreciably smaller than the loss that occurred during the curing period. In spite of the small over-all magnitude of the change, loss of nitrogen in the form of ammonia from fermenting tobacco is nevertheless a common observation, for ammonia can always be detected by its odor in the vicinity of a fermenting bulk.

TABLE 18. CHANGES IN NITROGENOUS COMPONENTS OF LEAVES DURING FERMENTATION

Data in grams per kilo of fresh weight.

Sample	Days from start of fermentation	Total nitrogen	Protein nitrogen	Soluble nitrogen	Nitrate nitrogen
FA	0	3.56	0.95	2.61	0.73
FB	13	3.48	0.95	2.53	0.76
FC	19	3.55	0.98	2.57	0.73
FD	27	3.44	0.98	2.46	...
FE	35	3.45	0.99	2.46	0.77
FF	43	3.49	0.95	2.54	0.79
FG	52	3.49	1.00	2.49	0.80
FH	126	3.43	0.98	2.45	0.83
FI	171	3.35	1.01	2.34	0.77
FJ	480	3.38	1.06	2.32	0.79
Mean		3.46	0.985	2.48	0.77
S.D.		±0.067	±0.034	±0.09	
C.V. (%)		1.9	3.4	3.8	

A further indication that the nitrogen content of this set of samples was not entirely constant is furnished by the coefficient of variation, which amounts to 1.9 per cent. Sets of 10 samples of tobacco leaves collected by the statistical method for experiments in which no opportunity for loss of nitrogen occurs usually have a coefficient of variation of the nitrogen content of 1.5 per cent or less, rarely greater.

Protein Nitrogen

The protein content of the leaves apparently underwent no change during fermentation. The data in Table 17 and the plot in Figure 3 give no indication of any activity that can be attributed to proteolytic enzymes. On the contrary, the data suggest a small increase in the form of nitrogen that remains insoluble when the tissue is successively extracted with hot alcohol and hot water in preparation for the determination of the protein nitrogen. Whether or not this small apparent increase is significant in the present case is open to question. Nevertheless it suggests the possible occurrence in Connecticut wrapper tobacco of a behavior which is far more fully developed in Pennsylvania filler tobacco. Frankenburg (7) has observed a marked increase in insoluble nitrogen in the course of fermentation and he attributes this to a reaction between amino acids and phenolic or quinone-like substances with the production of water-insoluble nitrogenous substances. This view is supported by data that show substantial increases in such water-insoluble substances together with losses of amino nitrogen. If an analogous reaction occurs at all in Connecticut wrapper tobacco, it does not become significant during the actual fermentation in the bulk and is at the limit of detection even after prolonged storage of the leaf.

Soluble Nitrogen

The difference between the total nitrogen and the protein nitrogen is plotted in Figure 3 as the soluble nitrogen. Direct determinations of this quantity are given in Table 11. Taking into account the slight irregularities in the curve for total nitrogen, it seems clear that no significant change took place in the soluble nitrogen during the actual fermentation in the bulk. However, during the subsequent storage period, a slight decrease in soluble nitrogen became apparent. This change is probably the resultant of a number of reactions as will become clear from the consideration of the data for the forms of soluble nitrogen.

Nitrate Nitrogen

The data for nitrate nitrogen are plotted in Figure 5. They are somewhat irregular owing to the deficiencies of the analytical method, and whether or not the indication of a small increase in nitrate during fermentation is to be taken seriously is problematical. The change, if real, is small. Nitrate is clearly not an active metabolite of tobacco leaves at any stage of the curing or fermentation processes. The apparent drop during the "mulling" stage may be compared with the change of similar magnitude between the fourth and eighth days of curing; it is doubtful if either is beyond the limits of accuracy of the determination. The present result is in conformity with Frankenburg's experience with Pennsylvania filler tobacco: he reports that only small and erratic changes of the nitrate nitrogen content are observed.

Ammonia Nitrogen

The behavior of the ammonia nitrogen is shown in Figure 5, the data being given in Table 19. Although the level of the ammonia remained constant throughout the latter part of the curing period, an immediate increase in ammonia took place when the tobacco was humidified and packed into the bulk for the fermentation process. A continuous increase in ammonia occurred during the first three turnings of the bulk, but it then remained constant for the rest of the period of actual fermentation. However, it again increased during the periods of cool storage and of mulling although no further change took place during the period of storage in the bale. These changes in the content of ammonia indicate that a reaction occurred whereby ammonia was liberated from some other component or components of the tissues. An examination of the curves at once suggests that the most likely source is the hydrolysis of the amide groups of asparagine. The curves for asparagine amide nitrogen and for ammonia are remarkably symmetrical in detail and the sums of the individual data for these quantities are shown in Table 19. The mean is 0.596 ± 0.033; that is to say, the sum is constant to within plus or minus about 6 per cent. An appreciable part of the variability of this sum arises from the fact that the ammonia did not increase during the period that the leaves were stored in the bale whereas the asparagine amide nitrogen continued to diminish throughout this time. It is possible that ammonia evaporated from the baled leaves to a sufficient extent during the period of 309 days to compensate for the presumed increase from the hydrolysis of the small remaining portion of asparagine. At all events, the constancy of the sum of the ammonia and the asparagine amide nitrogen throughout almost the entire fermentation and storage period is strong evidence that the greater part of the increase in ammonia that took place was due to the hydrolysis of asparagine.

Asparagine Amide Nitrogen

The plot of the data in Figure 5 indicates that asparagine underwent hydrolysis during the first part of the fermentation process and that nearly one-half of the amount initially present disappeared. Hydrolysis then apparently ceased, although the data for the last three turnings of the bulk are a little irregular owing to what appears to be a somewhat high value for the sample analyzed at 106 days, i.e. after 43 days of fermentation. During the protracted period of storage, hydrolysis of asparagine continued so that only a trace survived at the end of the period of storage.

The behavior of the asparagine is of the greatest interest and is also extremely difficult to explain. That hydrolysis of amide groups should occur during the fermentation of the leaves in the bulking process is perhaps not surprising. It may be assumed that an asparagine-hydrolyzing enzyme was present which was able to decompose a part of the asparagine under the conditions of temperature and moisture content that obtained in the mass of leaves in the bulk at the start of this phase of the process. The puzzling aspect of the findings is, however, that this reaction came to an end while there was still an appreciable quantity of asparagine present notwithstanding the fact that the bulk was rebuilt three more times and that the temperature it was allowed to reach was nearly as high (see

TABLE 19. CHANGES IN SOLUBLE NITROGENOUS COMPONENTS DURING FERMENTATION

Data in grams per kilo of fresh weight.

Sample	Days from start of fermentation	Ammonia nitrogen	Glutamine amide nitrogen	Asparagine amide nitrogen	Amino nitrogen		Sum: ammonia N + asparagine amide N	"Peptide" nitrogen
					Free	Total		
FA	0	0.172	0.045	0.408	0.59	0.73	0.580	0.14
FB	13	0.248	0.040	0.339	0.53	0.70	0.587	0.17
FC	19	0.279	0.028	0.305	0.55	0.68	0.584	0.13
FD	27	0.379	0.021	0.233	0.47	0.59	0.612	0.12
FE	35	0.378	0.020	0.248	0.48	0.59	0.626	0.11
FF	43	0.377	0.017	0.270	0.49	0.63	0.647	0.14
FG	52	0.378	0.009	0.230	0.48	0.64	0.608	0.16
FH	126	0.417	0.021	0.190	0.45	0.57	0.607	0.12
FI	171	0.479	0.000	0.110	0.41	0.51	0.589	0.11
FJ	480	0.479	0.018	0.045	0.40	0.53	0.524	0.13
Mean							0.596	0.13
S.D.							±0.033	±0.02
C.V. (%)							5.5	17

Table 16) as that attained during the first three stages. The moisture content of the samples as removed from the bulk (see Table 17) was also unchanged. That hydrolysis of asparagine ceased is supported by the independent data for the increase in ammonia nitrogen, for this also remained constant.

The source of the asparagine-hydrolyzing enzyme is by no means obvious. Green tobacco leaves contain an enzyme system capable of synthesizing asparagine rapidly and efficiently, the nitrogen being apparently obtained as ammonia from the oxidative deamination of the amino acids. However, this reaction ceased abruptly at the time the leaves passed into the brown stage of curing and asparagine then remained practically constant throughout the remainder of the curing period. The conditions were obviously favorable for the stability of asparagine, and it is important to note that the far more sensitive glutamine which was present in small amount was only partially decomposed during this time. Under the circumstances, then, how can the prompt establishment of the reverse process of asparagine hydrolysis be accounted for when the leaves were slightly humidified and piled in close contact in the bulk? The answer to this question lies at the basis of our understanding of the nature of the fermentation process. Unfortunately, only a speculative explanation can be given.

To those who maintain that the fermentation of tobacco is to be accounted for in terms of the activity of the specific intrinsic enzyme systems of the leaves, these systems being reactivated by the moderate increase in humidity that is established when the bulk is first built and their effects being promoted by the increase in temperature, the explanation of the hydrolysis of asparagine in the fermenting leaves would be that the enzyme system responsible for the synthesis of asparagine in the green leaves at the start of curing is reversed under the new conditions. Synthesis of asparagine takes place at the expense of energy supplied by the respiratory system present in the living leaf. Lacking this supply of energy owing to the death of the cells, the reaction proceeds in the direction of hydrolysis, and ammonia and, presumably, aspartic acid therefore accumulate.

There are several weaknesses in this argument. In the first place, energy *is* made available in the complex system of fermenting leaves, as is evidenced by their increase in temperature. Nevertheless, although available, it is clearly not in a form which is able to provide for maintenance of the level of asparagine in the system. If, as seems possible, the synthesis of asparagine is a reaction which involves the participation of adenosine triphosphate, exhaustion of the supply of this coenzyme could well account for the observations.

Hydrolysis of amide groups implies the presence in the system of an active specific amidase. In experiments carried out many years ago, culture of tobacco leaves in water in darkness under conditions such that the life of the cells is maintained as long as possible (43) gave no evidence of the presence of an active amidase in this tissue. On the contrary, synthesis of asparagine continued for 279 hours at which time the leaves had become flaccid and had turned yellow or brown with only small areas of green color remaining. Only when the evidence of autolytic changes had become extreme after more than 300 hours was there a small decrease in the asparagine amide nitrogen. In the present experi-

ment, it may be noted that after eight days of curing asparagine reached a maximum which was maintained for four more days. At this point the leaves in the curing barn still retained about 40 per cent of moisture. During the next four days, the moisture content dropped to 14 per cent, but, throughout the greater part of this interval, the moisture content must have been high enough to permit the activity of many of the intrinsic enzymes. Nevertheless, the asparagine amide nitrogen diminished to only a negligible extent. The activity of the asparagine synthesizing system had ceased after eight days of curing; for eight succeeding days there was only the smallest indication of the existence of an asparagine-hydrolyzing mechanism. It seems clear, therefore, that a strong case cannot be made for the existence in tobacco leaves of an asparagine-hydrolyzing enzyme of any marked degree of activity. Nevertheless, shortly after the cured leaves were packed into the bulk, the presence of such an enzyme became obvious.

The evidence, such as it is, thus points to the possibility that the capacity of the system to hydrolyze asparagine was acquired after the leaves were packed into the bulk. This suggests, in turn, that the amidase had its origin in the microbiological flora which thereupon developed.

If this argument is acceptable, there remains the problem of accounting for the cessation of asparagine hydrolysis after the bulk had been turned three times. There is little in the available data to guide speculation on this point. It seems unlikely that the microbiological flora suddenly diminished to such an extent that further action of the amidase was too slow for detection, or that the nature of the population suddenly changed. There is nothing to support the view that part of the asparagine was for some reason available for decomposition while a part was not. On the contrary, the remaining asparagine was almost completely hydrolyzed during the ensuing protracted period of storage. Furthermore, until another similar experiment has been carried out and the detailed behavior of the samples in the present experiment has been confirmed, there is no compelling reason to assume that the curves of Figure 5 do indeed represent what invariably happens when Connecticut wrapper tobacco is fermented. The present data must therefore unfortunately be left as a further example of how little is really understood about the chemical events that occur during the fermentation of tobacco. A convincing and thorough account cannot be given of even so simple a chemical reaction as the hydrolysis of the amide groups of asparagine; all that is known is that this reaction occurs.

Amino Nitrogen

Table 19 shows the quantities of amino nitrogen present in extracts of the samples both before and after severe acid hydrolysis. These figures are plotted in Figure 5 as "free" and "total" amino nitrogen, the latter figure being the amino nitrogen present after acid hydrolysis. The data indicate that reactions took place during the first 27 days of fermentation (from 63 to 90 days of total processing) whereby a small proportion of the amino nitrogen present disappeared as such. For the rest of the period of actual fermentation, the free amino nitrogen remained substantially constant, but a small further loss occurred during storage and mulling; there was no further loss on storage in the bale. The curve for

total amino nitrogen parallels that for free amino nitrogen rather faithfully, there being only one major discrepancy, Sample FC at 19 hours of fermentation, the free amino nitrogen determination of which appears to be out of line. Since these are independent analytical determinations, and the general course of the curves is similar, the two groups of data support each other and lend themselves to an interesting although speculative interpretation. The difference between the amino nitrogen before and after acid hydrolysis presumably represents amino acids combined as peptides, and the successive differences are accordingly shown in Table 19 as peptide nitrogen. Although there is some variation, this is irregular. It may therefore be inferred that the samples contained approximately 0.13 gm. of peptide nitrogen and that the peptides represented by this quantity are substances that were liberated from the protein or otherwise synthesized early in the curing operation and were stable throughout the subsequent period of fermentation. This observation indicates that proteolytic enzymes of the general type of peptidases capable of hydrolyzing certain of the possible peptides produced were not present in the tissues. The inference that has already been drawn, from the stability of the protein throughout the period of fermentation and storage, that enzymes capable of attacking the intact protein (i.e. enzymes of the pepsin and trypsin type) were absent is thus extended to include some at least of the better known types of peptidolytic enzymes.

The detailed behavior of the amino nitrogen is not so easily accounted for. Amino nitrogen to the extent of about 0.1 gm. disappeared during the first three turnings of the bulk but then remained constant. This recalls the behavior of the asparagine amide nitrogen, but although the fate of the nitrogen liberated from the amide groups could be adequately accounted for in terms of an equivalent increase in ammonia, the fate of this additional quantity of 0.1 gm. of nitrogen is much less clear. The increase in ammonia nitrogen was inadequate to account both for the loss of amide nitrogen of the asparagine and for this quantity as well. Accordingly, oxidative deamination cannot be invoked as the explanation of the fate of the small quantity of amino acids that disappeared; such a reaction would give rise to the production of ammonia. Neither is it possible to assume that condensation reactions occurred to give products of any usual type, for such products, if soluble, should have been hydrolyzed in the preparation of the extracts for the determination of total amino nitrogen and the curve for this quantity would not have fallen in conformity with the curve for amino nitrogen. If the products of the hypothetical condensation products were insoluble, there should have been a corresponding increase in the curve for protein nitrogen. The data for protein nitrogen in Table 17 do not conform with this view.

Loss of this nitrogen from the fermenting tissues either as ammonia or, which seems even less likely, as free nitrogen gas, is not impossible although the data for total nitrogen in Table 17 do not render this probable. Unfortunately, the total nitrogen cannot be determined with sufficient accuracy to render the loss of so small a quantity as 0.1 gm. readily apparent on the curve. In any case, the loss of precisely the same quantity of ammonia as might have been produced if the amino nitrogen had been oxidatively deaminized would be difficult to understand when at the same time ammonia was being liberated by the hydrolysis of amide groups and retained quantitatively in the tissue.

The contrast between the behavior of the fermenting leaves and the curing leaves is sharply illustrated by these data for the amino nitrogen. The drop in the curve for amino nitrogen in the interval between 6 and 16 days of curing (Figure 5), considered in its relationship with the curves for asparagine amide nitrogen and ammonia nitrogen in the same interval, furnished evidence for an easily apprehended sequence of chemical reactions. These reactions conform with the classical theory of amide metabolism. Unfortunately, there is no such well-established theory to help in the understanding of what occurred in the early phase of the fermentation process, and whether or not the behavior of the asparagine amide nitrogen is correlated with that of the amino nitrogen remains to be discovered. Nevertheless, the facts that both amino nitrogen and asparagine amide nitrogen underwent fairly rapid change for some 27 days and that both then remained at constant levels for the rest of the treatment in the bulk suggests that there is some common correlating influence at work.

The additional loss of amino nitrogen during the periods of storage and mulling remains to be mentioned. The compression of the time scale in Figure 5 gives a distorted view of the speed of this reaction; it was, in fact, extremely slow and involved rather less than 0.1 gm. of nitrogen whereas the hydrolysis of asparagine in the same period and in the additional period of storage in the bale was more than twice as great. There is no suggestion that the change gave rise to ammonia, but the total nitrogen of the samples did appear to diminish during this stage of the process. It is possible that some ammonia was lost during the long storage period.

Changes in Acidity during Fermentation and Storage

The data for the pH of the samples are shown in Table 20 and are plotted in Figure 6. During the whole period of fermentation, the pH scarcely changed at all, the variation around the mean value of pH 5.54 of the seven observations made while the samples were in the bulks being negligible. However, during the period of mulling the pH rose by 0.3 pH units and the higher pH was maintained during the period of nearly a year that the last sample was stored in the bale. Although there was an increase in ammonia nitrogen during the mulling period (see Figure 5), the quantity involved was only 0.062 gm. or 4 m.eq. and this is entirely inadequate to account for the rise in pH observed in view of the high buffering capacity of the organic acids present in the tissues. A titration curve of a sample showed that to alter the pH from 5.4 to 5.9 would require approximately 17 m.eq. of alkali. Furthermore, as will become clear, there was no loss of ether-soluble organic acids during this period. Accordingly, the rise in pH observed during mulling cannot be accounted for in terms of a simple neutralization phenomenon nor in terms of loss of titratable acidity. The complexity of the system is manifestly too great for simple explanations to be applicable.

Alkalinity of Ash

The alkalinity of the ash is one of the most satisfactory analytical determinations, from the standpoint of accuracy and reproducibility, avail-

TABLE 20. ACIDITY AND ORGANIC ACIDS

Data expressed in milliequivalents per kilo of fresh leaf.

Sample	Days from start of curing	Days from start of fermentation	pH	Alkalinity of ash	Total organic acids	Citric acid	Malic acid	Oxalic acid	Undetermined acid
FA	63	0	5.50	294	286	90.0	74.8	46.3	75
FB	76	13	5.48	293	286	90.5	65.9	46.4	83
FC	82	19	5.52	291	294	88.3	71.6	45.7	88
FD	90	27	5.59	294	294	88.0	70.6	45.7	90
FE	98	35	5.57	291	278	84.5	65.4	46.2	82
FF	106	43	5.56	287	285	87.6	68.2	46.5	83
FG	115	52	5.58	291	278	89.1	65.4	46.0	78
FH	189	126	5.63	285	274	82.4	62.3	46.8	83
FI	234	171	5.87	286	269	85.2	63.3	46.0	75
FJ	543	480	5.83	306	276	90.9	59.0	45.7	80
Mean				291.8	282.0	87.7	66.7	46.2	81.7
S.D.				±5.9	±8.4	±2.8	±4.7	±0.36	
C.V. (%)				2.0	3.0	3.2	7.1	0.8	

able for the study of samples of tobacco. Reference to Table 12, in which the determinations on the samples subjected to curing are given, shows that the mean value of 296.8 was determined with a coefficient of variation of only 2.7 per cent. If the coefficient of variation of the fresh weight of 1.4 per cent (Table 3) can be taken as an approximate indication of the error due to sampling alone, it is clear that the analytical method leaves little to be desired.

The observations on the FA — FJ series of samples recorded in Table 20 are even more satisfactory. The samples were collected from a stand of plants adjacent to those used for curing and the coefficient of variation of the fresh weight was 1.32 per cent (Table 3). The alkalinity of the ash of these samples was 291.8 $\pm$ 5.9 m.eq., the coefficient of variation being only 2 per cent. The difference between the mean values of the two sets of samples was also negligible for practical purposes.

The plot of the values in Figure 7 (broken line) emphasizes the small variations among the samples because of the large scale used; nevertheless only the last determination in the sample stored in the bale is notably out of line with the others, and, since this single determination was made many months after the routine analysis of the other members of the set, analytical variation doubtless played a part in the apparent discrepancy.

Total Ether-Soluble Acidity

The data for the total ether-soluble organic acids are shown in Table 20 and are plotted in Figure 7. The curve is obviously closely similar to that for the alkalinity of the ash and, save for two cases, lies slightly below it. The correspondence of the data is remarkably close as is evident from the mean values at the bottom of the table.

The present set of determinations is unusually constant, the coefficient of variation being only 3 per cent. The actual titration of the total acidity is unsatisfactory from the analytical point of view, for the analytical error is frequently large. Nevertheless it is clear that little or no alteration in the quantity of ether-soluble acids occurred during the process of fermentation or in the subsequent period of storage. These substances appear to be almost completely stable with respect to the total quantity present throughout the entire process.

Oxalic, Malic, and Citric Acids

The results of the determinations of the three main organic acid components of the tissues are shown in Table 20 and are plotted in Figure 7. Oxalic acid remained unchanged throughout the process and, since the analytical method is excellent, the data yield a horizontal straight line. The coefficient of variation of this set of figures is less than 1 per cent and is thus smaller than the assumed sampling error. Both malic and citric acids also appear to have remained constant. The irregularities in the curves are no greater than should be anticipated from the less accurate analytical methods available for these substances and it is clear that, whatever the chemical changes are that take place in tobacco leaves during the normal fermentation process, they do not involve the chief ether-soluble organic acids.

This result is of considerable significance. In living green leaves, the organic acids are among the more reactive of the metabolites and emphasis has already been placed upon the striking transformation of malic acid into citric acid that occurs in this type of tobacco during the first few days of curing. This extensive change having been completed, the organic acids undergo no further apparent change. The enzymes which catalyze reactions in which organic acids are concerned are either inactivated or the conditions have become such that there is no further scope for their activity. Nevertheless, it is somewhat surprising that nothing at all should happen to the main organic acids during fermentation. If, as some authorities hold, fermentation is brought about by the metabolism of the microbiological flora growing on and within the tissues, one would anticipate that the organic acids would be utilized to some extent at least, particularly as the soluble carbohydrate content of the tissues had been reduced to negligible amounts in the process of curing. However, the failure to observe such effects cannot be advanced as an argument against the microbiological hypothesis of fermentation since so little is known regarding the nature of the substrates essential for the life of the organisms concerned. The present data strongly suggest that, whatever the significance of the microbiological flora may be, these organisms do not bring about chemical transformations of the ether-soluble organic acid components of the tissues.

Lipides

The quantities of ether-extractable substances present in the samples are shown in Table 21 and the data are plotted in Figure 8. The results obtained by the A.O.A.C. method (1)[1] in which ethyl ether was used, are somewhat irregular but suggest a slow and continuous loss. The check results in which petroleum ether was used give a somewhat more satisfactory curve and likewise indicate slight losses of these components during the fermentation and storage of the leaves. Both sets of data are identical for the samples taken towards the end of the fermentation process and throughout the storage period, but the A.O.A.C. method indicates the presence of slightly larger amounts of ethyl ether-soluble components in the early stage of fermentation although the difference is obviously close to the limit of significance. The most important conclusion, however, is that the fat-solvent soluble substances in tobacco leaves are not affected to any great extent, at least with respect to the total quantity present, by the process of fermentation and the subsequent storage. Notwithstanding that such factors of quality as aroma are fundamentally altered by the treatment of the leaves, no striking evidence has come to light that this alteration is reflected by change in the total quantity of ether-soluble components.

Crude Fiber

The data in Table 21 plotted in Figure 8 indicate that the main structure elements of the tissue, the cellulose and other components

[1] We are indebted to the Department of Analytical Chemistry for the determinations by the A.O.A.C. method.

TABLE 21. LIPIDES AND CRUDE FIBER

Data in grams per kilo of fresh weight.

Sample	Days fermented and stored	Lipides: A.O.A.C. method	Lipides: Petroleum ether-soluble	Crude fiber
FA	0	2.52	2.33	7.11
FB	13	2.52	...	6.98
FC	19	2.57	2.20	6.91
FD	27	2.75	...	7.01
FE	35	2.53	2.10	7.09
FF	43	2.11	...	7.18
FG	52	2.12	2.15	6.96
FH	126	2.01	...	7.37
FI	171	1.95	1.93	7.43
FJ	480	2.08	2.17	7.17
Mean				7.12
S.D.				±0.17
C.V. (%)				2.4

estimated by the conventional A.O.A.C. method[1] for crude fiber undergo no significant change during fermentation and storage. The data both for the samples subjected to curing and to fermentation can be adequately expressed by a horizontal straight line if consideration is given to the probable accuracy and reproducibility of the analytical method. The data for each group of samples give a mean with a coefficient of variation of 2.4 per cent which may be regarded as highly satisfactory. Accordingly, it may be concluded that none of the components of the crude fiber fraction are affected by the fermentation process as applied to this type of tobacco.

Cooperative Analyses

In the course of personal discussions during the early phases of the present investigation, Dr. W. G. Frankenburg of the General Cigar Co., Lancaster, Pennsylvania, was kind enough to offer to have a study of some of the samples made by analytical methods that have been developed in his laboratory. The preliminary analyses of Samples A1, D1, J1, FB, and FG, which were selected so as to reveal the main course of the transformations that occur, were of such interest that the work was subsequently extended to the examination of all of the curing and most of the fermentation series.

The techniques employed in the Lancaster laboratory are in many respects quite different from those in use at this Station. In addition to the determinations that are customarily made on water extracts of tobacco, methods have been devised to obtain data for such items as the nitrogen that is adsorbed by the magnesium oxide during the distillation of the

[1] We are indebted to the Department of Analytical Chemistry for the determinations by the A.O.A.C. method.

ammonia, for the increase in ammonia nitrogen over that derived from the amides as a result of a distillation in the presence of sodium hydroxide, for nitrogen precipitated by silicotungstic acid in addition to that derived from alkaloids or alkaloid transformation products, for ammonia and amino nitrogen in such precipitates after hydrolysis, for nicotine, and oxynicotine, and for a number of other fractions. Nitrate is determined by a Devarda's alloy procedure and all total nitrogen determinations are made with the use of a prolonged digestion period. The dissection of the water-soluble fraction is thus considerably more detailed than has been attempted in the present work, and complete sets of comparative data for all determinations are accordingly not available. Nevertheless, a number of the determinations that are carried out in the two laboratories are essentially similar and the data for these items are shown in Table 22 arranged in parallel columns to facilitate comparison in detail. Only six of the samples from the FA — FJ series have been examined at Lancaster at the present writing.

Examination of Table 22 shows that the agreement between the results for total nitrogen is completely satisfactory. The mean of the values from the Lancaster laboratory is 3.65 ± 0.04 (C.V. $= 1.06\%$) while the mean of the values from this laboratory is 3.68 ± 0.07 (C.V. $= 1.9\%$). The smaller coefficient of variation of the Lancaster data suggests a slightly higher degree of precision in the method used.

The total nitrogen of the residue after extraction of the soluble components is reported from Lancaster as "insoluble nitrogen." This corresponds closely to the determination of protein nitrogen used in this laboratory and the data indicate that essentially the same quantity is being measured.

The agreement in ammonia nitrogen is for the most part extremely close in view of the difference in techniques used. Save for the determinations of the small quantities present in the first few samples, no significance can be attached to the differences and the interpretation of the data as a whole leads to identical conclusions. There are slight discrepancies in the results for glutamine amide nitrogen but all of the quantities are small. The Lancaster data do not lead to the conclusion that there is an appreciable hydrolysis of glutamine during the later stages of curing as is indicated by the results in this laboratory, but both sets of data show that much of the glutamine disappears during fermentation. There are also some discrepancies in the determination of asparagine amide nitrogen inasmuch as the Lancaster figures do not reach so high a maximum as do those from this laboratory. Nevertheless, the form of the curves that express the two sets of data is closely similar and there would be no essential difference in the interpretation.

The determinations of pH are in close agreement, indicating that the techniques and apparatus of the two laboratories give nearly identical results. Perhaps the most striking observation, in a test such as this, is the agreement between the figures for nitrate nitrogen. The two laboratories use widely different methods for the reduction of nitrate to ammonia before distillation and titration, and in this laboratory the nitric acid is extracted from the tissue with ether before reduction. Nevertheless the results are for practical purposes identical; both sets show a similar irregular variation of this component and lead to the conclusion that there is no essential change in the content of nitrate during the curing

TABLE 22. COMPARISON OF ANALYTICAL RESULTS OBTAINED IN THIS LABORATORY WITH THOSE OBTAINED IN THE LABORATORY OF THE GENERAL CIGAR COMPANY, INC.

Data expressed as grams per kilo of initial fresh weight.

Sample	Total nitrogen		Insoluble or protein nitrogen		Ammonia nitrogen		Glutamine amide nitrogen		Asparagine amide nitrogen		pH		Nitrate nitrogen	
	CAES	GCC	CAES	GCC	CAES	GCC	CAES	GCC	CAES	GCC	CAES	GCC	CAES	GCC
Curing Series														
A1	3.80	3.72	2.27	2.18	0.022	0.008	0.013	0.005	0.019	0.016	5.36	5.32	0.82	0.77
B1	3.76	3.65	1.88	1.86	0.036	0.022	0.040	0.032	0.078	0.093	5.49	5.50	0.81	0.76
C1	3.73	3.68	1.33	1.33	0.037	0.029	0.050	0.045	0.292	0.283	5.57	5.50	0.84	0.80
D1	3.66	3.68	1.07	1.01	0.10	0.089	0.041	0.041	0.435	0.424	5.68	5.70	. . .	0.80
E1	3.62	3.65	1.01	1.01	0.17	0.149	0.042	0.056	0.467	0.422	5.74	5.60	0.79	0.83
F1	3.69	3.65	1.02	1.01	0.227	0.200	0.026	0.047	0.465	0.383	5.77	5.70	0.81	0.80
G1	3.65	3.60	0.98	0.995	0.230	0.220	0.035	0.048	0.449	0.400	5.73	5.48	0.79	0.76
H1	3.65	3.64	0.97	0.995	0.226	0.194	0.035	0.050	0.449	0.398	5.69	5.70	0.81	0.82
I1	3.59	3.60	0.99	0.985	0.237	0.222	0.036	0.050	0.454	0.414	5.66	5.60	0.78	0.74
J1	3.60	3.60	0.97	0.975	0.206	0.168	0.018	0.051	0.426	0.417	5.53	5.54	0.77	0.76
Fermentation Series														
FA	3.56	3.45	0.95	0.99	0.172	0.173	0.045	0.047	0.408	0.414	5.50	5.41	0.73	0.77
FB	3.48	3.40	0.95	0.94	0.248	0.240	0.040	0.014	0.339	0.322	5.48	5.48	0.76	0.72
FE	3.45	3.41	0.99	1.02	0.378	0.372	0.020	0.037	0.248	0.162	5.57	5.50	0.77	0.73
FG	3.49	3.40	1.00	1.01	0.378	0.370	0.009	0.019	0.230	0.196	5.58	5.58	0.80	0.75
FH	3.43	3.41	0.98	1.01	0.417	0.388	0.021	0.019	0.190	0.146	5.63	5.49	0.83	0.74
FJ	3.38	3.36	1.06	1.07	0.479	0.476	0.018	0.030	0.045	0.047	5.83	5.59	0.79	0.76

and fermentation processes. The two methods are obviously equally reliable.

Owing to fundamental differences in the treatment of the solution in preparing the extracts, the agreement between the determinations of amino nitrogen in the two laboratories is not close in detail although the general behavior revealed is the same. The methods are not designed for the same purpose and the data are accordingly not reported. However, both sets lead to similar interpretations.

The outcome of this cooperative study is particularly gratifying. The close agreement gives increased confidence in the fundamental accuracy of the methods and in the technical skills available in both laboratories.

Part III

THE VARIATION IN THE COMPOSITION OF DIFFERENT GRADES OF CONNECTICUT WRAPPER TOBACCO

The data that have been presented in the tables and figures in the previous pages indicate the progress of some of the chemical changes that occur in Connecticut shade-grown tobacco when the leaves are subjected to the customary technical process of curing and fermentation followed by a period of storage. The samples used for these analyses were selected in such a way that each sample within a set resembled each of the other samples as closely as possible in composition at the time the leaves were picked. Some of the data, particularly those for ash, alkalinity of ash, and oxalic acid, that is, for factors that did not change during processing, can be accurately represented by horizontal straight lines. This result furnishes strong evidence that the groups of samples were indeed initially identical in composition within remarkably narrow limits of variation. The leaf material employed for the samples was thus extremely uniform. In view of this, it might be supposed that a sample of these leaves, on examination by a skillful tobacco grader at the end of the process, would be found to fall into a narrowly circumscribed number of technical grades or even all into one grade.

In order to test to what extent this hoped-for possibility might be realized, a sample of 400 leaves was collected at the same time as the other samples and from the same leaf positions on an adjacent block of 100 plants. This 400-leaf sample, which received the designation FER, was treated in exactly the same manner as the others. It was strung on laths for curing and was fermented along with the other samples and was then stored for 74 days. On January 27, 1951, the 400 leaves of this sample were graded by an expert grader at the warehouse of the Imperial Agricultural Corporation and the grading was checked and corrected with respect to the assignment of a few of the leaves by Mr. A. H. Green. The graded samples were then taken to the New Haven laboratory and prepared for chemical analysis by the same techniques as those used for the other samples with the following exceptions.

The leaves within each grade were counted and measured, and were weighed at the moisture content they possessed at the time of grading. The midribs were carefully cut from the blades and the blade and midrib tissues were separately dried in the oven and brought to equilibrium with air at 50 per cent relative humidity at 24° C. Accurate weights of all samples were then obtained and the tissues were ground for analysis and preserved as described for the other samples. Table 23 gives a record of the grades, the number of leaves that fell into each grade and of the weights.

It is at once evident that the leaves were sorted into a considerable number of grades although more than one-half of them were placed in two grades, LC2 and YL. However, 79 per cent by weight of the leaves were placed in the top four brown grades listed in the Key and an additional 5 per cent, being slightly greenish in color, were placed in the LV grade which commands a price equal to that of the LC grade. The leaves were thus of high quality with respect to grades.

TABLE 23. RECORD OF GRADING OF 400 LEAVES CURED AND FERMENTED IN PARALLEL WITH THE 40-LEAF SAMPLES

Fresh weight 12961 gm.; final weight 1184 gm.

Grade (in order of value)	Number of leaves	Weight of graded leaves	Weight distribution within grades	Oven dry weight			Equilibrated dry weight	
				Per leaf	Blade tissue	Midrib tissue	Blade tissue	Midrib tissue
		gm.	per cent	gm.	gm.	gm.	gm.	gm.
LL	17	56.75	4.8	2.703	37.30	8.65	39.60	9.15
LC	76	235.25	19.9	2.488	152.2	36.85	161.55	38.90
LV	20	60.90	5.1	2.445	38.15	10.75	40.45	11.35
LC2	104	319.40	27.0	2.499	205.55	50.35	218.15	53.10
YL	116	324.75	27.4	2.249	209.55	51.35	222.30	54.20
YL2	26	64.35	5.4	1.998	42.55	9.40	45.20	10.00
AL	16	58.10	4.9	2.938	37.95	9.05	40.20	9.55
XL	14	35.30	3.0	2.039	22.30	6.25	23.65	6.65
XX	11	29.65	2.5	2.159	18.95	4.80	1.15	0.30
Total	400	1184	100		764.50	189.45	782.25	193.20

The grading system employed may require explanation. Tobacco grading is an art that is learned by long experience. There is much that is esoteric and difficult to communicate, but the major factors that are appreciated by the grader in making his almost instantaneous judgments are color and uniformity, "finish," texture, "feel," which among other qualities involves elements of elasticity and thickness, and shape and freedom from damage. The Key for the assignment to the more important grades of Connecticut wrapper tobacco, for which we are indebted to Dr. P. J. Anderson of the Tobacco Laboratory of this Station, shows how the several qualities influence the judgment of the grader. The prices mentioned serve only to indicate relative values among the several grades.

KEY TO GRADES OF SHADE TOBACCO AND RELATIVE PRICES

A Sound grades, leaves not broken or torn
  B Thin leaves
    C Brown grades. Predominantly light brown
      D Not variegated with light yellow patches
        E Highest quality, silky texture, very "spready," *uniform in color,* round tips, inconspicuous veins, no blemishes ..... L ($7.20)
        E Same as L but *colors slightly less uniform* or veins may not be quite as inconspicuous ..... LL (6.60)
        E Inferior to the first two grades in one or more of the following: (1) duller finish, (2) less uniform color, (3) more conspicuous veins, (4) a few blemishes if not too conspicuous ..... LC (5.40)
        E Looks like LC but with *greater tolerance for the four characteristics* listed above. Also may have a narrower shape LC2 (4.20)
      D Variegated with light yellow patches
        E Characteristics of the first three above except for the yellow patches ..... YL (2.70)
        E Poor quality due to dull finish, poor elasticity, non-uniform colors, narrow shape, pointed tips or blemishes YL2 (2.10)
    C Green grades
      D All the characteristics of L or LL except that the leaves have a *greenish* or *olive cast* ..... LV (5.40)
      D Characteristics of LC but with *green cast* LV2 (4.20)
  B Thicker leaves (heavy body)
    C Brown grades
      D Uniform brown color, good finish, good shape, no blemishes ..... AL (2.10)

D Like AL but inferior in one or more of the following: (1) less uniform color, (2) duller finish, (3) more prominent veins, (4) some blemishes AL2 (1.50)

C Green grades

D Darker green and heavier body than LV and LV2 but not as heavy as ML and TOPS (below) ..... VL (1.80)

D Like VL but with duller finish or poorer shape or with some blemishes VL2 (1.20)

D Heavier and darker than VL but not too narrow and pointed ..... ML (.90)

D Very heavy, *leathery*, very dark green to black, short, narrow with pointed tips ..... TOPS (.60)

A Damaged grades

B Broken or torn on one side but with *one sound side* of suitable quality for a wrapper ..... XL (1.80)

B *Both* sides of leaf damaged ..... XX (.24)

Table 24 shows the distribution of the leaves with respect to length, the vertical columns under each length designation giving the distribution among the grades of the leaves of each length. This information is plotted as a block diagram at the left in Figure 11. The distribution with respect to length is somewhat skewed. The mean length is 15.6 inches but the median length is 15.2 inches; that is to say, on the average, half of the leaves are less than 15.2 inches long and half of them are longer than 15.2 inches. The blocks are subdivided so as to show the number of leaves that fell into each of the five best grades. The unlabeled portion at the top of each block shows the number of leaves in grades other than those indicated.

TABLE 24. DISTRIBUTION OF LEAF LENGTHS IN GRADED SAMPLES

Figures are number of leaves of designated length.

Length (inches)	12	13	14	15	16	17	18	19	Total	Mean length
LL	..	..	..	3	10	4	..	..	17	16.1
LC	..	1	5	17	30	22	1	..	76	15.9
LV	..	..	..	2	5	7	5	1	20	16.9
LC2	1	3	15	26	39	18	1	1	104	15.6
YL	..	9	22	35	37	12	1	..	116	15.2
YL2	..	2	12	5	5	2	..	..	26	14.7
AL	..	..	..	3	8	4	1	..	16	16.2
XL	..	1	1	6	5	1	..	..	14	15.3
XX	..	2	2	4	2	1	..	..	11	14.8
Total	1	18	57	101	141	71	9	2	400	

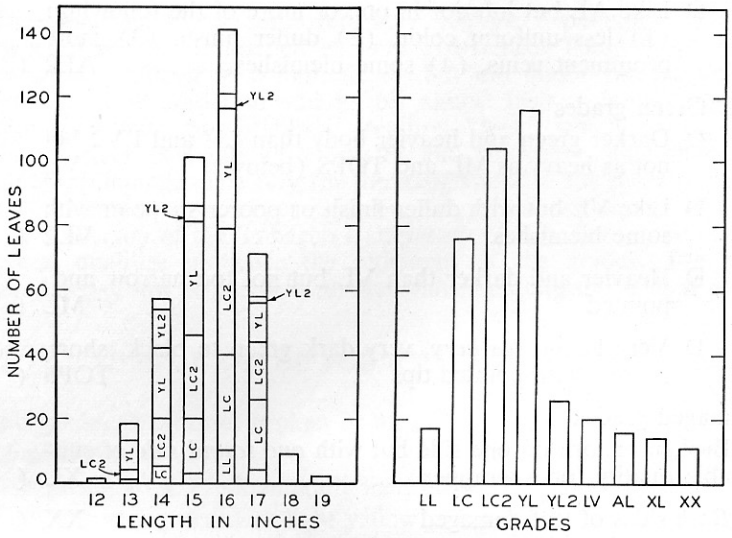

FIGURE 11

Distribution of leaves of the graded sample (sample FER) according to length and also among grades. The lowest subdivisions of the block for 15 and 17 inch leaves show the number of leaves of grade LL.

The distribution of the 400 leaves within the several grades is shown by the block diagram at the right in Figure 11. The predominance of leaves in the LC, LC2, and YL grades is clearly shown as well as the small proportions of leaves in the YL2 and AL grades. There were 313 leaves in the top four brown grades and 20 in grade LV. Thus, 83 per cent of the leaves by number were placed in the most valuable categories, a result which agrees with the calculation of 84 per cent by weight in these same grades. There were approximately equal although small numbers of leaves in the valuable LL and LV grades but none of the leaves in the 400-leaf sample was placed in the highest possible category designated L. Approximately 6 per cent of the leaves were more or less damaged and therefore fell into the X grades; in view of the frequent handling of the samples during the fermentation and storage operations, this is inevitable.

Returning to the data in Table 23, it is of interest to note the oven-dry weights per single leaf within the several grades. The mean oven-dry weight of the 400 leaves was 2.39 gm. per leaf. The leaves of the three top brown grades were all somewhat heavier than this average but those of the YL grade were somewhat below it. The thicker leaves of the AL grade were notably heavier than the average and the leaves of the inferior grades were appreciably lower.

To what extent the results of the grading of this small sample represent what may be anticipated from the study of the entire crop of lower leaves from a significant area of land is problematical. The main point with respect to the present investigation was to establish some measure of the quality of the leaves that were employed in the chemical

analyses of the samples subjected to curing and fermentation. It is clear that these samples were of high quality if the assumption is accepted that the 400-leaf sample collected at the same time as the curing and fermentation samples was fairly representative of the leaves grown on adjacent plants.

Expression of Data

The determinations of the components of the samples subjected to curing and to fermentation were all calculated to the common basis of the weight in that quantity of tissue that would have been obtained at each stage of processing if the original weight of the fresh leaves in each sample had been exactly 1,000 gm. This method of expression furnishes direct information on the magnitude of the chemical changes that occurred. The entire scheme for the selection and management of the samples was designed to facilitate this method of expression.

The FER sample was of necessity handled differently. Although the fresh weight of the entire lot of leaves and both oven-dry weights and equilibrated dry weights of the blade and the midrib tissue of the several graded samples were obtained, there is no way, in advance of the actual curing and fermentation operations, to separate the green leaves into the grade samples that the initial sample of 400 leaves will subsequently yield. Only if this could be done could the fresh weight of the grade samples be secured and the necessary factors obtained for the calculation of the results of the analysis of these samples in terms of a kilogram of initial fresh weight of the leaves from which they were derived.

Only an approximate solution of the problem can be reached. If the factors for the individual samples in the fermentation experiment are examined (Table 16), it will be noted that they are moderately constant. The mean is 0.7854 ± 0.0121, the coefficient of variation being only 1.5 per cent. It would therefore seem that an over-all factor could be computed from the data for the FER samples that would have a moderate degree of validity. The necessary figures are given in Table 23. If 12,961 gm. of fresh leaves yielded 782.25 gm. of fermented blade tissue, 1,000 gm. would have yielded 60.35 gm. and, accordingly, multiplication of the percentage of any component in the blade tissue by the factor 0.6035 will yield the number of grams of that component in the fermented blade tissue derived from 1 kilo of the fresh leaves. Similarly, since a total of 193.2 gm. of midrib tissue was obtained, multiplication of the percentage of any component in the midrib tissue derived from the fermented leaves by the factor 0.1491 will give the number of grams of that component in the midribs derived from 1 kilo of whole green leaves.

However, the application of these factors in the actual case involves the assumption that the behavior of each of the several grade samples was exactly the same with respect to the quantities, distribution and ultimate fate of the several components initially present, an assumption that can scarcely be entirely justified in view of the fairly wide range of quality represented in the different grades.

In order to illustrate the outcome of calculations of this kind, the data in Table 25 have been collected. The percentages of water, ash, and, by subtraction, of organic solids in the blade and midrib portions of the grade samples have been multiplied by the appropriate factors and the

sums of the water, ash, and organic solids in the two parts of the leaves obtained. These are shown in the three columns at the right. The means at the bottom should be compared with the mean values for the FA — FJ series shown in Table 17. The mean ash of the FA — FJ samples was 15.33 ± 0.22 gm. per kilo, whereas the mean ash of the grade samples was 14.49. Similarly, the mean value of the organic solids in the FA — FJ series was 57.04 ± 1.05 which is to be compared with the mean value from the FER sample of 54.9. The discrepancies are large and it must be assumed either that the 400-leaf FER sample was actually lower in ash content than were the samples of the FA — FJ series as well as being lower in organic solids, or that there is a fallacy in the method adopted for the calculation of the data in Table 25. In view of this uncertainty, it seems wisest to forego the advantages in the calculation of the data from the grade samples in terms of grams per kilo and to report the actual percentage values as obtained.

Ash, Moisture, and Organic Solids

The percentage values for the ash and moisture content of both blade and midrib parts of the grade samples are shown in Table 26. Examination of the means and standard deviations at the foot of the table shows that the variation within grades was extremely small. The constancy of the moisture content indicates that there is no striking difference among the grades in the water-holding capacity of the tissues after the samples had been brought to equilibrium at a relative humidity of 50 per cent at 24° C. Furthermore, there is no obvious difference in the ash contents of the samples with the possible exception of the ash in the somewhat greenish leaves of the LV sample. Both blade and midrib tissue of this sample were lowest in ash content of the entire group.

The organic solids content of the samples was also constant within unexpectedly narrow limits as is shown by the small coefficient of variation. It is clear that no discrimination among the several grades can be made in terms of the gross composition with respect to ash and organic solids. However, the proportion of water-soluble solids shows a moderately large variation among the grades. The blades of the LL grade are the highest and those of the LV grade the lowest and it may be noted that the thicker leaves of the AL grade are also high in soluble solids. On the other hand, the midrib tissue of the LV grade is the highest in soluble solids, and that of both the LL and YL2 grades is low.

The results of the analysis of the ash of the grade samples by the spectrographic method are shown in Table 27 and are given in full detail for the blade tissue. The LC2 leaves were low in potassium and calcium and the LL and AL grades were high in these components. Among the trace elements, aluminum and copper varied most widely among the samples, the YL2 grade being outstandingly high in both. However, if the data are taken as a whole, potassium and calcium alone showed variability which may be reckoned as really important with respect to the over-all composition of the ash. The range for potassium was from 4.7 per cent in the LL grade to 3.8 in the LC2 grade; that is to say, two of the grades differed as much as 1 per cent of the dry weight in potassium content. The calcium content ranged from 2.95 (in the LC2 grade) to 3.6 per cent (in the AL grade), a difference of a little more than 0.6 per cent. These are the major components of the ash.

TABLE 25. WATER, ASH, AND ORGANIC SOLIDS CONTENT OF GRADED SAMPLES EQUILIBRATED AT 24° C. AND 50 PER CENT RELATIVE HUMIDITY

Data expressed as grams per kilo of initial fresh weight.

Sample	Blades Factor A = 0.6035			Midribs Factor A = 0.1491			Whole leaf		
	Water	Ash	Organic solids	Water	Ash	Organic solids	Water	Ash	Organic solids
LL	4.71	11.29	44.4	1.11	3.52	10.3	5.82	14.81	54.7
LC	4.85	10.92	44.5	1.07	3.61	10.2	5.92	14.53	54.7
LV	4.77	10.38	45.2	1.08	3.46	10.4	5.85	13.84	55.6
LC2	4.68	10.98	44.7	1.09	3.58	10.2	5.77	14.56	54.9
YL	4.67	11.23	44.5	1.08	3.53	10.3	5.75	14.71	54.8
YL2	4.82	11.10	44.4	1.11	3.49	10.3	5.93	14.59	54.7
AL	4.80	10.86	44.7	1.10	3.52	10.3	5.90	14.38	55.0
XL	4.83	10.74	44.8	1.06	3.59	10.3	5.89	14.53	55.1
XX	4.96	10.80	44.6	1.07	3.58	10.3	6.03	14.38	54.9
Mean							5.87	14.49	54.9
Mean FA — FJ samples (Table 17)								15.33	57.0

TABLE 26. WATER, ASH, ORGANIC SOLIDS AND WATER-SOLUBLE SOLIDS OF GRADED SAMPLES

Data expressed as percentage of equilibrated dry weight.

Grades	Blades				Midribs			
	Water	Ash	Organic solids	Water-soluble-solids	Water	Ash	Organic solids	Water-soluble solids
LL	7.80	18.7	73.5	52.9	7.46	23.6	68.9	44.1
LC	8.08	18.1	73.8	52.0	7.20	24.2	68.6	44.4
LV	7.90	17.2	74.9	49.4	7.26	23.2	69.5	45.8
LC2	7.76	18.2	74.0	51.4	7.31	24.0	68.7	44.2
YL	7.74	18.6	73.7	51.1	7.29	23.7	69.0	44.4
YL2	7.98	18.4	73.6	50.4	7.45	23.4	69.1	44.0
AL	7.96	18.0	74.1	52.5	7.38	23.6	69.0	44.9
XL	8.01	17.8	74.2	...	7.08	24.1	68.8	...
XX	8.22	17.9	73.9	...	7.17	24.0	68.8	...
Mean	7.93	18.1	74.0	51.4	7.29	23.8	68.9	44.5
S.D.	±0.15	±0.46	±0.42		±0.13	±0.34	±0.26	
C.V. (%)	1.9	2.5	0.6		1.8	1.4	0.4	

TABLE 27. SPECTROGRAPHIC ANALYSIS OF ASH OF GRADED SAMPLES

Data expressed as percentage of the equilibrated dry weight except for copper and boron which are in parts per million.

Blade tissue	K	Ca	Mg	P	Mn	Fe	Al	Zn	Na	Cu	B
LL	4.70	3.50	0.96	0.26	0.012	0.059	0.049	0.022	0.051	26	29
LC	4.12	3.30	0.98	0.26	0.013	0.061	0.052	0.022	0.048	24	29
LV	4.46	3.05	0.90	0.25	0.012	0.061	0.054	0.014	0.038	30	22
LC2	3.79	2.95	0.92	0.25	0.012	0.069	0.066	0.025	0.050	26	32
YL	3.85	3.20	0.99	0.25	0.011	0.066	0.067	0.026	0.051	20	32
YL2	4.10	3.40	1.02	0.23	0.013	0.095	0.074	0.017	0.050	55	31
AL	4.67	3.60	0.91	0.24	0.012	0.058	0.050	0.016	0.042	30	27
Mean	4.24	3.28	0.95	0.25	0.012	0.067	0.059	0.020	0.047	30	29
Blades mean × 0.805	3.41	2.64	0.76	0.20	0.009	0.054	0.047	0.016	0.038	24	23
Midribs mean × 0.195	2.84	0.28	0.08	0.04	0.000	0.005	0.007	0.002	0.030	6	3
Whole leaf	6.25	2.82	0.84	0.24	0.009	0.059	0.054	0.018	0.068	30	26
Mean of samples FA — FJ	5.80	2.84	0.88	0.26	0.010	0.057	0.050	0.031	0.059	26	29
Mean of samples A1 — J1	5.13	2.77	0.93	0.28	0.012	0.073	0.089	0.030	0.066	26	41

Complete analyses of the midrib tissues of these samples were also obtained and it is accordingly possible to make a comparison between the composition of the ash of the FER samples and that of the other two series. Inasmuch as the FER samples at the time they were graded consisted, on the average, of 80.5 per cent by weight of blade tissue and 19.5 per cent of midrib tissue, it is possible to compute the quantities of ash components in both blade and midrib fractions from the means of the separate analyses. This has been done in the lower part of Table 27. The sums of the percentages of ash components in the two parts of the leaves given in the third line from the bottom show the percentages in the whole leaves, and these figures may be compared with those in the last two lines which show the mean values obtained from the analysis of the FA — FJ and the A1 — J1 series, these values being also expressed in percentage of the equilibrated dry weight.

The grade samples as a whole were higher in potassium than the FA — FJ and the A1 — J1 samples but the agreement in composition for the other components is strikingly close with the possible exception of the aluminum and boron in the A1 — J1 set. Inasmuch as the three sets of samples were taken from adjacent areas in the same field, it is clear that the composition of the soil with respect to the minor elements was uniform and that the plants absorbed closely similar amounts of these elements. However, there is an obvious variability with respect to the uptake of potassium. This may possibly be a matter of slightly uneven distribution of fertilizer in the field.

Total Nitrogen, Protein Nitrogen, and Soluble Nitrogen

The results of the determinations of nitrogen in Table 28 show differences among the several grades that would appear to be significant. The low coefficients of variation for ash and organic solids in Table 26 indicate that the samples differed little with respect to these factors but the higher coefficients in Table 27 suggest that there are in fact real

TABLE 28. TOTAL NITROGEN, PROTEIN NITROGEN, SOLUBLE NITROGEN AND NITRATE NITROGEN OF GRADED SAMPLES

Data expressed as percentage of equilibrated dry weight of blade tissue.

Grades	Total nitrogen	Protein nitrogen	Water-soluble nitrogen (difference)	Nitrate nitrogen	Water-soluble nitrogen (direct)
LL	4.17	1.28	2.89	0.61	2.68
LC	4.40	1.40	3.00	0.64	2.86
LV	5.14	1.82	3.32	0.65	2.99
LC2	4.22	1.42	2.80	0.64	2.63
YL	4.22	1.40	2.82	0.67	2.72
YL2	4.15	1.48	2.67	0.66	2.60
AL	4.31	1.46	2.85	0.52	2.81
XL	4.60	1.64	2.96	0.73	...
XX	4.19	1.46	2.73	0.65	...
Mean	4.38	1.48	2.89	0.64	2.75
S.D.	±0.32	±0.16	± 0.19	± 0.06	
C.V. (%)	7.3	10.6	6.6	9.0	

differences in fundamental composition. Outstanding among the other samples are the leaf blades of the LV grade; these are the highest in total nitrogen as well as in protein and soluble nitrogen, although the nitrate nitrogen is close to the average for the entire group. In contrast, the somewhat more valuable leaves of the LL grade are lowest in protein nitrogen and are also among the lowest in total nitrogen and in soluble nitrogen. Since the protein nitrogen can be determined with considerable accuracy and precision, it is of interest to place the grade samples in the order of increasing protein nitrogen and to compare this order with that of decreasing relative value. The results are as follows, the damaged grades being disregarded:

Order of increasing protein nitrogen LL LC YL LC2 AL YL2 LV

Order of decreasing commercial value LL LC LV LC2 YL YL2 AL

It is to be noted that four of the grades occupy the same position in both series and that two are out of order by only one position. The greenish grade LV alone is widely out of agreement in position. The LC and LV grades have equal value and this is true also for the AL and YL2 grades so that the closeness of the agreement in order has perhaps been a little overemphasized; nevertheless, with the exception of the LV grade, a hint is given of a relationship between protein nitrogen content and grade value; in the brown grades, high value appears to go with low protein content[1] which is, in turn, an evidence of completeness of digestion of the protein during the curing process.

The exceptional case of the greenish leaves of the LV grade is of interest from the chemical point of view. It would appear that these are leaves in which the destruction of the chlorophyll has not been as nearly complete as it is in the brown grades and this would appear to be true also of the protein content. Furthermore, they are leaves which are unusually high in total nitrogen content and they were, on the average, longer (see Table 24). They must have been unusually high in protein from the beginning, for they are also higher than all of the other grades in soluble nitrogen, much of which is derived from the protein during curing. The digestion of the protein during the early stages of curing had thus gone further with respect to quantity than it had in the other grades but nevertheless had not diminished the residual protein to the level found in the brown grades. These conclusions point in turn to actual physiological differences in the leaves that ultimately find their way into the LV grade.

It would be of the greatest interest to be able to correlate the leaves of the LV grade with some such factor as position on the plant. However, inasmuch as there were only 20 of these leaves in the entire sample of 400 taken from 100 plants, one cannot tell whether or not they represent, for example, the lowest leaves or, alternatively, the highest, for there were 80 other leaves in the entire sample that also occupied this position. The matter can be settled only by an experiment in which a similar sample is collected and each individual leaf identified by label with respect to plant

[1] Dr. W. G. Frankenburg (personal communication) has observed an analogous relationship in comparing the protein content of both Pennsylvania filler tobacco and Connecticut shade tobacco of high and low quality. It should be noted that the present results refer to tobacco grown under uniform conditions with respect to soil and nutrition.

of origin and position on the plant. At present the only suggestion that can be made is that these 20 leaves differed in some way in maturity from the others at the time of collection.

The nitrate nitrogen of the samples requires little comment save to point out the low value in the AL grade and the high value in the XL sample. Aside from these two results, the data are remarkably constant.[1]

Soluble nitrogen was determined by subtracting the protein nitrogen from the total nitrogen and also by direct determination in the water extract of the tissue. The latter values are consistently somewhat lower owing to the presence in the leaves of water-insoluble nitrogenous components that are extracted by alcohol during the preparation of the samples for the determination of protein nitrogen. The differences are small and are negligible for present purposes. Reference may be made to Table 11 for analogous data on the FA — FJ series; the differences in the present case are similar to those there recorded.

Soluble Nitrogenous Components

Table 29 shows results of the analyses of the water extracts of the samples for different forms of nitrogen. Since the samples were graded before being subjected to the process of mulling, it would be useful if the data could be compared with those for sample FH which was taken at the same stage in the process. Direct comparison with the figures in Table 19 cannot be made since the present data are expressed as percentages whereas those in Table 19 are given in grams per kilo of initial fresh weight. However, if the assumption is made that the mean factor for the conversion of the data of the FA — FJ series may be applied to the figures for percentage from the FER samples, an approximate idea can be secured of the magnitude involved. The last line of Table 29 contains the figures so calculated and these may be compared with the data for sample FH in Table 19. The value for ammonia nitrogen, 0.42 gm. per kilo, is practically identical with that for sample FH, indicating that the decomposition reactions leading to the formation of ammonia were approximately the same in the two lots of leaves. Similarly the figure for asparagine amide nitrogen, 0.23 gm. per kilo, is close to 0.19 gm. per kilo found for sample FH. Accordingly the decomposition of the asparagine had progressed to about the same extent in both lots of leaves. There is also a close agreement between the data for free and for total amino nitrogen, the mean of the samples being only a little higher in both components than the FH sample, and the "peptide" nitrogen is almost identical.

These data for the soluble forms of nitrogen are probably a rather sensitive index of the progress of the fermentation, for the curves in Figure 5 show that both ammonia and asparagine amide nitrogen changed rapidly and progressively in the early stage of the process. The conclusion appears to be justified, therefore, that the leaves of the FER samples were graded at a stage in the manufacturing process such that their com-

[1] It should perhaps be emphasized that the present samples were grown on soil heavily and *uniformly* fertilized with nitrogenous fertilizer. Differences in nitrate content between samples or among grades would therefore not be anticipated. However, samples that represent tobacco from fields differently treated would be expected to differ in nitrate content.

TABLE 29. SOLUBLE NITROGENOUS COMPONENTS OF GRADED SAMPLES

Data expressed as percentage of equilibrated dry weight of blade tissue.

Grades	Ammonia nitrogen	Glutamine amide nitrogen	Asparagine amide nitrogen	Amino nitrogen — Free	Amino nitrogen — Total	"Peptide" nitrogen	Sum of ammonia and asparagine amide nitrogen
LL	0.494	0.014	0.318	0.64	0.80	0.16	0.81
LC	0.566	0.000	0.326	0.67	0.79	0.13	0.89
LV	0.569	0.043	0.243	0.76	0.94	0.18	0.81
LC2	0.519	0.000	0.272	0.60	...	...	0.79
YL	0.525	0.006	0.263	0.60	0.75	0.16	0.79
YL2	0.510	0.013	0.217	0.55	...	...	0.73
AL	0.569	0.011	0.313	0.69	...	...	0.88
Mean	0.536		0.293	0.64	0.82	0.16	0.83
Mean[1] as gm. per kilo	0.42		0.23	0.50	0.64	0.13	

[1] It is arbitrarily assumed that the mean factor A 0.7854 for the data of the FA — FJ series may be applied to convert the present percentage data to gm. per kilo. This disregards the fact that the midribs were not removed before analysis of the FA — FJ series.

position resembled rather closely that to be anticipated from the fermentation experiment, and that the curves that represent the changes in composition of the FA — FJ series can with a moderate degree of safety be assumed to represent what had occurred in the FER samples up to the time that the leaves were graded.

It remains, therefore, to consider the differences among the several grades. With respect to free ammonia nitrogen, the LL grade was the lowest and the LV grade the highest, a conclusion that might be anticipated from their respective contents of soluble nitrogen shown in Table 27. Since free ammonia is presumably an index of the extent of amino acid and thus of protein decomposition, one might expect that the samples with the highest soluble nitrogen would be highest in ammonia, and this is so. However, the level of the asparagine amide nitrogen places these two samples in the reverse order. The LL sample that was lowest in ammonia is highest in asparagine amide nitrogen while the LV sample that was highest in ammonia is low in asparagine amide nitrogen, only the YL2 sample being a little lower. In the consideration of the FA — FJ samples, a reciprocal relationship between the behavior of the ammonia and the asparagine amide nitrogen was pointed out; Figure 5 shows that the curve for the one falls while that for the other rises. The effect was accounted for in terms of the hydrolysis of the asparagine amide groups to ammonia. This reciprocal relationship also appears to hold for the present samples and, in the case of the LL and LV samples in particular, it holds with some precision; the sums of the free ammonia and asparagine amide nitrogen of each of these samples are identical, both being 0.81 per cent. The right hand column in Table 29 shows the sums of the free ammonia nitrogen and the asparagine amide nitrogen of all of the samples and it is clear from the approximate constancy of the sums that, with the exception of the YL2 grade, the reciprocal relationship between these

two quantities holds with fair accuracy. This in turn indicates the validity of the general conclusion drawn from the fermentation experiment that the increase in free ammonia represents essentially the decomposition of the amide group of asparagine.

The free amino nitrogen is a measure of the amino acids produced by the decomposition of the proteins. It includes the amino nitrogen of the asparagine and, in fact, between 40 and 50 per cent of the free amino nitrogen is in all cases that of asparagine, the mean being 46 per cent. The remainder represents other amino acids which survived the oxidative deamination reactions that took place during the processing of the leaves. It is to be noted that the proportion of free amino nitrogen in the LV grade is outstandingly high, that in the YL2 grade is the lowest.

The total amino nitrogen after acid hydrolysis was obtained for only the four most valuable grades. Each of these appeared to contain a relatively constant quantity of what has been designated "peptide" nitrogen and the amount found conforms with that observed in the FA — FJ series of samples (see Table 19).

Acidity and Ether-Soluble Organic Acids

The pH of the grade samples, as in shown in Table 30, was practically constant and did not differ to any significant extent from that of the fermented samples throughout the period of actual fermentation (see Table 20). Although a moderate increase in pH was observed to occur in the samples subjected to mulling and storage, the grade samples were collected before this process was carried out.

TABLE 30. ACIDITY AND ORGANIC ACIDS OF GRADED SAMPLES

Data expressed as milliequivalents per 100 gm. of equilibrated dry weight of blade tissue.

Grades	pH	Alkalinity of ash	Total organic acids	Citric acid	Malic acid	Oxalic acid	Undetermined acid
LL	5.50	390	389	136	111	65	77
LC	5.60	384	384	137	101	64	82
LV	5.50	343	360	123	69	67	101
LC2	5.55	382	386	131	104	67	84
YL	5.55	384	398	134	103	69	92
YL2	5.60	390	388	131	106	66	85
AL	5.60	382	386	132	105	64	85
XL	...	361	368	125	85	70	88
XX	...	384	388	133	109	68	79
Mean	5.56	378	383	131	99	66.6	86
S.D.		±12	±12	±4.7	±14	±1.9	±7.2
C.V. (%)		3.2	3.0	3.5	14	2.9	8.3

The alkalinity of the ash was remarkably constant for all of the grade samples with the exception of that of the LV leaves; these were distinctly lower in alkalinity and it is to be noted that that of the XL sample was also

lower than the others. If these two values are omitted, the mean of the remaining seven is 385 ± 3.4 (C.V. = 0.9 per cent). There is no apparent reason why the XL leaves should have been out of line with the others in alkalinity of ash, but the LV leaves have been found to be exceptional in many respects and were also the lowest in total ash content (Table 26).

The total ether-soluble organic acids were found to correspond closely with the alkalinity of the ash throughout the series, the acids of the LV sample being the lowest. The figures given are in each case the average of duplicate determinations and, because of the experimental difficulty of the titration, the discrepancies between the duplicates were in a few cases fairly large and averaged as much as 5 per cent. Nevertheless, the mean values given are moderately satisfactory although the coefficient of variation (3 per cent) is misleadingly small as a measure of the reproducibility.

The citric acid content of the samples was remarkably constant, the variability being no greater than might be anticipated from the general accuracy of the analytical method employed. However, the LV leaves were the lowest in citric acid of the entire group and the XL sample was also low. Citric acid thus followed the indications given both by the determinations of the alkalinity of the ash and of the total organic acidity.

The results of the determinations of malic acid conform with some accuracy to those of citric acid. Most of the grade samples were identical in malic acid content within the limits to be expected of the analytical method, but the LV leaves were outstandingly low in this acid and there is little question of the validity of the result. The XL sample was also well below the mean of the other samples (106 m.eq. per cent for seven samples).

On the other hand, oxalic acid was substantially constant throughout the set of grade samples: there is no evidence that either the LV or the XL leaves differed in oxalic acid content from the other samples. The undetermined organic acid, being calculated in each case by difference from the results of the determination of the total acidity and those for citric, malic, and oxalic acids, is not known with sufficient accuracy for any valid conclusion to be drawn from the results although the figure for the LV leaves suggests that this sample may have been the highest in the acids of this group.

It is unfortunate that the present data cannot be converted with accuracy to milliequivalents per kilo of initial fresh weight of the leaves so that a comparison with the results of the analysis of the FA — FJ samples can be established. Using the mean factor A (0.7854) of the FA — FJ series, it is found that the alkalinity of the ash of the grade samples corresponds with 297 m.eq. per kilo which is satisfactorily close to the mean value 292 of the FA — FJ series (Table 20). However, the mean citric, malic, and oxalic acid contents of the grade samples appear to be respectively, 103, 78, and 55 m.eq. per kilo and these figures are not in satisfactory agreement with the respective mean values 88, 67, and 49 (Table 20) for the FA — FJ series. Nevertheless, the ratios of these figures are relatively constant (103 : 88 = 1.17; 78 : 67 = 1.16; 55 : 49 = 1.12) so that it may be inferred that, if the correct conversion factor were known, the mean organic acid composition of the two sets of samples would be found to be closely similar.

Lipides and Crude Fiber

The data in Table 31 for the lipides indicate that the grade samples were uniform in composition with the exception of the leaves of the YL2 grade. These appear to have been definitely low in residual lipide content. The leaves of the LV grade were also a little below the average. It is interesting to note that the thicker grade AL leaves were the highest in lipide content; no other of the components examined was present in unusual proportions in these leaves.

TABLE 31. LIPIDES AND CRUDE FIBER OF GRADED SAMPLES

Data expressed as percentage of equilibrated dry weights of blade tissue.

Sample	Lipides: A.O.A.C. method	Crude fiber
LL	3.43	6.90
LC	3.36	7.12
LV	3.20	7.00
LC2	3.55	7.26
YL	3.46	6.74
YL2	3.07	6.95
AL	3.60	6.72
Mean	3.38	6.96
S.D.	±0.19	±0.19
C.V. (%)	5.6	2.8

The crude fiber was also substantially uniform throughout, the leaves of the LC2 grade being the highest and those of the AL grade the lowest.

Determinations of lipide and crude fiber were made on some of the samples of midrib tissue. As might be expected, lipide was low in this tissue, the mean content of the LC, LC2, and YL grades being 0.74 per cent. Fiber was, however, high, the mean value for the same three grades being 18.4 per cent with little variation among the three.

The Question of Chemical Differences among Grades

As has been pointed out, the factors that lead to the assignment of individual fermented tobacco leaves to the various grades are to a considerable extent esthetic in nature. The user of wrapper leaves is concerned with uniformity of color, with texture, and with "finish." In addition, importance is attached to such matters as a size and shape that permit the maximum number of wrappers to be cut from a single leaf. The buyer is willing to pay a high price for material that satisfactorily meets his requirements, for the purchaser of the box of cigars demands uniformity in appearance. He obtains satisfaction if a high standard in this respect is met. Such matters are of wide general importance, for the appearance of many if not most agricultural crops is a large factor in the price they bring; it is instinctive to associate high quality with good appearance.

The chemist may be allowed his doubts that, weight for weight, small green peas or deep red beets are superior in nutritive properties to the larger peas or paler beets that can be bought for appreciably less. He is faced, nevertheless, with the fact that such differences in appearance are real factors in the marketing of agricultural products and it is abundantly clear, from the relative valuation of the various grades of wrapper tobacco, that appearance is a vital factor in the tobacco industry. To what extent, then, can the chemist account for differences in grades of tobacco?

Consideration of the data of the present experiment leads to the conclusion that, with present analytical methods, a clear discrimination can be made only with respect to differences between the brown grades on the one hand and the greenish leaves of the LV grade on the other. Throughout the tables, the LV grade with few exceptions gave chemical results that were appreciably different from those given by the brown grades. Although too much significance might not be assigned to a single observation such, for example, as that the LV leaves were outstandingly low in alkalinity of ash or outstandingly high in residual protein content, the combination of so many such observations indicates that the leaves which ultimately fell into the LV grade were physiologically different from the start of the curing process. Whether or not this was a matter of their maturity at the time of picking cannot be decided from the present evidence but is open to later experimental study. In this connection, it may be significant that the leaves in the LV grade were on the average longer than those of any other grades (Table 24).

With respect to chemical differences among the brown grades, little can be said. Although there must in fact be chemical differences at the level of the cell between two leaves one of which has been cured and fermented to a uniform brown shade while the other is variegated with yellow patches, a difference which involves a 100 per cent variation in value, these differences are so small as to defy detection by the present methods when the whole leaf is ground up for analysis. It is possible that chemical study of the pigments would reveal significant differences and it is quite likely that investigation of isolated patches of leaf tissue of different color might be rewarding. This, however, has not yet been attempted. The discovery of the physiological or chemical factors that control the behavior of different regions of the same leaf with respect to the alteration of the pigments during curing and fermentation is a challenging and extremely difficult problem for which no immediate solution appears to be forthcoming.

Part IV

RECAPITULATION AND DISCUSSION

The Chemical Reactions that Occur during Curing

Although incidental discussion of the evidence for various chemical reactions that occur in tobacco leaves during the curing process has been presented along with the data, it may be useful to recapitulate and summarize the information that has been obtained. As was pointed out in the introduction, the main event, at least in terms of quantity of substance involved, is the evaporation of water from the leaves. The concentration of the solutes in the cell sap increases after four days by a factor of 2 and after eight days by a factor of about 5; at 12 days only one-eighteenth of the original water content remains. In spite of this, however, many of the chemical changes that occur closely resemble those that have been observed in tobacco leaves that have been cultured in water in darkness under conditions such that serious loss of water from the system did not occur (44).

From many points of view, the most fundamental chemical change that takes place is the loss of organic solids from the leaves. This amounted to about 16 per cent of the organic solids initially present and the reaction was practically complete at the end of eight days when the leaves had, for the most part, passed through the yellow and into the brown stage. The phenomenon is attributed to respiration of the still living cells of the leaf, and its termination is evidence of the death of the cells. The initiation of the formation of brown spots was observed in a few of the leaves as early as the fourth day, and had become moderately extensive in many of the leaves by the sixth; by the 12th day only a few leaves retained substantial areas of yellow color, most of them having become entirely brown. These changes in color correlate rather precisely with the behavior of the organic solids and, by inference, with the phenomenon of respiration. After 12 days there was only a small further loss of organic solids and, it may be noted, there was little evidence of extensive subsequent chemical change in the composition of the leaves. The chemical reactions that had been going on with striking rapidity during the first few days of the curing process came to an end and the subsequent changes were slow and difficult to demonstrate.

The browning reaction itself is not at all well understood. Analogy with such phenomena as the color change in freshly cut surfaces of certain fruits suggests that the browning reaction arises from the activity of oxidizing enzyme systems of the tyrosinase type. The wounded cells at the surface of the cut fruit contain phenolic substances which are oxidized by the oxygen of the air with the formation of brown pigments. Perhaps a more striking analogy is furnished by the so-called "fermentation" of tea leaves. Tea leaves, after being gathered and allowed to wilt, are rolled either between the hands or by machine, a process whereby mechanical damage is done to many of the cells. This operation suppresses evidence of normal respiration and permits the penetration of phenolic substances of the tannin type into the damaged cytoplasm where oxidation occurs. Roberts (28) holds that the primary effect is the enzymatic oxida-

tion of ascorbic acid with the formation of dehydroascorbic acid and hydrogen peroxide. Tannin, which makes up some 20 per cent of the solids, is then oxidized by the effect of peroxidase and hydrogen peroxide presumably to substances containing *o*-quinone groupings which may then polymerize with the formation of a series of products of color ranging from reddish-brown to dark brown. The dehydroascorbic acid is supposed to become reduced again in the course of the oxidation of carbohydrate components of the tissue and can then renew its catalytic role. A further complication of the system arises from the possibility that ascorbic acid may react with the *o*-quinone to form dehydroascorbic acid and a catechol. Thus there are two possible fates of the *o*-quinone immediately on its formation; it may polymerize or it may be reduced to the phenolic form. However, the net effect, inasmuch as a part of the *o*-quinone may be expected to polymerize on each cycle of reaction, is the ultimate transformation of much of the tannin into its colored polymerization products.

The fermentation of tea is a rapid process and is completed in a few hours; the product, on being dried, becomes the tea of commerce. The chemistry and particularly the nature of the enzymatic reactions during the fermentation of tea have been studied in detail in recent years by Roberts (see a long series of papers of which (29) is the most recent). To what extent these researches, of which the above statement is a brief summary, may bear upon the problem of the browning reaction in curing tobacco leaves is questionable. Tea fermentation takes place in deliberately damaged leaves and, because of the high concentration of phenolic substances present and of the physical conditions under which the process is carried out, is a rapid reaction. The browning of tobacco leaves takes place in undamaged, although greatly dehydrated, tissue and is, in comparison, a slow reaction. The proportion of tannins including polyphenols and phenolic acids in the freshly harvested leaves of the cigar type, according to Frankenburg (5), is low, being only about 2.5 per cent of the dry weight, and little is known of their chemical nature save that rutin (3, 5, 7, 3′, 4′-pentahydroxyflavone-3-rutinoside) is present to the extent of about 1 per cent or less. The part taken by this substance in the browning reaction is quite unknown. However, the reaction begins only after the dehydration of the leaves to a point where extensive disorganization of the structure has taken place and this may be the factor which permits contact between reactants that have previously been held apart. To this extent, the initiation of the reaction has an analogy with that which takes place in tea leaves after deliberate mechanical damage.

As was pointed out in the introduction, the interpretation of the data obtained by the available methods of analysis in terms of specific chemical reactions that can be expressed by equations is possible only in a few cases. The best that can be done in general is to point out types of reactions that may be supposed to bring about the specific changes in composition observed. This applies particularly to the observation that nitrogen is lost from the leaves during the curing process. Sufficient accuracy has not yet been attained either in sampling or in the analytical operations to permit conclusions as to where in the process this loss begins to become significant. That it is unlikely that nitrogen in any form can escape from the leaves early in the process when the tissues are still more or less highly hydrated has been mentioned. Nevertheless, a loss of nitrogen did occur and it seems probable that the

reaction began at some time after the leaves had become brown and were rather thoroughly dried out. The loss then continued throughout the fermentation and storage periods and the most likely hypothesis to account for it is the extremely slow evaporation of ammonia together with a little of the nicotine.

A scarcely more satisfactory account can be given of the behavior of the protein in the early stages of the curing process. It is generally held today that the level of the protein in a living plant tissue is the resultant of two opposing and continuous processes, protein synthesis and protein decomposition under the action of proteolytic enzymes. Synthesis requires the provision of energy, presumably in part at least derived from the respiration of carbohydrates, whereas hydrolysis liberates energy and may thus be presumed to be spontaneous. The balance between these two reactions must be a rather sensitive one, for loss of protein can be detected in tobacco leaves within a few hours after they have been detached from the plant (unpublished observations), and factors other than and in addition to the mere maintenance of a certain level of respiration are doubtless involved. It seems clear, however, that the hydrolytic process assumes dominance shortly after the leaves are picked, and the rate and extent of the decomposition of the protein during curing are closely similar to what is seen in leaves cultured in water in darkness. The factors introduced by the dehydration of the tissue and the corresponding concentration of the solution in which the hydrolytic process is going on do not seem to exert any great influence until dehydration has become extreme. Hydrolysis then ceases and that part of the leaf protein which has survived the early stage remains apparently completely unaffected throughout the rest of the curing period and also throughout the fermentation operation. It is probable that the proteolytic enzymes become inactivated as the leaves pass into the brown stage, but experiments designed to test this hypothesis have not yet been attempted.

Correlated with the rate of decomposition of the protein is the rate of accumulation of soluble forms of nitrogen, and it is of more than passing interest that only a little of this soluble nitrogen is in the form of peptide nitrogen. The hydrolysis of that part of the protein which is attacked by the enzymes is evidently complete to the amino acid stage or nearly so. This implies the presence of enzymes of a type capable of hydrolyzing all kinds of peptide bonds.

The behavior of the protein and of the amino acids produced from it is closely similar to that observed many years ago by Schulze in his investigations of the behavior of the protein of lupine seeds during germination. Why this should be so is, of course, a question for which no answer can be offered. The fact remains that there are enzyme systems present in the sprouting seed and also in the mature leaf which are analogous in function. Amino acids produced by the hydrolysis of the protein are promptly and efficiently deaminized with the formation of ammonia. The ammonia then combines with a non-nitrogenous substance, which is assumed on general chemical grounds to be oxaloacetic acid or a closely related substance, with the formation of asparagine, aspartic acid being a probable intermediate in the chain of reactions. Chemical equations that represent the main successive steps can easily be written although it remains for

future research to define the details of the reactions,[1] their relationships to the supporting reactions whereby the non-nitrogenous precursor of the asparagine is produced, and to discover the enzymes and co-factors whereby each phase of the successive changes is catalyzed.

In order to illustrate the significance and magnitude of the changes that occurred in the forms of soluble nitrogen, the data in Table 32 have been assembled from the figures in Table 9. The increases, after 8 and after 16 days of curing, of the asparagine, glutamine, and ammonia nitrogen and of the amino nitrogen other than that of the two amides have been set out and added. The sums are, respectively, 1.057 and 1.127 gm. These sums agree almost exactly with the separately determined increases in the soluble nitrogen of 1.08 and 1.14 gm. Accordingly, the change in the soluble nitrogen can be quantitatively accounted for in terms of the increases in the four components mentioned. Furthermore, the change in the asparagine nitrogen alone accounts for 71 and for 67 per cent of the protein nitrogen that had disappeared at the expiration of each of these two periods; the sums of the four components account, respectively, for 84 and 87 per cent of the protein nitrogen that disappeared. These are extraordinarily high figures. They suggest either that the decomposition of the products of hydrolysis of the protein was even more complete than would have been supposed on the assumption that deamination of the amino acids produced was the only reaction that occurred, or that amino acid components of the tissue present from the start became involved in the transformations. The latter view is the more probable. In either case, the efficiency of the chemical changes is remarkable.

Synthesis of asparagine ceased after eight days of curing. Loss of organic solids from the system had become extremely slow at this time, and was no longer detectable after 12 days. Thus the source of energy and presumably also the source of the precursor essential for the synthesis of asparagine became exhausted at the same time that the synthesis could no longer be detected. Regarding the nature of the precursor, only one fact

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[1] The reactions which are assumed to occur may be outlined as follows:

$$\underset{}{\text{Protein}} \rightarrow \underset{\text{Amino acids}}{\text{R-CH(NH}_2\text{)-COOH}} \qquad (1)$$

$$\underset{\text{Amino acids}}{\text{R-CH(NH}_2\text{)-COOH}} + \tfrac{1}{2}\text{O}_2 \rightarrow \underset{\text{Keto acids}}{\text{R-CO-COOH}} + \underset{\text{Ammonia}}{\text{NH}_3} \qquad (2)$$

$$\underset{\text{Oxaloacetic acid}}{\text{COOH-CH}_2\text{-CO-COOH}} + \text{NH}_3 + \text{H}_2 \rightarrow \underset{\text{Aspartic acid}}{\text{COOH-CH}_2\text{-CH(NH}_2\text{)-COOH}} + \text{H}_2\text{O} \qquad (3)$$

$$\underset{\text{Aspartic acid}}{\text{COOH-CH}_2\text{-CH(NH}_2\text{)-COOH}} + \text{NH}_3 \rightarrow \underset{\text{Asparagine}}{\text{CONH}_2\text{-CH}_2\text{-CH(NH}_2\text{)-COOH}} + \text{H}_2\text{O} \qquad (4)$$

Reaction (1) represents the hydrolysis of the protein to a mixture of amino acids. Reaction (2) represents the oxidative deamination of the amino acids with the production of ammonia. Reaction (3) is the reductive amination of oxaloacetic acid to aspartic acid and reaction (4) the amidation of aspartic acid to asparagine. Each of these reactions is the summation of what is doubtless a more or less complex sequence of reactions, each step of which is controlled by enzymes. For example, between reactions (3) and (4) there is probably a series of steps involving phosphorylation and interaction with adenosinetriphosphate.

TABLE 32. CHANGES IN FORMS OF SOLUBLE NITROGEN DURING CURING

Data as grams per kilo of fresh weight.

	After 8 days	After 16 days
Change in asparagine amide N × 2	0.896	0.860
" " glutamine amide N × 2	0.058	0.044
" " ammonia N	0.048	0.208
" " corrected amino N	0.055	0.015
Sum	1.057	1.127
Change in soluble N	1.08	1.14
Decrease in protein N	1.26	1.29
Increase in asparagine N as percentage of decrease of protein N	71	67
Increase in four components as percentage of decrease of protein N	84	87

is definitely known, namely that it is a non-nitrogenous substance. The only likely *nitrogenous* precursor would be aspartic acid derived from the protein by direct hydrolysis, and the quantity available on any reasonable assumption of the aspartic acid content of the proteins of the leaf is far too small. Furthermore, all experimental evidence from other tissues than the tobacco leaf, and especially from seedling tissues, argues against such an explanation. That the precursor is oxaloacetic acid or a closely related substance is, however, a speculation although there is much evidence in its favor.

It is a matter of assumption that the reaction undergone by the amino acids immediately after their liberation from the protein is an oxidative deamination. The products of such a reaction would be ammonia and the corresponding α-keto acid. Concerning the subsequent fate of the keto acids, little or nothing is known although it is quite probable that further oxidation takes place, and in a few cases, for example the rhubarb leaf (45), evidence has been obtained that these substances may furnish a part of the substrate of respiration. The fate of the ammonia is, however, quite clear; it is promptly converted into asparagine. The puzzle presented by the behavior of asparagine in curing tobacco leaves, as well as by the data of other studies of plant tissues under analogous circumstances, is to account for the fact that asparagine, and to a somewhat lesser extent glutamine, should be stable in a *milieu* in which all other α-amino acids are attacked and destroyed by the enzyme systems present. The cyclic structure proposed by Steward and Thompson (34) for asparagine may be a possible explanation of the difference between the behavior of this substance and that of the other amino acids. Such a suggestion remains, however, a speculation.

The behavior of the glutamine and the fact that only a relatively small quantity of this amide is formed during the early stage of the curing process both suggest that glutamine is a primary product of the hydrolysis of the protein in the tissues. Like asparagine, it appears to enjoy a higher degree of stability than do the other amino acids, but glutamine in comparison to asparagine is not a stable substance in solution. That the quantity present should gradually decrease over the long period of

curing and subsequent fermentation is not surprising and no specific mechanism other than slow spontaneous hydrolysis need be invoked to account for its behavior.

With regard to the behavior of the other major soluble nitrogenous component of the leaves, the nitrate, little comment is necessary. Nitrate accumulates in the leaves in considerable quantities under the agricultural conditions used for the production of shade tobacco and is the source of the nitrogen for the synthesis of the nitrogenous organic substances that are produced during growth. Although little is known about the sequence of reactions whereby this highly oxidized form of nitrogen is reduced, presumably ultimately to ammonia, and made available for synthesis, it seems clear from the data that have been presented that reduction of nitrate does not occur to a measurable extent during the curing process. Although the curve in Figure 5 indicates a moderate degree of irregularity in the amount present, the variations do not exceed those to be anticipated from the analytical method used and there is no clearly defined trend in spite of the fact that the last observation is the lowest.

The changes in the pH of the leaves during the curing process are small. There was an initial rise of the order of 0.4 pH units representing a gradual decrease in acidity which continued during the period of maximal chemical activity in the first eight days. Subsequently, the pH remained fairly constant for the greater part of the time the leaves were in the curing barn although the trend of the observations was downward, representing a minor increase in acidity. It has not been found possible to account for these changes in terms of the alterations in the content either of the ammonia or of the organic acids of the tissues. The system that controls the pH reaction of the leaves is manifestly a complex one and the buffering effect of the organic acid components is high in the vicinity of pH 5.4, the reaction of green tobacco leaves.

The alkalinity of the ash of the leaves and the total ether-soluble organic acidity were essentially constant throughout the curing period. That the alkalinity of the ash should be constant is to be anticipated, for there is no mechanism whereby the inorganic components which are ultimately determined in the ash can change. In tobacco leaves cured on the stalk, migration of inorganic components out of the leaf into the stalk is possible, and changes in the alkalinity as well as in the total quantity of the ash may be expected. But the samples of leaves taken for the curing experiment constitute a closed system save for the exchange of volatile or gaseous substances with the atmosphere. Ash components must remain constant.[1]

Although the total ether-soluble organic acidity was constant over the greater part of the curing period, there was a small but probably significant loss of organic acids during the first four days. This was a period when the still living cells were subjected to maximal physiological stress, and respiration was proceeding at what seems to have been a high rate. The carbohydrate components, especially the small quantity of starch, were speedily exhausted and it seems likely that use was made of a small

[1] Frankenburg has drawn attention (6, 7) to a mechanism whereby the ash content of tobacco leaves may diminish, especially during fermentation. The transformation of the "gums" on the surface of the fresh leaf into products that are no longer sticky and which may become detached during the frequent handling and shaking of the bunches of leaves may lead to actual mechanical loss of ash components. This was not observed in the present experiment.

proportion of the organic acids. However, some factor that limited the extent of this reaction became operative after the fourth day and no further detectable change in the total ether-soluble organic acids occurred.

Notwithstanding the very moderate change in the total quantity of the organic acids present, an alteration of the greatest importance took place in the composition of the organic acid fraction. Malic acid diminished rapidly during the first 12 days of the curing period and citric acid increased. All of the evidence of the present experiment together with observations from many experiments in which tobacco leaves were cultured in water or in various culture solutions supports the view that malic acid is transformed into citric acid. Conclusive proof of this relationship has recently been obtained by Burris and his associates with the use of radioactive carbon.

The extent of the reaction during the first two days of curing is closely similar to that observed when tobacco leaves are cultured in darkness in water for the same period. The ultimate total change during curing involved a substantial proportion of the organic solids of the leaves, for malic acid equivalent to 9 per cent of the organic solids disappeared, its place being taken by citric acid equivalent to 6.6 per cent of the solids initially present. Thus this chemical change ranks with the formation of asparagine with respect to the proportion of the leaf solids involved. It was a major reaction.

The disappearance of ether-soluble components during the early phase of curing was also a reaction of considerable importance in terms of the quantity involved. The loss amounted to 2.4 gm. in eight days or almost exactly half of the lipides present at the start. This is 3.4 per cent of the organic solids initially present. In the absence of analytical methods whereby the composition of the mixture of ether-soluble material can be determined in a routine manner, no definite information has been obtained regarding the nature of the substances that disappeared. A substantial part doubtless consisted of chlorophyll, for about 1.8 gm. per kilo of this substance is present in tobacco leaves and all visible green color vanished from the ether extracts after about six days. Nevertheless, it is possible that a part of the products of the decomposition of chlorophyll would be extractable from the tissue by ether so that the estimated loss of 1.8 gm. of chlorophyll does not necessarily account for the greater part of the total loss of lipide material.

Only negligible quantities of reducing substances calculated as glucose remained in the leaves after six days of curing. The small residual quantity may well represent substances other than sugars which have the capacity to reduce the sugar reagent. An estimate of the total quantity of simple carbohydrates which disappeared in this period is 2.5 gm. per kilo. In addition, about 0.6 gm. of starch also vanished. Thus the total amount of carbohydrate material which was presumably used up in respiration during the first six days is a little more than 3 gm. or about 4.4 per cent of the organic solids initially present. Thus this chemical change is also one of important relative magnitude although the details of the chemical reactions that occurred are by no means clear.

The Chemical Reactions that Occur during Fermentation

The nature of the chemical changes detected by the analytical methods that were employed for the study of the leaves subjected to

fermentation can be briefly recapitulated. For the most part there was remarkably little alteration in the apparent chemical composition of the leaves, notwithstanding that fermented tobacco leaves are different from cured leaves in appearance, "feel," odor, and in all of the factors that go to make up the concept of tobacco "quality." Whatever the changes in composition may be which bring about the marked and unmistakable difference between cured and fermented tobacco, they are clearly subtle in their nature and do not lend themselves to detection by the methods of analysis presently used. This observation is in itself of considerable importance. It is obvious that the methodology to be employed in future research on fermentation, as this operation is carried out with Connecticut wrapper tobacco, must be altered. The methods that were developed and found valuable for the study of the chemical changes that occur particularly in the early stages of curing do not concern themselves with the detection of the chemical changes in the components of the leaves which are responsible for the development of the characteristic appearance, "feel," and color of the fermented leaves. No attention has been given to the nature of the substances to which the unique odor of fermented tobacco is due, although it is clear that specific volatile components must be slowly generated or other components that mask the characteristic odor must be decomposed during the process. Attention must also be given to the nature of the components which, on being burned, give rise to the aroma which is valued so highly. The study of fermentation, in a word, is a different problem from the study of curing, and specific chemical methods must be developed which are suited for the detection of the nature of the changes that occur. Until this is done, the chemistry of fermentation will remain for the most part a field for speculation.

The present investigation has shown that there is no detectable change in the composition of the fermenting leaf with respect to its content of ash, protein, crude fiber, nitrate nitrogen, and ether-soluble organic acid components. The small quantity of what the analytical evidence suggests is "peptide" nitrogen also does not change.

There are small but more or less continuous losses of total nitrogen, soluble nitrogen, and amino nitrogen which may well be measurements in part at least of the loss of the same substance or group of substances. They were accompanied by a small decrease of organic solids and probably of lipide material. The small quantity of glutamine amide nitrogen that survived the curing operation also disappeared during fermentation, an observation that is not surprising in view of the well-known instability of glutamine.

Only two of the components of the fermenting leaves for which analytical methods are available showed what may be termed major alteration in the amount present. Asparagine amide nitrogen dropped during the first phases of the actual fermentation in the bulk, remained constant throughout the rest of this part of the treatment but then diminished almost to zero during the protracted storage period. Free ammonia nitrogen increased in a manner that was correlated in almost exact detail with this diminution of the asparagine amide nitrogen so that the conclusion could be drawn that the increase in the free ammonia was the result of the hydrolysis of the asparagine amide nitrogen. This change clearly suggests the presence of a specific amide hydrolyzing mechanism and raises the question of the nature of this mechanism. Is it the result of the

activation of a specific hydrolyzing enzyme system present in the leaves from the beginning or is it evidence of the specific decomposition of asparagine by the microbiological flora which developed during the early stage of the fermentation? Decision between these possibilities is difficult. Neither hypothesis accounts at all well for the cessation of both amide hydrolysis and the formation of free ammonia after the bulk had been turned three times (see Figure 5) and for the renewal of these two processes during the long period of storage. However, the stability of the asparagine in the later phases of curing and up to the point at which fermentation in the bulk was established is an argument against the hypothesis that the decomposition of the asparagine is caused by an intrinsic leaf enzyme system which, with only a minor increase in the moisture content of the leaves, became activated during the first three turnings of the bulk.

Any attempt to interpret the present observations is necessarily highly speculative but may lead to a better understanding of how little is really known about the fermentation process. Fortunately, the meaning of some of the analytical results is clear. The constancy of the ash and of the alkalinity of the ash is to be expected if no opportunity is afforded, during the treatment in the bulks, or in storage, for transfer of material into or out of the samples or for mechanical loss. It would appear that this essential requirement for the validity of the present analytical results is indeed satisfied. Had there been significant interchange of solid components either by diffusion or otherwise, some variability of the ash would doubtless have been detected since certain of the components, for example, the potassium, could conceivably migrate if the moisture content of the leaves were high enough and adjacent leaves were sufficiently closely pressed together. Examination of the data from this point of view shows only one point at which the least suspicion may be entertained that transfer of ash components may have occurred, namely during the long period when the last sample collected (sample FJ) was compressed into a bale which stood for nearly a year. The potassium content of the FJ sample was appreciably higher than any of the others, being 5.34 gm. per kilo as compared with the average of the 10 samples of 4.56 gm. per kilo. The next highest value was 4.83 gm. per kilo in sample FB. The total ash content of the FJ sample was also the highest (Table 17) being 15.8 gm. per kilo, the average of the 10 samples being 15.3 $\pm$ 0.2. The other samples showed no pattern of variation and thus the hypothesis that migration of ash components may have occurred rests upon the analysis of a single sample. Further experimentation would be required to demonstrate that migration of ash components did indeed occur during the storage of the bale. The present experiment merely shows that this was a possibility.

The constancy of the protein throughout the fermentation and storage periods indicates that the activity of the proteolytic enzymes had ceased either because they had been destroyed early in the curing period or that the physical conditions essential for activity were at no time established during fermentation. The former view seems the more likely since proteolytic enzymes are themselves proteins and might well have become denatured under the conditions brought about by the extensive dehydration of the tissues.

The present data are of particular interest since they do not show the phenomenon repeatedly observed by Frankenburg (6) of an increase

in apparent protein nitrogen after fermentation. Frankenburg's data were obtained from Pennsylvania Seed leaf tobacco which had been subjected to the severe fermentation process employed for the preparation of filler tobacco. Under such conditions, an increase is invariably noted in insoluble nitrogen, whether determined by extraction with water or with the conventional reagents employed for the determination of protein. The result is interpreted by him as an evidence of the formation of insoluble compounds of, presumably, amino acids and quinone-like components of the leaves. Frankenburg has pointed out that an analogous phenomenon is observed in the case of the fermentation of tea, a leaf tissue notably high in polyphenolic substances which give rise to quinones on oxidation. The fermentation process as used for the treatment of Connecticut wrapper tobacco apparently is not sufficiently severe to afford an opportunity for such reactions to occur to a significant extent.

The constancy of the components determined as crude fiber is to be expected. Frankenburg's data show that even the far more severe fermentation employed for filler tobaccos does not bring about changes in the quantity of the cellulose and lignin which make up the crude fiber, and the present observations are, like the data for the ash, evidence of the satisfactory degree of constancy in the initial composition of the samples achieved by the sampling methods used.

However, the constancy of the total ether-soluble organic acids, and particularly of the chemically reactive substances malic and citric acids throughout the fermentation operation was not anticipated and does not conform with Frankenburg's observations on filler tobacco. He has found that substantial losses of both malic and citric acids occurred during fermentation and that oxalic acid increased slightly. Again, the more severe conditions of the fermentation of filler tobacco appear to have been responsible.

The small loss of total nitrogen that was observed is to be expected and is presumably due to the evaporation of some of the ammonia and of some of the alkaloids. Frankenburg has demonstrated the presence of both ammonia and nicotine in the gases withdrawn from the middle of a fermenting case of tobacco. Loss of these components would be reflected in the data for the soluble nitrogen as was observed. However, there was also a loss, although a small one, of amino nitrogen. This implies the activity of some mechanism that attacked the amino acids and either decomposed them, liberating ammonia, or that brought about a combination of some substance with the amino nitrogen to give products that do not respond to the nitrous acid method for the determination of α-amino nitrogen. Frankenburg places considerable emphasis upon the latter possibility in the case of the fermentation of filler tobacco, the reactive substance being supposed to be an ortho-quinone which condenses with the amino group.

With respect to the small losses of organic solids and lipide components, little can be added to what has already been pointed out. Loss of organic material with the ultimate production of carbon dioxide and water and with the absorption of oxygen and accompanied by the generation of heat has been repeatedly demonstrated during fermentation whether conducted on the commercial scale or in small experimental samples. The effects have been variously interpreted as evidence for metabolic changes catalyzed by the intrinsic enzymes of the leaves, as evidence for

the metabolism of the microbiological flora and as evidence for purely chemical reactions involving oxidations under the influence of such catalysts as heavy metal-containing compounds or polyphenolic substances. Each view of the process has its advocates and decision among the three possibilities as explanations of the fundamental reactions of fermentation remains for the future. As has been pointed out by Frankenburg, all three types of catalyst may contribute a share to the total effect and differences in technique of fermentation and in type of tobacco may well influence the relative significance of the several possible modes of action. A general theory of tobacco fermentation is still to be developed.

The Composition of the Graded Samples

In spite of the high degree of uniformity in the initial composition of the individual samples collected for the study of curing and fermentation, the leaf material used for the present investigation was not uniform as a whole as judged by the criteria applied by the tobacco grader. The leaves of the FER sample, after curing and fermentation, were found to fall into nine different grades, although 84 per cent by weight and 83 per cent by number were placed either in the best four brown grades or the one greenish grade used in classifying this lot of leaves. Most of the leaves were thus of relatively high quality, but the uniformity that was the hoped-for outcome of the careful selection of the plants and of the leaf positions chosen for the experimental material was not achieved.

As in the case of the study of the samples collected during the course of the fermentation experiment, it became apparent that the analytical methods used are not entirely appropriate for the investigation of the differences between leaves that are placed in the several commercial grades. These differences are indeed considerable when expressed in terms of the relative value of the graded leaves, but, for the most part, the differences in the chemical composition of the material are too small to be effectively demonstrated. The one grade that stood out from the others in composition was the LV sample which consisted of 20 leaves or 5 per cent of the entire lot. These leaves were the highest or the lowest of all of the grades with respect to many analytical components. It became clear that the leaves which ultimately fell into this grade must have been different from the start in many ways. However, aside from the fact that the LV leaves were, on the average, longer than those in the other samples and thus were obtained from what must been somewhat larger green leaves, no means was found to detect in advance of curing and fermentation those leaves that would fall into this grade.

In general, a high dry weight per leaf was associated with a high grade rating. An exception to this was provided by the thicker leaves of the inferior AL grade, for these were the highest in dry weight per leaf. Accordingly, an attempt to sort fresh leaves in terms of weight would fail to group them satisfactorily into high and low grade material.

The only analytical criterion which appeared to correspond well with the relative value of the leaves was the content of protein nitrogen. When the samples were placed in the order of increasing protein nitrogen they were also, with the exception of the LV grade, very closely in the order of decreasing relative value. Thus, *in the brown grades,* low protein content appears to go with high grade rating. Inasmuch as the level attained by

the protein nitrogen is determined during the first week or 10 days of the curing process, it is clear that fundamental effects upon the ultimate value of the crop may be exerted by the rate and extent of the decomposition of the protein during this short but critical period, should it develop that the generalization regarding the protein content has any wide degree of validity. In this connection, the greenish leaves of the LV grade provide the outstanding exception. These were considerably higher in protein nitrogen than were any of the brown grades. Since they were also highest in soluble nitrogen, they must have been unusually high in protein nitrogen from the start, for the level attained by the soluble nitrogen is determined by the quantity of protein that had been decomposed during the early phase of curing. The leaves of the LV grade when green thus contained such a large quantity of protein that, in spite of the fact that more of it was decomposed during curing than was the case in the leaves ultimately assigned to the brown grades, they still retained more protein at the end than did the brown grades. The impression is thus given that the leaves

TABLE 33. RELATIVE COMPOSITION OF GRADE SAMPLES

I	No clearly evident difference among grades:	
	Water content after equilibration	
	Organic solids	
	Nitrate nitrogen	
	Ammonia nitrogen	
	Glutamine amide nitrogen (traces only present)	
	Peptide nitrogen	
	pH	
	Oxalic acid	
	Undetermined ether-soluble organic acid	
	Crude fiber	
II	Apparent differences among grades: no correlation with relative value:	
	Ash	LV lowest; LL highest
	Water-soluble solids	LV lowest; rest indistinguishable
	Potassium	LC2 lowest; LL highest
	Calcium	LC2 lowest; YL2 highest
	Total nitrogen	YL2 lowest; LV *highest*
	Soluble nitrogen	YL2 lowest; LV *highest*
	Asparagine amide nitrogen	YL2 lowest; LC highest
	Amino nitrogen	YL2 lowest; LV highest
	Alkalinity of ash	LV lowest; rest indistinguishable
	Total organic acids	LV lowest; rest indistinguishable
	Citric acid	LV lowest; rest indistinguishable
	Malic acid	LV *lowest;* LL highest
	Lipides	YL2 lowest; LC2 highest
III	Apparent differences among grades possibly correlated with value:	
	Protein nitrogen	LL lowest; YL2 highest of brown grades; LV exceptionally high

which found their way into the LV grades were initially more vigorous or more vegetative than the others. Although these terms are vague, they perhaps convey a suggestion as to why these leaves behaved differently from the others.

In order to summarize briefly the results of the analytical studies of the grade samples, the information in Table 33 has been collected. The upper part of the table lists those analytically determined factors which failed to show differences among the grades that could be regarded as in any way significant. In assembling these items, attention has been paid not only to the constancy of the values found but also to the probable accuracy of the method used. With respect to data for glutamine amide nitrogen, although the leaves of the LV grade may have contained a small residual quantity of glutamine while some of the other grades contained none, little significance can be attached to the observation in view of the known properties of this substance.

The second part of the table shows the analytical factors with respect to which the grade samples appeared to show differences. In some cases these differences were outstanding and beyond all doubt significant; these cases are emphasized by the use of italics. In most cases, however, the differences were small, and a far more elaborate analytical study coupled with proper statistical analysis of the data would be required to demonstrate significance. Nevertheless, the grade that was the highest or the lowest in each of these analytical components is indicated and attention is at once attracted by the number of items in which the LV grade was either highest or lowest. The weight of cumulative evidence with respect to the factors that did not vary over wide limits supports the three cases where this grade was outstandingly different in composition from the others.

It will be noted that the inferior YL2 grade also appears a number of times in the table but in no case are the data for the other grades such that a correlation is at all likely with the relative value of the grades. In several cases, the LV grade is different from the brown grades but among themselves these are indistinguishable from each other.

The single case in which there is an apparent correlation of an analytically determined quantity, namely protein nitrogen, with the value of the several grades is shown at the bottom of the table. Here again, the LV grade provides an exception for, although a moderately valuable grade, it is by far the highest in protein nitrogen.

It is obvious that the study of the differences among grades of tobacco cannot be profitably pursued by the analytical methods used in the present investigation. Because of the importance of color and especially of evenness of coloration in the assignment to grades of widely different value and also of the importance of thickness of the leaf, texture and "finish," it would seem that the development of physical methods of testing would hold out more promise as a means of discriminating among grades than do the present chemical methods. However, it is likely that a comprehensive chemical study of the pigments of the fermented tobacco leaf, especially those substances to which the brown and yellow colors are due, might be helpful. At present, there seems to be no substitute for the trained eye and sensitive hand of the experienced tobacco grader in making these all-important judgments upon which the value of the crop rests.

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SUMMARY

Connecticut shade-grown tobacco is subjected to the process of curing in large barns in which the individually picked leaves are hung for a period of about two months; the leaves are then packed into rectangular "bulks" in which the process of fermentation is allowed to take place. The fermented tobacco is graded and stored in cases for several months, for a part of the time at a warm temperature when the process of "mulling" occurs, and it is finally compressed into bales in preparation for the market. The present bulletin describes the results of an investigation of the chemical changes that take place in the leaves at each stage of this process.

A sampling method was devised whereby a series of small samples of green leaves could be collected under conditions that furnished assurance that the individual samples were initially as nearly identical in chemical composition as possible. One of these samples was immediately dried and prepared for analysis, and single samples were then withdrawn from the process at successive stages. Analyses of the entire set of samples then gave information concerning the changes in composition which occurred. In addition, a large sample of similar leaves was collected and processed along with the small samples and the leaves were graded. A study of the composition of the several grades was made in order to assess the magnitude of the chemical differences among them.

The major rapid chemical changes that occur are restricted to the first 8 to 12 days of the curing operation. Subsequent changes are slow although for the most part continuous. During curing, about 97 per cent of the initial water evaporates from the leaves, the greater part of this during the first 12 days at which time the leaves had become brown in

color. During the remainder of the curing period, the color became more uniform over the entire surface of the leaves, but the chemical composition changed only a little.

The most important chemical events of the early stage of the curing process are the loss of some 16 per cent of the organic solids through respiration of the still living cells, the decomposition of about one-half of the protein with the production of soluble nitrogenous substances and the formation of a substantial quantity of asparagine from these soluble products by a complex series of enzymatic reactions. These reactions involve deamination of the amino acids with the production of ammonia and the formation of asparagine by condensation of ammonia with a non-nitrogenous precursor.

The precursor may have been in part formed as a metabolic product of the carbohydrates but insufficient material was available from this source to account for all of it. Some other source of the precursor must be sought and the hypothesis is advanced that the residues of the deamination of the amino acids may have contributed to the supply.

During the early stage of curing, there was a loss of about one-half of the ether-soluble components; this loss was associated with the disappearance of the chlorophyll.

The total ether-soluble organic acids changed very little during curing but there was an outstanding alteration in the relative proportions of malic and citric acids; reactions occurred whereby malic acid in an amount equivalent to about 9 per cent of the organic solids of the leaves disappeared and citric acid equivalent to about 6 per cent of the solids took its place. The relative quantities are such as to suggest that two moles of malic acid are converted into one mole of citric acid.

The carbohydrate components including the starch practically disappeared during the first 12 days of curing, the traces of reducing substances which remained probably being other than carbohydrate in chemical nature.

Among the minor chemical changes that occurred were the slow and continuous loss of a small quantity of nitrogen, possibly owing to the evaporation of a little ammonia and nicotine, and a small loss of ether-soluble organic acids and the formation of a small proportion of oxalic acid which occurred during the first few days.

There was no significant change in the ash components nor in the crude fiber, and the protein that remained after the rapid digestion process of the first 8 to 12 days subsequently underwent no further change.

In contrast to the marked alteration in many components of the leaves during curing, the changes that occurred during fermentation were for the most part small and took place slowly. There was a small and continuous loss of organic solids for which no clear explanation can be given. A small loss of nitrogen was probably due to the evaporation of ammonia and nicotine. The protein remained unchanged as did the nitrate. However, there was a marked increase in the ammonia and an almost complete loss of asparagine amide nitrogen. Amide hydrolyzing enzymes are evidently present which ultimately decompose all of the asparagine that was synthesized during the early phase of curing. The destruction of the asparagine and the formation of the ammonia are closely correlated but the reaction was not continuous, for hydrolysis of asparagine ceased

after the fermentation was about one-half complete and was reestablished only during the extended period of storage of the leaves.

The ash content remained constant as would be expected, but the complete stability of the ether-soluble organic acids which was observed was not anticipated. There was no detectable change in any of the acids for which analytical methods are available. This finding is of considerable importance since the organic acids are especially reactive components of plant tissues and that they underwent no change during a technical process so fundamental in its outward effects as the fermentation of tobacco suggests that the kinds of reactions that occur are unlike those encountered in fresh leaves.

The crude fiber of the leaves did not change and there was only a small loss of ether-soluble lipide components.

Analysis of the several grades into which the leaves of the large separate sample were sorted showed that there are only small and for the most part insignificant differences in composition among the brown grades. The only component which appeared to be at all well correlated with the relative values of the several brown grades was protein nitrogen; high commercial value was associated with low protein content. The greenish leaves of the LV grade, which are rated as being of moderately high commercial value, were, however, markedly different in composition from the brown grades. They were conspicuously high in protein nitrogen and soluble nitrogen and low in malic acid content; with respect to a number of other components, these leaves were either the highest or the lowest although the differences were less outstanding. The inference appeared to be justified that the leaves that found their way into the greenish grade were physiologically different from the others at the time they were picked.

The general conclusion appears to be justified that the chemical methods that have been developed for the study of the changes that take place in the curing process and in tobacco leaves subjected to culture in water or solutions of nutrients are not well adapted for the study of the changes that occur during fermentation. The chemistry of fermentation is a different problem from the chemistry of curing and a different methodology will be required for the detection of the more subtle and possibly less extensive alterations in composition, important though these are in the development of an acceptable commercial product.

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