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CONTENTS

1. INTRODUCTION

The chemical problems presented by the changes that occur when tobacco leaves are subjected to the commercial curing process have a scientific interest that far transcends their importance in the tobacco industry. There is probably no feature of the landscape in the fall of the year more striking than the brilliant coloration of the leaves. The changes that give rise to this coloration are chemical and the colors are a visual reminder of the progress of extensive intracellular alterations that may be summed up in the term katabolism. They are alterations that involve release of stored-up energy, destruction of the chemical integrity of the tissues and ultimate death. The study of these intracellular changes is a matter of the greatest importance, particularly the study of those changes that occur during the early stages when the water content of the tissues has not seriously diminished and the reactions may be supposed to follow paths that do not differ fundamentally from the normal. The intact leaf is the seat of innumerable chemical reactions some of which result in the synthesis of new substances; others result in their decomposition. Reactions that represent both anabolism and katabolism go on side by side. By a study of the detached leaf one may hope to detect the nature of some of these changes since, after the removal of the leaf from the plant, the reaction products do not have the opportunity to pass into the circulation of the plant and their concentration in the tissue therefore increases.

In the present study we have been interested chiefly in chemical changes that are frankly katabolic in nature. Very little appears to be known about the behavior of the substances in the cells of excised leaves after extensive desiccation has taken place, since it has been a primary consideration, in most investigations of this nature, to preserve the turgidity of the cells by immersing the cut petioles in water or nutrient solution. Furthermore few studies of excised leaves have been extended beyond the time when conspicuous changes due to autolysis supervened. Our studies have been made on leaves picked from the plant and hung in a dark shed in such a way that a free circulation of air over the surface of the leaf might take place; a maximum opportunity was therefore presented for the evaporation of water and the evolution of gaseous products of the chemical reactions that occurred. The

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

changes were allowed to continue until experience showed that little further alteration was to be expected.

The selection of the tobacco leaf for these investigations was made largely as the result of practical considerations. In order that the tissue may be killed before extensive change due to the removal from the plant can occur a large, thin leaf is desirable. The leaves selected should be uniform in size and as free as possible from petioles, buds, or structures other than leaf blade and vascular tissue. If elaborate studies are planned it is also necessary that the leaves should be of uniform genetic origin and that the plants should be grown under carefully controlled conditions that are to a large extent reproducible from year to year. No plant available to us fulfilled these conditions so well as tobacco. The data that are described in the following sections were secured upon samples of Connecticut shade-grown tobacco that were collected from a large field (Field 10) at the Tobacco Substation in Windsor. The field was fertilized as follows:

	Lbs. per acre
Cottonseed meal	1400
Castor pomace	400
Calcium nitrate	100
"Calurea"	150
Potassium nitrate	150
Potassium carbonate	200
Precipitated bone	200
Lime	400

2. EXPRESSION OF THE DATA

The data obtained in most studies of the composition of leaf material have been expressed in terms of percentage either of the dry or fresh weight of the leaves or, in the case of the nitrogenous components, in terms of percentage of the total nitrogen. This method of presentation admirably fulfills the requirements when the composition of a single lot of material is under consideration or when two or more lots of different material are to be compared one with another. The method is not desirable, however, when a comparison is to be established between two or more lots of the same material that have passed through procedures during which loss or gain of some of the components occurs and it is particularly objectionable when the fundamental datum, the dry weight or total nitrogen, changes. Under these circumstances the interpretation of percentages becomes difficult if not impossible. On the other hand, loss or gain of the components immediately becomes evident when the composition is expressed in terms of weight and the products from equal initial quantities of experimental material are compared.

Our study of the changes that occur during the curing of tobacco showed that both dry weight and total nitrogen underwent material alterations and, consequently, in order to present these changes in a form in which they may be readily appreciated, we have founded our data on the composition of five equal lots of leaves picked from the plants on the same day, and have expressed each constituent in terms of weight.

3. PREPARATION OF MATERIAL

The five lots of tobacco leaves were picked on August 1, 1929, and weighed exactly 50 kilos each. The material consisted of the eighth to eleventh leaves counting from the bottom of the plant; the first to seventh leaves had been removed at two earlier pickings. One lot (E) was immediately transported to the laboratory in New Haven and there extracted with hot water; the other four lots were strung on cord in the usual way and placed in the curing shed. Lot A was removed and taken to the laboratory on August 12 when the leaves had reached the "yellow" stage; Lot B was removed August 17 at the "brown" stage, that is, when the leaves first assumed a uniform brown color; Lot C was allowed to hang until September 20 when it had become "fully cured." Lot D was subsequently fermented by the Heber[1] process and reached the laboratory December 5, 1929.

Each lot received the same treatment at the laboratory. Immediately on arrival small samples were removed and a convenient quantity of the leaves was then dropped, at such a rate that the temperature was not depressed more than a few degrees, into a large volume of boiling water to which sufficient sulfuric acid was added, from time to time, so that the reaction should not become more alkaline than pH 4 to 5. This was done so as to prevent loss of nicotine during boiling and subsequent concentration of the extract. The leaves were boiled for about half an hour, or until the midrib was thoroughly softened, were then removed from the liquor, allowed to drain, and were pressed at the hydraulic press. Meanwhile more leaves were added to the boiling liquor and the process was repeated. In this way the entire lot of leaves was extracted and pressed out in a period of three hours. The residues of extracted leaves in the press cakes were shredded, ground in a meat chopper and again boiled in a large volume of

[1] In the Heber process a solution of secret composition is sprayed over the leaves, which are then packed tightly together and subjected to artificial heat. Fermentation proceeds much more rapidly than by the old "bulk" process. Our specimen was carefully marked and was put through this process along with several hundred pounds of tobacco derived from the same field.

distilled water maintained at pH 4 to 5 with sulfuric acid. After pressing, the operations of grinding and boiling were repeated once more. The thoroughness of this extraction can best be appreciated by a glance at the data of Table 8. The press cakes contained only about 1 per cent of the original nicotine and in two cases less than 0.5 per cent of the original nitrate of the leaves. In three cases no nitrate whatever remained in these cakes and in no case could ammonia be detected in them.

The three successive extracts of each lot of leaves were collected quantitatively, filtered from dirt and sand on paper pulp, and were then concentrated in a large glass-lined vacuum still. The weight of the dirt and sand was determined from the increase in weight of the paper pulp used in making the filter and was subtracted from the initial weight of the leaves. The correction was of the order of 50 gm. and was practically negligible. The extract, after concentration to approximately 8 liters, was allowed to settle, the clear fluid was decanted and the sediment, which consisted to the extent of 95 per cent of inorganic salts, was centrifuged off and washed thoroughly with water until free from nicotine. The washings were united with the main fluid which was then brought to exactly 8,000 cc. and preserved with toluene. The precipitate was dried and analyzed. In the interests of simplicity of presentation the results for the solids, ash, and nitrogen of this precipitate have been applied as corrections upon the similar data obtained from the analysis of the extract. The data have also been corrected for the small samples of each lot that were removed for analysis when the material reached the laboratory, and calculated to an initial weight of exactly 50 kilos.

4. THE CHANGES IN WATER AND SOLIDS DURING CURING

Data showing the major changes that occur during the curing of Connecticut shade-grown tobacco are given in Table 6. Since the changes up to stage C took place under constant conditions, namely during the process of curing in the tobacco sheds, it is proper to plot these against a time scale; this has been done in Figures 13 to 17. The subsequent change due to fermentation bears no temporal relationship to the previous changes and furthermore the addition of unknown substances during the Heber process renders the data of little comparative value. The curves have therefore been extended to indicate the nature of the changes due to fermentation only in certain cases.

The most outstanding change that occurs during the curing of tobacco is the diminution in total weight of the leaves. The original 50 kilos of fresh leaves contained 43.53 kilos of water and 6.47

kilos of solids. When fully cured the leaf tissue weighed only 6.68 kilos. By far the greater part of the 43.32 kilos, or 86.6 per cent loss in weight is, of course, represented by the evaporation of water from the tissues, but this is not the complete story. The original 6,474 gm. of total solids diminished to 5,187 gm., or by 1,287 gm. (19.8 per cent); consequently the 43.32 kilos loss is due to the evaporation of approximately 42 kilos of water or 96.4 per cent of the water originally present and further to the loss of approximately 1.3 kilos of solids. The inorganic solids remained constant within the limits of accuracy of the determinations and the entire loss therefore falls upon the organic solids. Turning for a moment to Table 7 it would seem that only a small part of the loss was due to diminution in the amount of water soluble organic solids. These apparently diminished by an amount between the limits 160 to 360 gm. during the curing, but, for technical reasons discussed in Section 11, the accuracy of the solids determination in these solutions is not great and these two figures merely suggest that about 200 gm. of organic solids disappeared from the water soluble fraction. Table 8 shows that a loss of approximately 1,000 gm. falls on the organic solids of the extracted leaf residue in the press cakes. These diminished from 3,125 gm. to 2,122 gm. The determinations of the solids in the press cakes could be conducted with great accuracy and there is consequently no doubt of the magnitude of the change. The conclusions to be drawn are that the fresh leaves lose the greater part of their water during curing, also that an amount of substance equivalent to nearly one-third of the hot water insoluble organic solids of the leaves vanishes entirely. The essential organic constituents of the press cakes are coagulated protein and insoluble substances many of which are related to the carbohydrates. During the process of curing, then, an extensive conversion of protein and carbohydrate from an insoluble into a soluble form occurred and nearly one-third of the total quantity of organic solids disappeared from the tissues. The term insoluble may be objectionable as here used since we do not know how much of the material was really insoluble. We employ it to refer to an *experimental* observation; material was present at one stage of curing in a form that was found to be insoluble after treatment with hot water; at a later stage some of the same material was so changed or converted that it became soluble in hot water.

The total loss of organic substance from the press cakes was of the order of 1,000 gm. (Table 8, $3{,}125 - 2{,}122 = 1{,}003$ gm.). As will be shown later (page 220) the probable loss of protein from the insoluble fraction was of the order of 689 gm. The balance of approximately 310 gm. of organic substance, that was converted into a soluble form, probably consisted mainly of originally insoluble substances related to carbohydrates. We must assume,

then, although with the same reservation respecting the exact meaning of the terms, that hydrolysis and solution of nearly 700 gm. of protein and 300 gm. of carbohydrate occurred which, together with about 200 gm. of substance that was water soluble from the beginning, underwent changes whereby approximately 1,200 gm. of substance passed off from the leaves in volatile form. Garner, Bacon and Foubert (9) likewise observed a substantial loss of weight in their experiments on the curing of tobacco.

The greater part of the loss of organic substance from the leaves is probably to be accounted for as carbon, oxygen and hydrogen evolved in the form of carbon dioxide and water, although a part may have consisted of other volatile carbon compounds. This view immediately raises the question of the carbon dioxide relationships during curing. Experiments in which the amount of carbon dioxide that is evolved from a known quantity of leaves as they undergo curing will alone show whether the losses of organic matter can be accounted for qualitatively and quantitatively as we have suggested above.

That an extensive loss of carbohydrate[1] material (Table 7, 348 — 65 = 283 gm.) took place is clear from the data of Table 7 on the total soluble carbohydrate. The determinations were made by means of the reduction of Fehling's solution and are expressed in terms of glucose. This is, of course, arbitrary because other substances than glucose may have been present which could reduce Fehling's solution and some of these may not have been of carbohydrate nature. Nevertheless, in spite of the difficulty of precise interpretation, it seems evident that a substantial destruction of carbohydrates occurred. Furthermore, it is certain that some of the material was either glucose, fructose, or mannose since 48 per cent of the indicated 348 gm. (Table 7) of soluble carbohydrate in the fresh leaf was actually isolated as phenylglucosazone. If the figures are taken as a true measure of the soluble carbohydrate in the tissues, the evidence points to the destruction of 209 gm. of reducing sugar during the first 12 days of curing and a subsequent destruction of 74 gm.; in all, 283 gm. or 81 per cent of the original 348 gm. A curve that illustrates this transformation is shown in Figure 13.

The reactions by which the indicated quantity of carbohydrate was destroyed can hardly be other than oxidative in nature. Direct measurement of oxygen consumption would be necessary, however, to prove this and to determine at what stage the oxidative reactions proceed with greatest intensity. It is perhaps suggestive, however, that, of the total loss of some 1,200 gm. of organic solids

[1] Tobacco leaves of the type investigated here contain remarkably little starch. This may have something to do with the fact that they are picked while still far from mature as this term is used in connection with other types of tobacco.

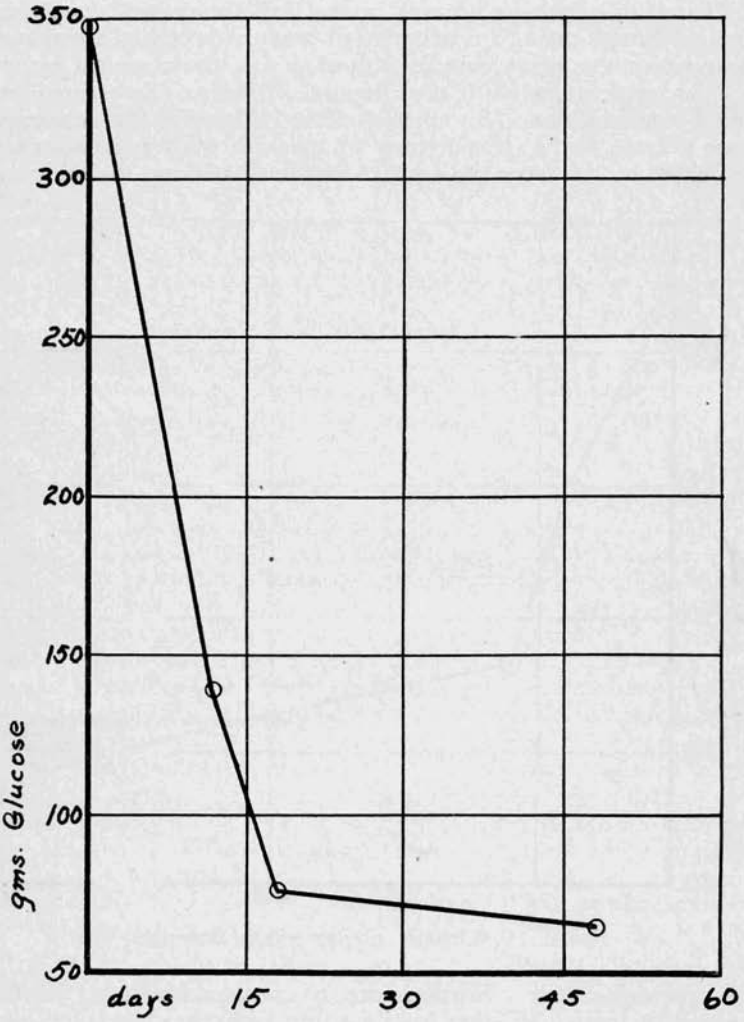

FIGURE 13. Changes in total soluble carbohydrate expressed as glucose.

during the whole curing process, more than 660 gm. vanished during the first 12 days.

In connection with the carbohydrate changes one further comment may be made. The "crude fiber," as determined by the conventional method, underwent no significant diminution in quantity. The cellulose framework of the leaf therefore appears to survive both curing and fermentation practically unchanged.

The ether soluble substances in the leaf diminished sharply in amount during curing; nearly 39 per cent of the initial weight of these substances disappeared. Knowledge of the chemical nature of this complex mixture is very limited. Behrens (2) pointed out that a waxy substance, an etherial oil and a lecithin-like substance were present in the ether extract of tobacco; the waxy substance he believed came from the surface of the cuticle and the oil from

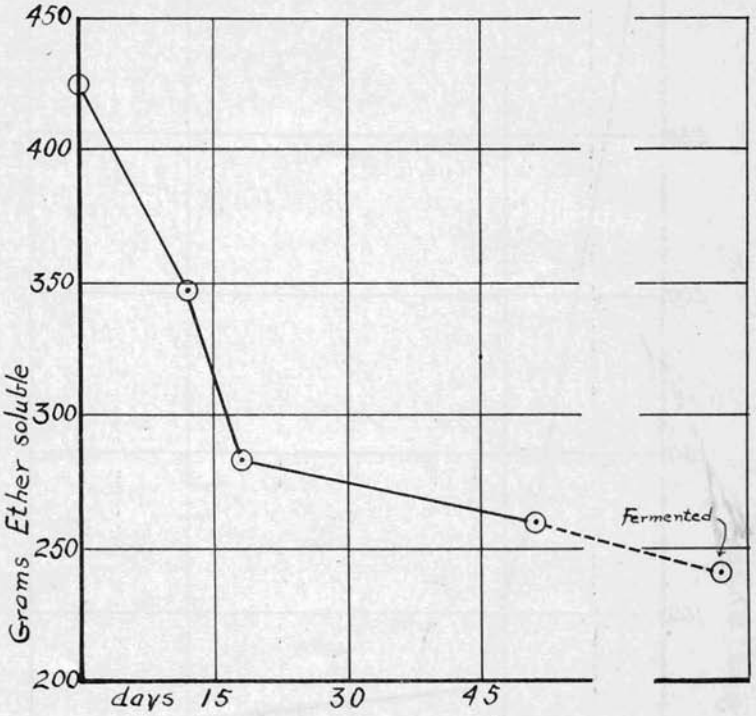

FIGURE 14. Changes in ether soluble material.

the glandular hair. Furthermore a considerable part of the material extracted by ether has its origin from the chlorophyll and other pigments of the leaves. Practically all of the small amount of available information regarding the ether soluble part of tobacco has been admirably summarized by Schmuck (18). Thorpe and Holmes (20) isolated a hydrocarbon of melting point 68° from tobacco and a few analogous substances have been described by others. Aside from measurements of the physical constants of the oily mixture practically nothing has been done which serves to give more than a limited view of its chemical nature.

The diminution in ether soluble material observed during the

early stages of curing is striking; 79 gm. or 18.5 per cent of the total quantity disappeared in 12 days and 142 gm. or 33 per cent in 18 days (Figure 14). It seems likely that a considerable part of this early rapid change is associated with the decomposition of the chlorophyll. Dunlap (8) has determined chlorophyll in tobacco leaves of the type employed in these studies. On the assumption that the chlorophyll content of our material was not dissimilar to that he employed, our 50 kilo specimens should each have contained 92.5 gm. of this pigment. The nature of the decomposition of the chlorophyll, that occurs during the yellowing of tobacco leaves in the early stage of curing, is not known with certainty but, on the assumption that decomposition to chlorophyllides and phytol under the action of chlorophyllase occurred, a rational explanation of some of the loss of ether soluble material can be advanced. One-third of the weight of chlorophyll consists of phytol, which is an ether soluble alcohol. The other two-thirds of the chlorophyll, if the above mentioned reaction occurred, should be converted to chlorophyllides (a + b). These substances, while probably not wholly insoluble in ether, are much less soluble than is chlorophyll itself. Consequently one may assume that about 60 gm. of substance which was previously soluble in ether was transformed to substances of very limited solubility in ether early in the curing process. These probable changes in the solubility in ether of a part of the chlorophyll account at most for less than half of the loss of ether soluble constituents of the leaves. In view of our ignorance of the composition of these substances, speculation on their fate is perhaps futile. Two obvious possibilities can be suggested however. Oxidation of some of the substances into compounds that are no longer soluble in ether may have occurred. Direct evaporation of some more volatile constituents may also have taken place. Loew has stated that etherial oil was present in the air of rooms in which tobacco fermentation was proceeding (11). It would seem, therefore, that evaporation plays some part in these very substantial changes.

Only a small additional loss of ether soluble material occurred during fermentation. This suggests that no essential change in the chemical nature of the ether soluble fraction took place during the latter process.

A point which illustrates the value of our method of presentation of the data arises in connection with the changes in the amount of ether soluble substances. The 50 kilos of fresh leaf contained 426 gm. of ether soluble material; the 6.68 kilos of fully cured leaf derived from this amount of fresh leaf contained only 260 gm. of ether soluble material. Thus almost 39 per cent of this group of substances vanished during curing. If the same results are expressed in percentages of the organic solids of the leaf before and after curing the apparent change is only from 8.07

per cent to 6.5 per cent, a loss that would attract scarcely more than passing attention and to which no especial significance could be attached.

5. NITROGEN DISTRIBUTION DURING CURING

The discussion of the data for the changes in the amount and form of combination of the nitrogen that occurs during the curing and fermentation processes is complicated by the fact that these data were obtained in two ways. The data of Table 6 were secured from samples of each lot of material withdrawn before the leaves were extracted with hot water. These samples were dried in a current of air at 60° and were then finely ground and analyzed. During the drying, definite losses of nicotine and apparent losses of ammonia and nitrate nitrogen occurred as is shown if the data of Table 6 are compared in detail with the data of Table 9. The data of Table 9 were secured by adding the data for the several forms of nitrogen, etc., determined in the respective hot water extracts as shown in Table 7, to the similar forms of nitrogen, etc., determined in the press cakes and shown in Table 8. In certain cases the sums so obtained give more reliable information than the direct determinations made upon dried samples of the leaves. In other cases the reverse is the case; a fuller discussion of this matter is deferred to Section 11.

The interpretation of the changes that took place during the fermentation process that was applied to the leaves of Lot D is difficult. Our specimen was carefully marked and was then fermented along with a large lot of tobacco derived from the same field. It was withdrawn at the end of the process but was found obviously to have acquired nitrogen and inorganic salts. During fermentation there is a possibility that soluble substances may pass by diffusion from one leaf to another, although extensive changes by this method are perhaps not to be expected. The addition of the solution of secret composition, which is a part of the Heber process, introduces an uncontrolled factor that seriously impairs the value of our experimental data. The figures are reported here only because, in a few cases, useful deductions can be drawn. A quantitative study of the chemical changes that occur during fermentation can be carried out only when a technique for fermenting small samples of cured leaf has been developed.

The total nitrogen of the tobacco leaves diminished markedly during curing. The data of Table 6 indicate that this loss proceeded rather rapidly at first (see Figure 15) and then less rapidly. The data of Table 9 suggest that the loss of nitrogen was slow at first and then occurred with great rapidity while the leaves passed from the yellow to the brown stage. Subsequent loss of

nitrogen was then again slow. It is difficult to judge which of these pictures of the process is more accurate, and statistical treatment of a much more elaborate series of data will be required

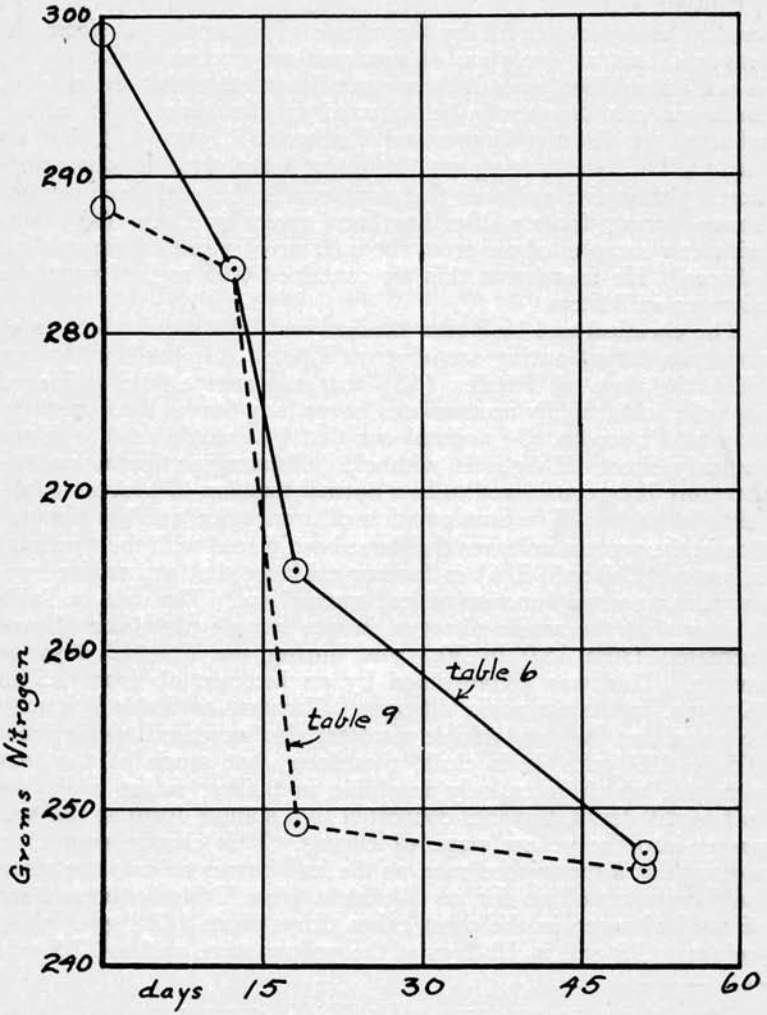

FIGURE 15. Changes in total nitrogen content of leaves.

before this can be determined. The essential fact of a marked loss of total nitrogen during curing is, however, clear.

Of the different compounds of nitrogen in tobacco with which we are familiar only two are volatile under the conditions that

exist during curing; these are nicotine and ammonia.[1] The total loss of nitrogen that occurs amounts to more than 40 gm., but of this only about 4 gm. can be accounted for as loss of nicotine (Tables 6 and 9). The balance of this loss of nitrogen can most readily be accounted for by the escape of ammonia gas from the leaves. That an evolution of ammonia may occur during curing is not improbable, since it is common knowledge that the odor of ammonia can frequently be detected in the vicinity of curing tobacco, see Garner, Bacon and Foubert (9, page 27), but no quantitative data bearing on this point have come to our attention. Qualitative evidence that ammonia is liberated from tobacco leaves during fermentation has been given by Loew (11) who withdrew samples of air from the interior of a pile of fermenting tobacco. He found that this air contained 0.05 mg. of ammonia nitrogen in 3 liters.

The chemical and biological mechanisms involved in the loss of ammonia during curing are of great interest. It has been known since the time of Pfeffer (13) that asparagine, the amide of aspartic acid, rapidly accumulates in seedling leaves during etiolation, and Borodin (3) pointed out that this amide readily forms in leafy shoots if light is withheld. Asparagine formation has therefore been considered to be a normal reaction of protein katabolism in leaves. The accumulation of asparagine and the diminution in the protein in leaves that have been placed with their petioles in water (Chibnall, 5, 6) is further evidence that asparagine synthesis is a normal function of leaf metabolism. The data in Table 6 show that the amide nitrogen in our sample of tobacco leaves increased from 13.0 to 30.3 gm. during the first 12 days of curing. This was accompanied by an increase of from 2.3 to 10.6 gm. in the ammonia nitrogen. Turning to Table 7 it may be noted that the total soluble nitrogen also increased, rising from 105 to 189 gm. It is clear, therefore, that much of the leaf nitrogen, which is entirely insoluble in boiling water while the leaf is still fresh, becomes converted to a soluble form as the leaf passes into the yellow stage of curing. This change continues, although at a diminished rate, as the leaf passes on into the more fully cured condition for, as is evident from Table 8, the amount of total nitrogen in the press cakes drops from 182.8 gm. initial weight to 95 gm. in 12 days at the yellow stage and to 72.5 gm.

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[1] Our attention has been drawn by Dr. W. W. Garner (personal communication) to the possibility that elementary nitrogen may escape from the leaves. The most obvious mechanism that could account for this would be the reduction of nitrate to nitrite which then reacted with amino groups with the evolution of gaseous nitrogen. Our observation that the nitrate content of the leaves did not diminish during curing is strong evidence against this view and, if elementary nitrogen did indeed escape from the leaves, some other mechanism must be invoked to account for it.

at the fully cured stage. Figure 16 represents the change graphically.

The simplest assumption that can be made to account for this involves a discussion of the chemical changes undergone by the protein that is found in a coagulated form in the press cakes after

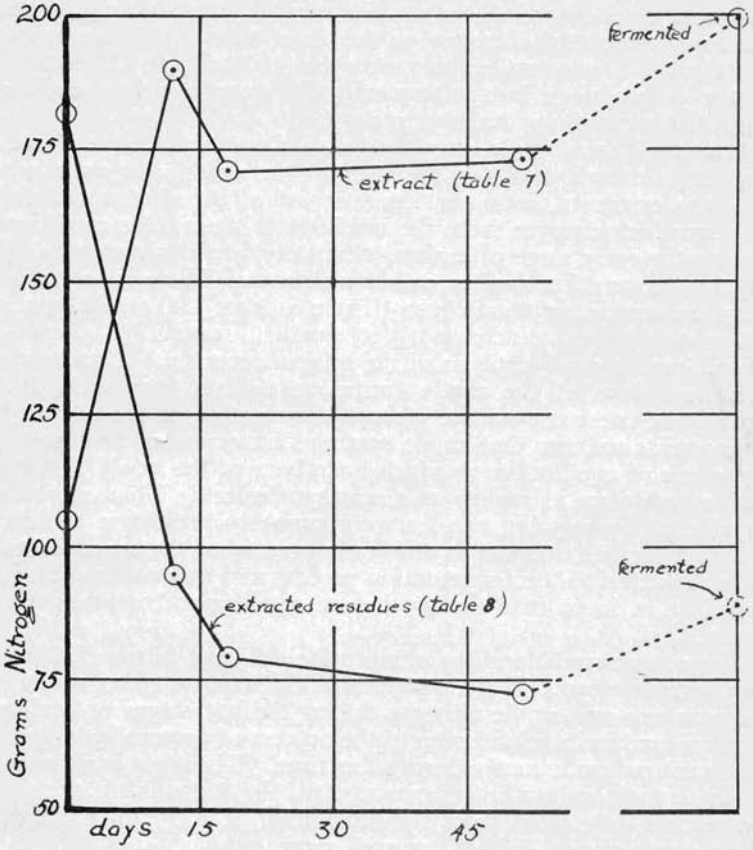


FIGURE 16. Changes in the nitrogen content of the extract and of the extracted residues of leaves.

the hot water extraction. It may be supposed that, during the early stages of curing, an enzymatic digestion of leaf protein goes on at a rapid rate with the result that a considerable part becomes converted to a form which is no longer coagulable. From the figures mentioned above (182.8 gm. initial, 72.5 gm. final) it is clear that a quantity of protein that contains about 110 gm. of

nitrogen undergoes this change. If the conventional conversion factor for protein (6.25) is here used it becomes evident that no less than 689 gm. of protein passed into a hot water soluble form as a result of the changes that occurred during the curing of this lot of leaves.

Data from Tables 7 and 8, plotted in Figure 16, make it clear that this digested protein is not represented quantitatively by an increase in the total nitrogen of the water extract. There is an initial rapid rise in total soluble nitrogen (from 105 to 189.4 gm.) at the yellow stage but subsequently the amount drops slightly and then remains approximately constant.

Consideration of the observations of Chibnall referred to above, and of our own data for the increase in amide and ammonia nitrogen, suggests that a considerable part of the nitrogen of the protein which changes from the insoluble to the soluble condition during the early stage of curing, ultimately becomes converted to asparagine, or other amides, and to ammnoia. There is an initial large increase in amide nitrogen (Table 6, from 13.0 to 30.3 gm.) but subsequently the increase is very small (Figure 17). Nevertheless continuous hydrolysis of the protein occurred. To account for the failure of the amide formation to keep step with this hydrolysis, one must assume prompt deamination of the liberated amino acids and also that amide synthesis either ceased or reached a position of equilibrium at which hydrolysis of the newly formed amide took place as rapidly as it was synthesized. That enzymes capable of hydrolyzing amides were probably present is evident from the marked decrease in amide nitrogen when the cured leaves were subjected to the fermentation process and this observation is therefore in favor of the view that an equilibrium condition was attained.

The complex relationships between the different forms of nitrogen become clearer if the remarkable constancy of the sum of the ammonia and amide nitrogen during the last stages of curing is considered in its relationship to the process of protein hydrolysis that went on continuously during this time. This sum is given in Table 1 together with the measure of the amount of protein hydrolyzed, calculated from the diminution of the total nitrogen of the press cakes (Table 8) at each stage.

TABLE 1. SUM OF AMMONIA AND AMIDE NITROGEN

	E Fresh leaf	A Cured 12 days	B Cured 18 days	C Cured 51 days	D Fermented
	gm.	gm.	gm.	gm.	gm.
Table 6	15.34	40.94	46.73	46.42	69.62
Table 9	13.92	42.5	47.37	47.62	70.04
Protein N hydrolyzed at end of each period, Table 8		87.8	16.6	5.85	

In the period from the 12 to the 18 day stages of curing there is only a small, although definite, increase in the ammonia and amide nitrogen; in the period from 18 to 51 days the sum of the ammonia and amide nitrogen does not change by an amount that is outside the limit of experimental error. Meanwhile, however, that is, in the period from 12 to 51 days, a quantity of protein that contained 22.5 gm. of nitrogen was hydrolyzed and passed into a soluble form. Furthermore, during the same time the total nitrogen of the water extract, in spite of the above mentioned increment of nitrogen due to the hydrolysis of the protein, diminished from (Table 7) 189.4 to 173 gm. One is forced to the conclusion that nitrogen escaped from the tissues. The constancy of the sum of the amide and ammonia nitrogen during the later stages of curing suggests that the amides and ammonia reached a concentration at which they were in dynamic equilibrium with the other substances present and that ammonia passed into the air surrounding the leaves. The chemical reactions are undoubtedly very complex and many side reactions may have occurred, but the essential facts are clear.

From the quantitative point of view we can make the following statements. For simplicity of presentation, and also because it is not certain that all of the ammonia passed through the amide stage, the sum of the ammonia and amide nitrogen is again considered. Of the 288 gm. of nitrogen in the 50 kilos of fresh leaves (Table 9), 15.3 gm. were present as ammonia + amide (Table 6) and 182.8 gm. (Table 8) as essentially insoluble protein. During curing, insoluble protein that contained 110 gm. of nitrogen (Table 8, $182.8 - 72.5 = 110.3$ gm.) was converted into substances that were soluble and, at the end, 46.42 gm. (Table 6, $14.21 + 32.21$) were present as ammonia + amide nitrogen. Meanwhile the total nitrogen in the leaves dropped from 288 to 246 gm. (Table 9), i.e., 42 gm. of nitrogen disappeared. Of this 4.2 gm. may be accounted for as volatilization of nicotine (Table 9, $18.51 - 14.34$), leaving 37.8 gm. which were probably lost as ammonia. Adding this to the ammonia + amide nitrogen present in the fully cured leaves (46.62 gm.), we get 84.2 gm., and deducting the ammonia + amide nitrogen originally present (15.3 gm.) approximately 69 gm. of nitrogen are left. This quantity therefore represents the nitrogen involved in the complex series of reactions that resulted in the formation of ammonia and of amides. It would be unsafe to assert that the 69 gm. of nitrogen came entirely from the 110 gm. of nitrogen in the hydrolyzed protein; other compounds present in the leaf may have played a part in the reactions. But, if our assumptions are sound, certainly 69 gm. out of the total of 288 gm. originally present, or 23.9 per cent of the whole nitrogen of the leaf, were involved. It is evident, therefore, that the reactions in which amides and ammonia

are formed are of great importance during the curing of tobacco leaf.

The significance of these reactions is further emphasized if they are considered in relation to the total probable protein content of the fresh leaf. No peptide nitrogen was demonstrable in the hot water extract; consequently all the original protein of the leaf probably remained in the press cakes after the hot water extraction. The total nitrogen of the press cakes amounted to 182.8 gm. and, of this, only insignificant traces (0.4 gm.) were present as nicotine and nitrate. Although not all of this nitrogen was in the form of protein, that the greater part of it was can be shown as follows. After total hydrolysis with 8 N sulfuric acid 104.2 gm., or 57 per cent of it, was converted into amino groups and 9.6 gm. or 5.3 per cent into ammonia. These are typical ratios for the amino and ammonia nitrogen of proteins after hydrolysis. A considerable part of the nitrogen (47.7 gm. or 26 per cent) was still insoluble after hydrolysis. This is not the place for a discussion of humin formation during the hydrolysis of proteins in the presence of carbohydrates, but it is generally true that a small proportion of the nitrogen of proteins is converted to an insoluble nitrogenous black material (humin) during hydrolysis and that this proportion is greatly increased if carbohydrates are present. So high a proportion as 26 per cent is unusual, but, on the other hand, the conditions of hydrolysis here employed were unusual. It is safe to conclude, therefore, that by far the greater part of the nitrogen of the press cakes was present as protein and, further, that this represented almost the whole of the original protein of the fresh leaf. Granting this, out of 288 gm. of nitrogen in the fresh leaf, nearly 182 gm. or 63.2 per cent, were originally present as protein. If it be assumed for the moment that little or none of the water soluble nitrogen in the extract underwent the conversion to amide or ammonia during curing, the 69 gm. of nitrogen that entered into these reactions represent a conversion of 37.8 per cent of the original protein nitrogen of the fresh leaf. The actual conversion of the protein nitrogen that occurred probably lies between this upper limit of 37.8 per cent and the lower limit of 23.9 per cent mentioned above.

These estimates of the proportion of the protein nitrogen of tobacco leaves that undergoes transformation to amides or ammonia during curing, are of great interest in connection with the views of Pfeffer (13), of Schulze (19), and of Prianischnikow (14, 15) on the function of asparagine in protein metabolism. Pfeffer believed that translocation of nitrogen in the intact plant occurs mainly through the agency of asparagine. He supposed that, during early life, this substance is formed from the protein of the seed endosperm; it migrates into other parts of the plant and is then, in the presence of carbohydrates, reconverted into protein.

This view was advanced long before knowledge of protein composition was more than rudimentary and its simplicity made a strong appeal to those investigators, such as Schulze, who were occupied with these problems. More recent investigations have served to emphasize the significance of asparagine in the metabolism of leaf proteins, although it became evident that breakdown of protein into amino acids under the action of proteolytic enzymes also played a part in the translocation of nitrogen in the plant. Prianischnikow (15), however, prefers to account for the presence of asparagine or other amides in etiolated seedlings on other grounds. His ideas are a development of those first advanced by Boussingault, who pointed out the close analogy between urea in the animal body and asparagine in plants. The animal converts much of the ingested nitrogen into urea, which is an amide, a neutral non-toxic substance, and is excreted. The plant converts ammonia into asparagine, which is likewise a neutral non-toxic amide. The plant, however, lacking organs of excretion, stores this substance in the tissues and may subsequently draw upon it as a source of nitrogen for synthesis. In short, asparagine, and by analogy, other amides, are used by the plant to detoxify the ammonia which is produced by the decomposition of the amino acids. Its formation is, therefore, a secondary process.

The protein metabolism of the mature green leaf was studied a few years ago by Chibnall who investigated the changes that occur when leaves are "starved," that is, when the leaf is detached from the plant and the petiole is placed in water. Under these circumstances translocation out of the leaf becomes impossible while those reactions which normally go on in the leaf are supposed to continue; the products of these reactions therefore accumulate. Chibnall (4, 5, 6) observed a marked increase in the water soluble nitrogen of the leaf, which implies a digestion of the protein into water soluble forms, and also a marked increase in the amide nitrogen. Our observations on the changes that occur during the early stages of curing of tobacco leaves are in complete agreement with these of Chibnall. The subsequent changes that occurred in our material after extensive dehydration of the leaves took place cannot, of course, be expressed in such simple terms. It is perhaps safe to suppose that many of the normal reactions of the leaf cells proceeded along accustomed paths during the first few days of curing; hydrolysis of the protein occurred, and the amino acids so produced were in part deaminized with the production of ammonia. The ammonia was then converted into asparagine in order to maintain the reaction of the tissues within proper limits, that is, in order to prevent toxic effects. Ultimately, however, the loss of water from the tissues brought about so high a concentration of the soluble substances that another method of eliminating the ammonia became possible; ammonia was liberated and escaped

from the cells in gaseous form. There seems little doubt that this really happened, but the exact mechanism is obscure. Prianischnikow (15) has reported that, during drying in the oven, ammonia escaped from lupine seedlings that had been grown on ammonium chloride nutrient solutions and in which the concentration of ammonia was unusually high. There is a certain similarity in the conditions he described and in our experiment. In both an excess of ammonia was present in the tissues; in both extreme desiccation occurred. The difference in temperature hastened the changes in Prianischnikow's seedlings and undoubtedly had other more specific effects but, essentially, conditions were analogous.

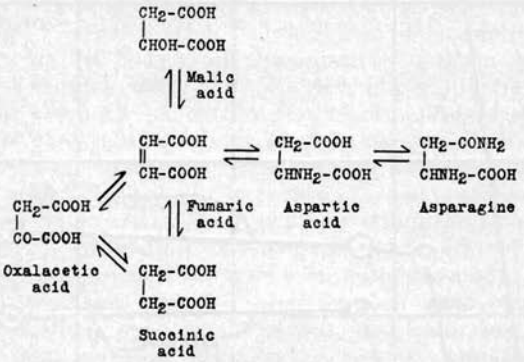
It is not easy to explain the loss of ammonia from tobacco leaves in terms of the acidity of extracts of the tissues. This remained remarkably constant throughout the period of curing, varying between the limits pH 5.4 and 5.7. Ammonium salts are not appreciably hydrolyzed at these reactions. One can only assume, therefore, that the cells became locally much more alkaline than this and, as a consequence, ammonia escaped.

The whole problem of protein metabolism in green leaves is in a notoriously unsatisfactory condition (17). The only methods available for its study involve the impression of an artificial constraint; the assumption is then made that the cell reactions continue to run along normal paths. While this may be so, it is generally true that living tissues react, when artificial chemical or other constraints are placed upon them, in a way that is calculated to minimize the damage to the entire organism. Consequently some of the reactions that are observed under artificial conditions may be entirely different from the normal reactions of the tissues. At present we can only hope that more accurate and sensitive methods will ultimately complete the picture we now see only in crude outline.

To recapitulate, our data on the chemical changes that occur during the curing of tobacco leaves suggest that hydrolysis of protein occurs; the amino acids are then in part, at least, deaminized, probably by oxidative hydrolytic processes, and much of the ammonia so formed combines with carbon compounds, possibly of carbohydrate origin, to form amino acid amides. Ultimately, owing to the desiccation of the tissue, the concentration of the amides and ammonia reaches a maximum. Hydrolysis of the amides with the production of ammonia occurs and evolution of ammonia furnishes a new means by which the cells eliminate this substance.

It may be of interest to refer at this point to a speculation, given by Robinson (17), upon the mechanism of amide formation in the tissues. It has long been suspected that the changes involved can proceed only in the presence of carbohydrates or their decomposition products. Prianischnikow (15) has shown that asparagine

formation in lupine seedlings is notably stimulated if sugar is added to the nutrient solution. α-Keto acids, such as pyruvic acid, are regarded, especially by Knoop (10), as of supreme importance in the synthesis of amino acids. Acids of this type are found among the products of carbohydrate as well as of amino acid decomposition and, consequently, these acids are, as it were, the common meeting ground of both carbohydrate and protein metabolism. Simple relationships between α-keto acids and hydroxyacids are well known and hydroxyacids are invariably found in green leaf tissue. A connection between asparagine and some of these acids may be imagined as follows.



This purely hypothetical scheme which, however, involves only reactions of known biological significance, serves to illustrate possible relationships between asparagine and fumaric, malic, and succinic acids, all of which have been isolated from tobacco leaves (25). The indicated relationship to oxalacetic acid is of less importance. This substance, or some analogous α-keto acid, might arise from the carbon chain of sugars, or from certain amino acids, by reactions that have been supposed to occur in cells.

6. THE AMINO NITROGEN

The amino nitrogen of the hot water extract from tobacco leaves increased very rapidly while the leaves passed from the fresh to the yellow stage. No significant amount of peptide nitrogen was detectable in the extract from the fresh leaf nor, indeed, at any stage of curing (see Section 12); consequently the rapid increase of amino nitrogen can only be attributed to the extensive hydrolysis of protein to which reference has already been made. The insoluble nitrogen of the press cakes diminished from 182.8 to 95.0 gm. in the first 12 days, that is, about 87 gm. of nitrogen passed

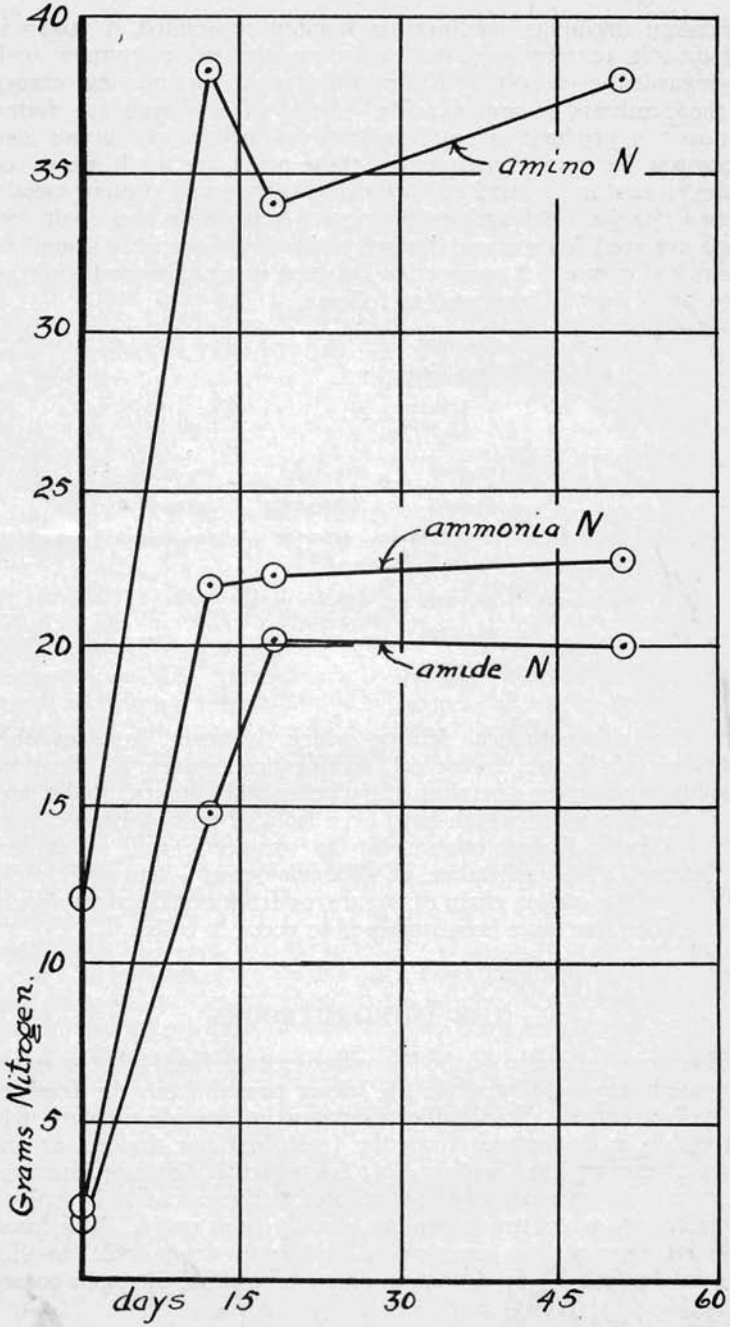


FIGURE 17. Changes in the amino, ammonia, and amide nitrogen of the extract (data from Table 7).

into a soluble form. The nitrogen of the water extract increased from 105 to 189.4, or by 84.4 gm., in the same period. No significant loss of nitrogen therefore occurred at this early stage. The amino nitrogen of the extract rose from 11.9 gm. to 38.2 or by 26.3 gm., the ammonia rose from 2.3 to 21.9 or by 19.6 gm. and the amide nitrogen rose from 1.98 to 14.8 or 12.8 gm. (Figure 17). It is highly probable that much of this increase in amide nitrogen represents a synthesis of asparagine (see Section 13). Of the 87 gm. of protein nitrogen that were converted to a soluble form we can allocate 26.3 + 19.6 + 12.8 or 58.7 gm. to the amino nitrogen of amino acids, to ammonia and to amide nitrogen. This leaves a balance of 27.3 gm. of nitrogen in forms for which we cannot account. The apparent absence of significant amounts of peptide nitrogen in the extract is interesting. It suggests that the hydrolysis of that part of the protein that was digested proceeded all the way to soluble amino acids and implies the presence of extremely active proteolytic enzymes. A relatively large part of the nitrogen of the protein was converted into ammonia or amides. A calculation similar to that on page 221 shows that 29.8 per cent[1] of the 86 gm. probably underwent this conversion.

The most striking feature of these data is, however, the proportion of the nitrogen of the converted protein in undetermined forms; it amounts to 31.7 per cent of the whole. There are three types of combination of nitrogen which may be present to account for this. Some of the nitrogen may have been present in peptide union in spite of our failure to detect this form of nitrogen. There are technical difficulties in the determination of peptide nitrogen[2] in these extracts which may not have been overcome. The second possibility is nitrogen in the form of ring structures with neutral properties of the type of proline or oxyproline. These two substances are well known protein constituents and they undoubtedly made up a part of the protein which passed into the soluble condition. The third possibility is nitrogen in the form of bases. One or more of the three well known basic amino acids arginine, histidine, and lysine are found in all proteins. Each of these bases contains α-amino nitrogen but each also contains nitrogen in other forms of combination to which the highly basic properties of these substances are due. The proportion of basic nitrogen has long been used as one of the means of characterization of proteins; it ranges in well known cases from 5 to 33 per cent. The protein from alfalfa leaves described by Osborne,

[1] Table 6, 10.64 + 30.30 = 40.94 gm. ammonia + amide N (A)
2.33 + 13.01 = 15.34 " " + " " (E)
40.94 — 15.34 = 25.6 " " + " " produced
= 29.8 per cent of 86 gm.

[2] See Section 12 for a discussion of peptide nitrogen determinations.

Wakeman and Leavenworth (12) had 23 per cent of its nitrogen in this form. A part of the basic nitrogen as usually determined is, of course, α-amino nitrogen and would therefore be included in the total amino nitrogen produced by the hydrolysis of the protein of the tobacco leaf. Although we have no definite information regarding the amino acid composition of the tobacco leaf protein either before or after conversion to the soluble form, it is clear that most of the 31.7 per cent of the converted nitrogen in undetermined forms can be satisfactorily accounted for as non-amino basic nitrogen and as imino nitrogen of proline and oxyproline.

The total amino nitrogen of the press cake, determined after this had been subjected to complete hydrolysis, decreased by 51 gm. (Table 8, 104.2 — 53.0 = 51.2 gm.) in the first 12 days of curing. Thus, due to the enzymatic hydrolysis of leaf protein, 51 gm. of potential amino nitrogen must have passed into a soluble form during the interval. If the hydrolysis of leaf protein were the only process that took place it is obvious that the leaf extract should show an equivalent increase of amino nitrogen. Instead of this the increase amounted only to 26.3 gm. (Table 7, 38.17 — 11.88 = 26.29 gm.), which corresponds to a disappearance of 24.7 gm. (51 — 26.3 = 24.7) of amino nitrogen from the extract. At the same time an accumulation of 28.6 gm.[1] of ammonia + amide nitrogen occurred. The amount of amino nitrogen that disappeared corresponds quantitatively, within the limits of experimental error, with the sum of the amounts of ammonia and amide nitrogen formed. It is clear, therefore, that approximately half of the amino nitrogen of the amino acids produced by the hydrolysis of the leaf protein during the first 12 days undergoes a transformation as a result of which the nitrogen appeared in the extract as ammonia and amide nitrogen.

The discussion of the changes in the amino nitrogen that occur during the curing of tobacco has been restricted, up to this point, to the changes that took place in the earliest stage of the process. After the leaves reached the yellow stage of curing the total amount of amino nitrogen in the extract remained, with minor fluctuations, constant (Table 7). The marked preliminary rise in amino nitrogen of the extract was followed by a decrease, during the next six days, of 4 gm. This apparent loss of amino nitrogen was recovered during the final 33 days (see Figure 5). Whether or not the drop has significance is not clear. It suggests

[1] This figure is calculated as follows: The increase in ammonia + amide nitrogen in the extract was 32.5 gm. (Table 7 (21.94 + 14.84) — (2.30 + 1.98) = 32.5 gm.). The amide nitrogen of the press cake diminished by 3.9 gm. (Table 8, 9.64 — 5.72 = 3.92 gm.). Consequently 28.6 gm. of ammonia + amide nitrogen (32.5 — 3.9 = 28.6 gm.) must have arisen from sources other than the amide nitrogen of the protein of the press cake.

that, temporarily, the process of oxidative deamination may have overcome the production of amino nitrogen by hydrolysis of the protein. Be this as it may, the approximate constancy of the soluble amino nitrogen over a long period, during which no less than 14.3 gm. of potential amino nitrogen (Table 8, 53.02 — 38.76) and 22.5 gm. of total nitrogen (Table 8, 94.99 — 72.51) passed into a soluble form, is very striking. In spite of the extensive conversion of nitrogen from the insoluble to the soluble form there was no appreciable change in the soluble amino nitrogen.

Attention has already been called to the similar constancy of the sum of the amide and ammonia nitrogen during the same period and the suggestion was made that these forms of nitrogen reach a type of equilibrium in which ammonia leaves the tissues at a rate approximately equal to that at which it is formed. The observation that the amino nitrogen likewise reaches a maximum is evidence for a close inter-relationship of all three forms of nitrogen. It is probable that the amino acids accumulate, by hydrolysis of the protein, until concentration of amino nitrogen is such that the rate of deamination equals the rate at which amino nitrogen is formed by hydrolysis. The ammonia produced by the decomposition of the amino acids reacts with other constituents of the cell sap, possibly of carbohydrate origin, to form amides; these in turn decompose and liberate ammonia which is evolved. It is, of course, also probable that a part of the ammonia produced from the amino acids passes off directly without going through the amide stage; our data do not permit a decision on this point. Regardless of the exact mechanism, it may be noted that the greater part of the loss of ammonia occurred after the leaves had reached the yellow stage and extensive desiccation had occurred. This is in marked contrast to the loss of organic solids described in Section 4, which was most pronounced during the first stage of curing.

7. THE NITRATE NITROGEN

The data of Table 7 indicate that the nitrate nitrogen changed very little during the process of curing. This specimen of tobacco leaves was unusually rich in nitrate, since it contained 40 per cent of the water soluble nitrogen, or 14 per cent of the total nitrogen of the leaf in this form. There is a suggestion that an increase of nitrate occurred during the first 12 days, the quantity indicated by analysis apparently increasing from 42.3 to 54.1 gm. Subsequently the quantity dropped to 42.8 gm. and there remained. If this increase is real it can only be accounted for by extraordinarily active oxidation processes. There is every reason to believe that oxidative processes did occur in the leaves, particularly during

the first 12 days. Evidence secured in connection with the determination of amide nitrogen in the presence of nitrates showed that fresh tobacco leaves contain an easily oxidized substance that promotes the reduction of nitric acid to ammonia when the mixture is boiled with hydrochloric acid (24). This easily oxidized substance vanished almost entirely during the first 12 days of curing and the most reasonable assumption is that it underwent oxidation although not necessarily at the expense of nitrate. But the oxidation of this substance is one thing; the oxidation of some precursor to nitric acid is quite another. No attempt was made to estimate nitrites in the tissue. If such were present the formation of additional nitric acid during the first 12 days might be simply accounted for. At present we can only conclude that there is evidence of a marked oxidative process during curing. The solution of the problem will have to await a repetition of the experiment to ascertain if the apparent initial increase in nitrate is real and will also require investigation of the possible precursors of this additional nitrate.

The marked increase in nitrate that occurred during fermentation can be accounted for either by migration of nitrate into our specimen from the large quantity of tobacco that surrounded it while fermentation was going on, or by the presence of nitrate in the solution used in the Heber process. As has already been pointed out there was an increase in the total nitrogen and inorganic salts of our material during this operation.

8. THE STABILITY OF THE AMIDES

In the discussion of the quantity of amide nitrogen in these samples of tobacco the data of Table 6 were used to the exclusion of those of Table 7. Table 6 gives analyses of the dried tissue; Table 7 gives analyses of a hot water extract that had been boiled for some time and was then concentrated in vacuo. The data of Table 7 therefore represent only the soluble amides while those of Table 6 include also the amide nitrogen of the protein in the leaf.

On the other hand, however, the data of Table 7 show that considerable hydrolysis of soluble amides to ammonia occurred during the operations of extraction and concentration and, consequently, do not furnish as accurate an idea of the real amounts of amides in soluble form as can be deduced from the data of Table 6 together with those of Table 8, which represents the analysis of the protein in the press cakes.

If the data for the amide nitrogen of the protein in the press cakes (Table 8) be subtracted from the data for amide nitrogen in the whole tissue (Table 6) the figures in the first line of Table 2 are obtained. In the second line are given the figures taken from Table 7 for amide nitrogen in the hot water extracts.

TABLE 2. AMIDE NITROGEN

	E Fresh leaf	A Cured 12 days	B Cured 18 days	C Cured 51 days	D Fermented
	gm.	gm.	gm.	gm.	gm.
Amide N (whole tissue—press cakes)	3.37	24.58	27.01	27.41	8.17
Amide N (hot water extract)	1.98	14.84	20.14	20.08	6.16

Comparison of the two rows of figures shows that in each case significant amounts of amide disappeared as such during the operation of preparing the extracts. The precise interpretation of these figures will have to await a much more extensive study of the composition of these solutions. The figures suggest, however, that some amide other than asparagine is present. Asparagine, while not entirely stable at pH 4, is by no means easily hydrolyzed at this reaction; furthermore, many hours of boiling at pH 3 are required to split off appreciable amounts of amide nitrogen from the protein gliadin.[1] Very considerable amounts of amide nitrogen were hydrolyzed, however, when these tobacco leaf extracts were boiled for a short time during the extraction and were subsequently evaporated in vacuum at low temperature, although the reaction of only one of them was more acid than pH 4.0.

Glutamine is much less stable than asparagine and the results suggest that this amide may have been present. More definite evidence must be obtained, however, before this conclusion can be drawn. Another possibility is suggested by the recent synthesis of the amide of hydroxyaspartic acid by Chibnall and Cannan (*7*). Although this substance has not yet been found in nature there is every reason to suppose that it, or its higher homologue, the amide of hydroxyglutamic acid which has not yet been synthesized, may sometimes occur. Such compounds would undoubtedly be less stable than asparagine.

9. THE "REST" NITROGEN

If the different assigned forms of nitrogen in the tables are added together and the sum deducted from the total nitrogen, the remainder represents that part of the nitrogen that is present in unknown configurations. The "rest" nitrogen of the extract increased markedly during the first 12 days of curing. As has already been pointed out, a part of this increase is doubtless due to the hydrolysis of the protein and the liberation of proline, oxyproline and the basic amino acids in a soluble form. The changes in rest nitrogen that occur are therefore to a considerable extent explicable. But the discovery of the nature of the rest

[1] Unpublished observation.

nitrogen of the fresh leaf extract presents a difficult problem. Certain nitrogenous substances that are well known constituents of plant extracts undoubtedly occur in tobacco; such are purines and the methylated base choline. Moreover, arginine and lysine also are probably present to some extent. Nevertheless there is no evidence whatever of the form of combination of the greater part of this nitrogen. A study of the detailed composition of these solutions is necessary before any conclusions as to the nature of the rest nitrogen can be drawn.

10. THE PERCENTAGE COMPOSITION

The discussion of the chemical changes that occur during the process of curing tobacco has been confined to data that represent absolute quantities of the different constituents. Many of the changes can be detected, however, if the data are calculated on a percentage basis. Table 5 shows the results of these calculations and, since this form of expression is perhaps more customary, the table may be of some assistance in obtaining a mental picture of the composition of these extracts.

If the total solids of the tissue are taken as 100, the proportion of inorganic solids rises from 18.5 to 23 per cent during the curing process, while the organic solids correspondingly decrease from 81.5 to 77 per cent. This change is a result of the loss of over a kilo of organic solids mentioned in Section 4. The proportion of water soluble organic substances varied between the limits 30.5 and 35.1 per cent in a somewhat irregular way. The great difficulty of drying such extracts to constant weight, and the impossibility of deciding at what point the loss of free water ceases and that of combined water or water of composition begins, is reflected in these data. No trustworthy conclusion can be drawn from them. On the other hand, the definite conclusion that a material loss of total solids occurred can be drawn from the data as presented in Table 6.

The proportion of total nitrogen in the tissue underwent very small changes. It varied between 4.62 and 5.07 per cent of the solids and shows no trend except perhaps the sharp rise that occurred during fermentation. The proportion of nicotine nitrogen likewise shows little change—a marked contrast with the data of Table 7 that reveal a definite loss of over 4 gm. or 22 per cent of the nicotine nitrogen originally present.

The striking increases in ammonia and amide nitrogen are well shown by the proportional variation of these constituents, but no clear indication of the origin of this nitrogen is given.

The ether soluble constituents drop from 6.58 to 5.01 per cent of the total solids and give evidence of a slight loss. As has

already been mentioned on page 215, the data of Table 6 show that that loss of ether soluble constituents is in fact very substantial. The proportion of crude fiber apparently increases from 8.83 to 10.83 per cent. Table 6 shows, however, that the crude fiber, as might be expected, really remained sensibly constant throughout the curing and fermentation processes.

The data in the three lower sections of Table 5 serve to show the distribution of the nitrogen in the water soluble and the insoluble fraction in terms of the total leaf nitrogen and also the distribution of the water soluble nitrogen in terms of itself. A detailed discussion of these data would add little to the conclusions already drawn and is therefore omitted.

11. SELECTION OF DATA FROM THE TABLES

In the above discussion what may have appeared to be an arbitrary selection of data from the several tables has occasionally been made. Table 6 contains results secured upon samples of tissue withdrawn before the hot water extraction was made; Tables 7 and 8 give analyses of the extract and the press cakes respectively, while Table 9 gives the sums of the data for each component in Tables 7 and 8. In certain cases the data in Table 9 differ from those of Table 6, although in most cases the agreement is all that could reasonably be expected when consideration is given to the technical difficulties of the analytical operations. In our discussion we have selected figures now from Table 6, now from Table 9. Where this has appeared arbitrary and no reason has been definitely assigned to the choice the explanation is simply that a consideration of the conditions under which the actual analysis in question was made and also of the magnitude of similar figures from samples at other stages of curing has convinced us that some obscure and uncontrolled error occurred and that the figure in one table is not as trustworthy as that in the other. A notable case is the figure for the total nitrogen of the fresh leaf (Table 6, 299 gm.; Table 7, 288 gm.). We have consistently chosen the smaller figure for our calculations in part because, in the two tables, the figures for the total nitrogen of the leaves that had been cured 12 days agree exactly as do those for the leaves that had been fully cured; the total nitrogen determinations of the fermented leaves also agree very closely. Thus in three of the five sets of data the total nitrogen as calculated from different analytical determinations are in substantial or exact agreement. The selection of the more probable value in the two cases that are not in agreement depends on a judgment of the probable accuracy of the different analyses. The figure 299 gm. for the total nitrogen of the fresh leaf (Table 6) is founded on an analysis

of a relatively few leaves arbitrarily selected from a mass that weighed 50 kilos. The material was extremely uniform, to be sure, but the opportunity for a sampling error is apparent. The figure of 288 gm. is the sum of nitrogen determinations made on an accurate aliquot part of a thoroughly mixed solution and a small sample removed from several kilos of air-dry leaf tissue that had previously been repeatedly ground and thoroughly mixed. Appreciable sampling errors are thus highly improbable in the latter case and our selection of 288 gm. as the probable total nitrogen of the fresh leaf and also of 249 gm. as the probable total nitrogen of the 18 day cured leaf is obviously proper.

The nicotine determinations in Table 6 are all lower than those of Table 9. The data of Table 9 are probably more accurate. In this case the possibility of sampling errors is of less importance than the possibility that nicotine volatilized from the leaf tissue during drying of the sample in the oven. No steps can be taken to prevent this when an intact leaf is dried, on the other hand the extracts were carefully acidified to pH 4 so that no loss of nicotine could occur during their concentration. That this precaution was adequate was shown by the failure to detect nicotine in the distillates.

The data for amide nitrogen in Table 6 are all higher than the similar data in Table 9. In this case Table 6 is probably nearer the truth although it is quite possible that some hydrolysis of amides occurred during the drying of the tissues at 60°. In our discussion of the metabolism of the amides we have for the most part employed the sum of the amide and ammonia nitrogen in the calculations of the quantitative relationships. This has been done because the sums when taken from Table 6 agree remarkably well with similar sums taken from Table 9.

12. THE DETERMINATION OF PEPTIDE NITROGEN

The estimation of peptide nitrogen depends on the determination of the increase in the amount of amino nitrogen that results after the extract has been subjected to severe acid hydrolysis. The determination is impossible if substances are present that react with amino groups during the acid treatment in such a way as to remove or condense with them. Nitrites might be expected to interfere with the peptide nitrogen determination by eliminating some of the amino groups, aldehydes might be expected to condense; inasmuch as both types of substance may occur in plant extracts it is clear that peptide nitrogen determinations may readily be seriously in error.

The determinations of peptide nitrogen by hydrolysis of tobacco extracts with 8 N sulfuric acid for 20 hours gave erratic results

difficult to interpret. These extracts contained considerable nitrate nitrogen and it was surmised that this might, by partial reduction to nitrite, act as an interfering substance. In order to study the effect of nitrates during the hydrolysis of peptide bonds, 2 gm. samples of edestin were boiled with 8 N sulfuric acid for 20 hours in the presence of 60 mg. of nitrate nitrogen as potassium nitrate. This was a proportion roughly equivalent to the nitrate content of the tobacco extracts examined. Amino nitrogen was determined by the method of Van Slyke (21) on suitable aliquots after removal of the ammonia. The results are shown in Table 3.

TABLE 3. TOTAL AMINO NITROGEN OF EDESTIN DETERMINED AFTER HYDROLYSIS IN THE PRESENCE AND ABSENCE OF NITRATE

Amino nitrogen from 2 gm. edestin		Loss of amino nitrogen in presence of nitrate
No nitrate nitrogen	60 mg. nitrate nitrogen added	
mg.	mg.	mg.
182	161	11.5
	173	4.95

It is evident that nitrate, in concentrations equivalent to that in our tobacco extracts, may cause an appreciable loss of amino nitrogen. To test this further a mixture of 2 gm. of edestin and 60 mg. of nitrate nitrogen was treated with iron and sulfuric acid according to the method described by Vickery and Pucher (23) for the reduction of nitrate to ammonia. After the reduction was complete the sulfuric acid concentration was increased to 8 N and hydrolysis and analyses for amino nitrogen were completed in the usual way. In this case 185 and 183 mg. of amino nitrogen were recovered, showing that, after the removal of the nitrate, accurate results could be obtained. In Table 4 are given data which show the amino nitrogen in the five tobacco leaf extracts before and after hydrolysis and also after hydrolysis of samples from which the nitrate had been removed by reduction. In the case of the fresh leaf extract there is an indication of an increase in amino nitrogen after hydrolysis in the absence of nitrate, which probably indicates the presence of a little peptide nitrogen in this extract. The extracts from the partially and fully cured leaves showed a depression of the amino nitrogen after hydrolysis and, in all but one experiment, the depression was greater after hydrolysis in the absence of nitrate. In view of these results we hesitate to interpret the increase found in the fresh leaf extract as a measure of the true peptide nitrogen. Furthermore, there is obviously some factor present that destroys amino nitrogen during hydrolysis and prevents accurate estimation of the peptide nitrogen. It is clear that this factor is something other than nitrate but further investigation will be required before it can be identified and its effects eliminated.

TABLE 4. THE AMINO NITROGEN OF TOBACCO EXTRACTS BEFORE AND AFTER REMOVAL OF NITRATE

The figures are gm. of amino nitrogen in the entire 8 liters of extract.

	E Fresh leaf	A Cured 12 days	B Cured 18 days	C Cured 51 days	D Fermented
	gm.	gm.	gm.	gm.	gm.
Before hydrolysis	11.29	36.0	32.28	36.08	24.24
After hydrolysis, nitrate present	11.90	30.7	28.1	30.7	....
After hydrolysis, nitrate removed	13.6	37.4	25.8	27.87	16.84
		27.9	25.83	26.72	17.51
		30.1			

13. THE ISOLATION OF ASPARAGINE FROM TOBACCO EXTRACTS

The clarification of the samples employed for the carbohydrate determination in the hot water extracts (Table 7) was conducted according to the procedure of West, Scharles and Peterson (26), who advise the removal, by precipitation with mercuric sulfate and barium carbonate, of substances that might interfere with the reduction of the copper. The nature of the precipitates obtained suggested the advisability of an investigation of their composition and, to this end, the material secured from 100 cc. samples each of the extracts from Lots A, B, and C (partially and fully cured leaf) were combined, decomposed by hydrogen sulfide and freed from sulfuric acid. The solution contained 0.802 gm. of nitrogen, of which 0.451 gm. was amino nitrogen (0.437 gm. calculated from data of Table 7) and 0.159 gm. of nitrate nitrogen. This left 0.208 gm. of nitrogen in unknown forms. The residue of the solution after analysis was evaporated to 10 cc. and treated with 100 cc. of absolute alcohol. The precipitate was dissolved in water, an excess of silver sulfate was added and the solution was neutralized to pH 7.0 with barium hydroxide. This was done to eliminate substances (organic acids?) that had been found to prevent crystallization of the nitrogenous compounds present. After removal of reagents from the filtrate this was concentrated to small volume. Crystals that resembled asparagine separated on cooling. These weighed 0.725 gm. when the weight was corrected for the aliquots previously removed from the solution for analyses. The crystals contained 12.04 per cent water of crystallization and 18.74 per cent of nitrogen. The theory for asparagine, $C_4H_8N_2O_3.H_2O$, is 11.99 per cent and 18.67 per cent respectively. The amount isolated contained 0.135 gm. of amide nitrogen or 62 per cent of the total amide nitrogen of the three samples as calculated from Table 7. When the difficulty is con-

sidered of isolating a soluble substance by crystallization from such a complex mixture it is clear that a large part of the amide nitrogen of these three extracts was probably present as asparagine.

14. METHODS OF ANALYSIS

Total solids. Leaf tissue. A representative sample of the leaves was spread on trays and dried in a current of warm air (60-70°). The sample was then weighed again and thoroughly comminuted in a suitable mill. A weighed sample of the powder so obtained was dried to constant weight over sulfuric acid in a vacuum desiccator. Preliminary experiments showed that sulfuric acid was more efficient as a dehydrating agent than calcium chloride. A sample dried over sulfuric acid lost 2.25 per cent of water; another sample dried over calcium chloride lost only 1.12 per cent of water. These observations suggest that a part of the water of the leaves is held very firmly and indicate that it is essential to specify the procedure used in obtaining the data for total solids.

Total solids. Extract. 10 cc. samples were evaporated to dryness on the steam bath and then dried to constant weight at 105° in platinum dishes.

Inorganic and organic solids. Dried samples of the leaf were ignited at as low a temperature as possible until an ash of constant weight was obtained.

Total nitrogen. This was determined in all cases by the reduced iron method of Pucher, Leavenworth and Vickery (16).

Nicotine. This was determined by the silicotungstic acid method (1, page 66).

Nitrate nitrogen. The method of Vickery and Pucher (23) was used.

Ammonia nitrogen. This was determined by the colorimetric method of Vickery and Pucher (22) in which the separation of ammonia from nicotine is effected by absorption of the ammonia on permutit.

Amide nitrogen. This was determined from the increase in ammonia produced by hydrolysis with 2 N sulfuric acid for six hours as suggested by Vickery and Pucher (24) in order to avoid possible errors introduced by the presence of nitrates.

Amino nitrogen. The Van Slyke nitrous acid method (21) was employed.

Carbohydrates. All extracts were clarified by the method of West, Scharles and Peterson (26) and sugar was determined in the filtrates by the Munson and Walker method (1, page 190).

Crude fat or ether extract and crude fiber. These determinations were made according to the standard methods (1, page 117).

15. RECAPITULATION AND CONCLUSIONS

The curing of tobacco leaves under standard conditions of commercial practice is accompanied by chemical changes that may be briefly recapitulated as follows.

1. Extensive dehydration of the tissues occurs. The fresh leaf contained 43.53 kilos or about 87 per cent of its weight as water, the fully cured leaf contained only about 1.49 kilos, or 24 per cent, of its total weight as water. Under the experimental conditions adopted 50 kilos of fresh leaf lost about 42 kilos of water or 96.4 per cent of the amount initially present in the tissue. If it be assumed that this water was derived from the cell sap, it is clear that the concentration of the soluble substances dissolved therein must have increased nearly 28 times.

2. A loss of organic solids took place that amounts to nearly 20 per cent of the total solids of the fresh leaf. The original 6.47 kilos of solids diminished to 5.19 kilos and this loss was found to fall, to a large extent, upon the organic solids of the extracted residues of leaf tissue in the press cakes, that is, upon the protein and originally insoluble carbohydrate of the leaf. The data indicate that a considerable part of the coagulable protein and insoluble carbohydrate passed into a soluble form and an equivalent quantity of substance then underwent transformation ultimately to carbon dioxide, water and ammonia or other volatile substances which escaped from the tissues. About 22 per cent of the nicotine also evaporated from the tissues.

3. More than 81 per cent of the apparent soluble carbohydrate of the fresh leaf, much of which was demonstrated to be a sugar that yields phenylglucosazone, disappeared as such. To what extent this sugar was converted to carbon dioxide and water or other volatile substances was not ascertained. If all of it were so converted the changes involved could account for only about one-quarter of the total loss of organic solids that took place.

4. The crude fiber of the leaves underwent no apparent change.

5. The ether soluble material diminished rapidly in amount and about 33 per cent of the initial quantity disappeared in the early stages of curing. The behavior suggests that some of the loss is due to a conversion of chlorophyll into substances some of which are only partly soluble in ether. Changes in other ether soluble constituents must also have occurred whereby these became insoluble in ether. Evaporation of ether soluble constituents of the leaf may also have occurred.

6. The changes in the distribution of nitrogen in the leaf indicate that much of the leaf protein underwent hydrolysis to amino acids and that these subsequently played an important part in the formation of amides and ammonia. The results suggest that oxidative deamination occurred. This was followed by amide

synthesis and at least 23.9 per cent of the nitrogen of the leaf was involved in changes of this kind. Probably nearly a third of the protein nitrogen of the leaf passed through this series of reactions.

7. A loss of 14.6 per cent of the total leaf nitrogen occurred. Only a small part of this (1.5 per cent) could be accounted for as the evaporation of nicotine; the remainder probably represents a direct loss of ammonia from the cells. The results suggest that amino, ammonia and amide nitrogen attained concentrations such that an equilibrium condition was reached in the sense that loss of ammonia compensated amino nitrogen formation from the hydrolysis of the protein. Although all of the amino nitrogen may not have passed through the intermediate step of amides it is clear that much of it did, and the isolation of asparagine suggests that this amide may have played the part of a detoxicating or neutralizing agent for the ammonia as has been suggested by Prianischnikow (15).

8. The nitrate nitrogen of the leaf underwent little apparent change during curing. It seems unlikely therefore that it shared indirectly in the deamination reactions and gave rise to a loss of nitrogen in the elementary form.

9. Evidence was secured of the presence of amides in curing tobacco that are much less stable than asparagine. The possibility that the amide of glutamic acid or of hydroxyaspartic or hydroxyglutamic acid may have been present is suggested.

As this manuscript was going to press a paper by Smirnow and Izwoschikow (A. J. Smirnow and W. P. Izwoschikow, Biochem. Z., **228**, 329 (1930)) came to hand, which records an important investigation of the changes that take place in the forms of nitrogen in tobacco leaves during the first five days of curing. The authors distinguish two types of protein in the green leaves, reserve protein which is easily decomposed by enzymes during curing, and plasma protein which is more resistant and is also of a different composition. They point out that the early stages present phenomena that are associated with starvation and are a continuation of ripening. They observe the material decrease in protein nitrogen, the increase in soluble nitrogen, amino nitrogen, ammonia, and amides, and a small decrease in nicotine that occurs. Their data are expressed in terms of milligrams of each form of nitrogen per 1,000 square centimeters of leaf area and also in percentage. The former of these methods has many of the advantages possessed by the method of expression employed in this bulletin.

The results of Smirnow and Izwoschikow and our own are mutually confirmatory; their studies have dealt with the very early stages of tobacco curing, ours have been chiefly concerned with the later stages of the process.

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TABLE 5. COMPOSITION OF TOBACCO LEAVES

	E Fresh leaf %	A Cured 12 days %	B Cured 18 days %	C Cured 51 days %	D Fermented %
Total dry leaf solids = 100%					
Inorganic	18.53	19.99	22.37	23.00	23.78
Organic	81.47	80.01	77.63	77.00	76.22
Water soluble inorganic	16.41	17.42	18.28	19.86	20.51
Water soluble organic	30.56	36.15	30.83	35.08	33.34
Total soluble solids	46.97	53.57	49.11	54.94	53.85
Total insoluble solids	53.03	46.43	50.89	45.16	46.15
Nitrogen	4.62	5.07	5.05	4.76	5.38
Nicotine N	0.250	0.294	0.294	0.249	0.298
Nitrate N	0.614	0.810	0.610	0.603	0.793
Ammonia N	0.036	0.190	0.279	0.274	0.875
Amide N	0.200	0.540	0.610	0.620	0.264
Ether Soluble	6.58	6.19	5.41	5.01	4.62
Crude Fiber	8.83	9.29	10.01	10.83	10.71
Total dry leaf nitrogen = 100%					
Total N of water extract	35.15	66.64	64.42	68.96	70.71
Nicotine N	6.11	6.49	5.46	5.74	5.59
Nitrate N	14.14	19.05	16.11	16.71	17.85
Ammonia N	0.77	7.72	8.36	9.19	20.70
Amide N	0.66	5.22	7.59	8.12	2.19
Amino N	3.97	13.41	12.82	15.35	9.08
Rest N	9.50	14.75	14.08	13.85	15.30
Total N of press cakes	61.14	33.43	29.52	29.33	31.77
Nicotine N	0.08	0.05	0.09	0.06	0.05
Nitrate N	0.07	0.10	0.00	0.00	0.00
Ammonia N	0.00	0.00	0.00	0.00	0.00
Amide N	3.22	2.01	1.90	1.94	2.00
Amino N	34.84	18.66	14.67	15.68	15.77
Rest N	22.93	12.61	12.86	11.65	13.95
Total water soluble N = 100%					
Nicotine N	17.38	9.73	8.47	8.20	7.91
Nitrate N	40.23	28.57	25.04	23.89	25.24
Ammonia N	2.19	11.58	12.97	13.14	29.26
Amide N	1.88	7.83	11.78	11.61	3.08
Amino N	11.28	20.15	19.90	21.89	12.83
Rest N	27.04	22.14	21.84	21.27	21.68

TABLE 6. COMPOSITION OF TOBACCO LEAVES FROM ANALYSIS OF SAMPLES OF DRIED MATERIAL

The figures are grams of each component in material derived from 50 kilos of fresh leaves.

	E Fresh leaf	A Cured 12 days	B Cured 18 days	C Cured 51 days	D Fermented
	gm.	gm.	gm.	gm.	gm.
Weight of leaf as received ..	50000	20350	11050	6680	6560
Total solids	6474	5604	5254	5187	5231
Inorganic	1199	1220	1175	1193	1244
Organic	5275	4384	4079	3994	3987
Total nitrogen	299	284.1	265.4	247.2	281.4
Nicotine N	16.18	16.47	15.44	12.91	15.59
Nitrate N	39.82	45.40	32.05	31.27	41.49
Ammonia N	2.33	10.64	14.67	14.21	45.81
Amide N	13.01	30.30	32.06	32.21	13.81
Ether soluble	426	347	284	260	242
Crude fiber	572	521	526	562	560
Reaction of water extract (pH)	5.38	5.62	5.74	5.55	5.86

TABLE 7. COMPOSITION OF THE HOT WATER EXTRACT FROM TOBACCO LEAVES

The figures are grams of each component in the extract from material derived from 50 kilos of fresh leaves.

	E Fresh leaf	A Cured 12 days	B Cured 18 days	C Cured 51 days	D Fermented
	gm.	gm.	gm.	gm.	gm.
Weight of leaf used in analysis	47570	47170	47420	47560	47370
Total solids	3044	3003	2581	2851	2821
Inorganic	1063	977	961	1031	1074
Organic	1981	2026	1620	1820	1747
Total nitrogen	105.1	189.4	171.0	173	199
Nicotine N	18.27	18.45	14.51	14.20	15.74
Nitrate N	42.28	54.13	42.83	41.31	50.23
Ammonia N	2.30	21.94	22.18	22.74	58.24
Amide N[1]	1.98	14.84	20.14	20.08	6.16
Amino N	11.88	38.17	34.02	37.94	25.55
Rest N	28.4	41.9	37.3	36.7	43.1
Total carbohydrate	348.0[2]	139.0	76.0	65.0	
Reaction of final extract (pH)	4.8	4.0	4.2	3.9	4.0

[1] Pure crystalline asparagine isolated.
[2] 48 per cent isolated as glucosazone.

TABLE 8. COMPOSITION OF THE EXTRACTED RESIDUES OF TOBACCO LEAVES IN THE PRESS CAKES

The figures are grams of each component in material derived from 50 kilos of fresh leaves.

	E Fresh leaf	A Cured 12 days	B Cured 18 days	C Cured 51 days	D Fermented
	gm.	gm.	gm.	gm.	gm.
Total solids	3390	2669	2488	2343	2432
Inorganic	265	251	240	221	287
Organic	3125	2418	2248	2122	2145
Total nitrogen	182.8	94.99	78.36	72.51	89.4
Nicotine N	0.24	0.15	0.23	0.14	0.13
Nitrate N	0.20	0.29	0.00	0.00	0.00
Ammonia N	0.00	0.00	0.00	0.00	0.00
Amide N	9.64	5.72	5.05	4.80	5.64
Amino N[1]	104.2	53.02	38.93	38.76	44.36
Rest N[1]	68.52	35.81	34.15	28.81	39.27
Total soluble N[1]	135.1	68.55	52.34	50.35	57.78
Total insoluble N[1]	47.7	26.17	26.02	22.11	31.64

[1] After hydrolysis with 8 N sulfuric acid.

TABLE 9. COMPOSITION OF TOBACCO LEAVES FROM ANALYSES OF EXTRACT AND EXTRACTED RESIDUES

The figures were obtained by adding the separate items in Tables 6 and 7, and represent the grams of each component in material derived from 50 kilos of fresh leaves.

	E Fresh leaf	A Cured 12 days	B Cured 18 days	C Cured 51 days	D Fermented
	gm.	gm.	gm.	gm.	gm.
Total solids	6434	5672	5069	5194	5253
Inorganic	1328	1228	1200	1252	1361
Organic	5106	4444	3869	3942	3892
Total Nitrogen	288	284	249	246	289
Nicotine N	18.51	18.60	14.74	14.34	15.87
Nitrate N	42.48	54.42	42.83	41.31	50.23
Ammonia N	2.30	21.94	22.18	22.74	58.24
Amide N	11.62	20.56	25.19	24.88	11.80
Amino N[1]	116.1	91.19	72.95	76.70	69.91

[1] After hydrolysis with 8 N sulfuric acid.