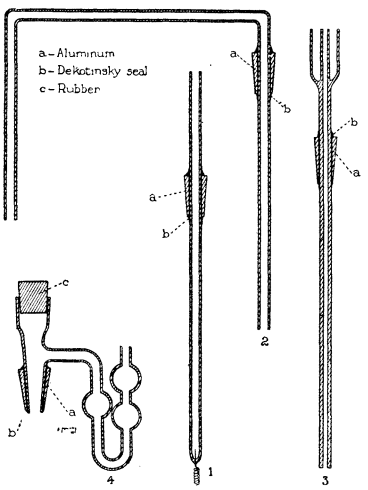

FIG. 3

oxygen. DeKhotinsky seals made as described are much more secure than if made without the collar of metal. Any other suitable material could be used in place of aluminum; this metal is easy to work on the lathe and has been found to be satisfactory in every way (Fig. 3).

Evidence that the copper furnace completely removes all traces of oxygen and that the construction of the electrode cells and movable burettes satisfactorily meets all requirements will not be included in this paper, but will be given in the papers dealing with the reaction of cobalt salts with cysteine, and the oxidation reduction potential of cysteine and glutathione.

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SPECIAL ARTICLES

THE CHEMICAL CHANGES THAT OCCUR DURING THE CURING OF TOBACCO LEAVES[1]

IT has long been assumed that the metabolism of excised leaves follows a relatively normal course for quite appreciable periods of time, and that differences that arise between the chemical composition of such leaves and the intact leaf are due to the cutting off of the food supply from the stem and to the interference with the outlet for the products of metabolism; storage of these products therefore occurs. Subsequently, however, the changes become more patently katabolic; extensive dehydration of the tissue takes place if water is not supplied and this brings in its train a whole series of complex changes, many of which can be recognized as the results of the activity of well-known types of enzymes.

The curing of tobacco under commercial conditions affords an excellent opportunity for the study of these katabolic changes, since the soluble substances in the large thin leaf of this plant can be readily extracted by means of hot water and adequate quantities of leaves of uniform size and development are easily secured.

In order that definite comparisons might be established between leaf tissue which had reached different stages of the curing process five 50 kg lots of leaves (8th to 11th leaf) were picked from the plants the same day (August 1, 1929). One of these lots was immediately extracted with boiling water, the other four lots were strung on cords in the customary manner and suspended in a curing shed. The second lot was removed and extracted at the expiration of 12 days when all the leaves had become yellow, the third lot at the expiration of 18 days when the leaves had first become a uniform brown color, the fourth at the end of 51 days when the leaves were pronounced "fully cured," and the last lot was subsequently fermented along with the main crop of tobacco from the same field. Each lot was extracted with hot water in the same way. The leaves were dropped slowly into a large volume of boiling water to which sufficient sulphuric acid was added from time to time to maintain the reaction at or near pH 4.0; loss of nicotine was thereby avoided. After boiling until the mid-ribs were soft the leaves were pressed at the hydraulic press. The residues were then ground in a meat grinder and re-extracted with boiling water. This process was repeated once more. The three successive extracts were collected quantitatively, were filtered and concentrated *in vacuo* to 8 l. Analyses of extracts and extracted residues were then carried out, the methods for total, ammonia, amide and nitrate nitrogen employed being those recently developed in this laboratory,[2] amino nitrogen was determined by Van Slyke's method and the total solids, ash, crude fiber, soluble carbohydrate and ether soluble solids were determined by standard methods.

Inasmuch as each lot of leaves was initially of the same weight and the leaves were individually of the same size[3] and age, comparison between the different lots could be established by calculation of the absolute

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[2] H. B. Vickery and G. W. Pucher, *J. Biol. Chem.*, 83, 1 (1929); *Ind. Eng. Chem., Anal. Ed.*, 1, 121 (1929); G. W. Pucher, C. S. Leavenworth and H. B. Vickery, *Ind. Eng. Chem., Anal. Ed.*, 2, 191 (1930); H. B. Vickery and G. W. Pucher, *J. Biol. Chem.*, 90, 179 (1931).

[3] The uniformity of the material is evident from the fact that the 12-day lot contained 2,014 leaves and the 18-day lot contained 2,011.

weight of each component at each stage of curing. Loss or gain of any component could then be readily determined. This method of calculation gives results that are much more easily appreciated than those founded on a percentage basis since the total solids and total nitrogen, which are customarily employed as the fundamental data, both underwent substantial changes.

After completion of the curing process the 50 kg of fresh leaves weighed only 6.68 kg. By far the greater part of the loss of weight was due to the evaporation of water. The fresh leaves contained 43.53 kg of water and 6.47 kg of dry solids, the fully cured leaves contained 1.49 kg of water and 5.19 kg of dry solids, consequently 42 kg or 96.4 per cent. of the water originally present had evaporated. The magnitude of the loss of solids is striking. Approximately 1.3 kg or 19.8 per cent. of the whole disappeared, and this loss was found to fall exclusively, as might be expected, on the organic matter of the leaf. A detailed discussion of the types of substances that contributed to this lost material can not be attempted in this place[4] but the data strongly indicate that more than half of the material that disappeared had its origin in protein.

There were 426 gm of other soluble material in the fresh leaves; after 12 days 347 gm were present and after 51 days only 260 gm remained. This represents a loss of about 39 per cent. of the initial quantity during the curing process. Much of the extensive early change in the quantity of ether soluble material present is probably associated with the destruction of the chlorophyll which was practically complete at the end of 12 days. The fresh leaves contained 92.5 gm of chlorophyll. On the assumption that decomposition to chlorophyllides and phytol occurred, two thirds of this amount should have been converted to substances of rather low solubility in ether at the time the chlorophyll was no longer recognizable in the leaf. During the entire curing process, however, other and quantitatively much more extensive changes also occurred. In how far these were due to conversions of ether soluble substances to ether insoluble substances rather than to direct loss from the leaf (*e.g.*, by evaporation) was not ascertained.

The amount of crude fiber in the leaves underwent no change, but the amount of soluble carbohydrate, estimated from copper reduction as glucose, diminished from 348 to 65 gm, a loss of more than 81 per cent. That a considerable part of this carbohydrate was either glucose, fructose or mannose was demonstrated by the isolation as phenyl-glucosazone of 48 per cent. of the indicated 348 gm of sugar in the extract from the fresh leaf. If it be assumed that all the loss of carbohydrate was due to oxidation to carbon dioxide and water, or other volatile substances, the quantity that disappeared accounts for only about one quarter of the total loss of organic matter during curing.

Of the 288 gm of nitrogen in the fresh leaves 105 gm were soluble in hot water but, during the first 12 days, enzymatic hydrolysis of coagulable protein occurred and the amount of nitrogen soluble in hot water was increased by 84 gm. Peptide nitrogen could not be demonstrated to be present in these extracts and it is therefore probable that the protein underwent complete digestion to amino acids. During the same period a rapid rise in ammonia and amide nitrogen took place, the sum of these increasing from 4.3 to 36.8 gm. Extensive deamination of the amino acids followed by amide synthesis must, therefore, have occurred. These observations are in complete agreement with those of Chibnall[5] on starved runner bean leaves.

The magnitude of the quantities of nitrogen involved in these conversions can best be appreciated from a consideration of the data for the whole curing period. The total nitrogen of the leaves decreased from 288 gm to 246 gm but evaporation of nicotine accounted for a decrease of only about 4 gm. The remaining 38 gm therefore represent nitrogen that escaped from the leaves in some other form of combination, in all probability as ammonia. Inasmuch as the nitrogen in the water insoluble residues of the leaves diminished from 182.8 to 72.5 gm, an amount of protein that contained approximately 110 gm of nitrogen was converted into a form soluble in hot water. Calculation from the sum of the ammonia and amide nitrogen present at the beginning and end of the curing process, with due allowance for the 38 gm of nitrogen that escaped from the tissues, showed that no less than 69 gm of nitrogen passed through the ammonia or amide nitrogen stage. While it is improbable that all this was originally in the form of protein and was derived from the 110 gm of nitrogen in the protein that was digested, it is evident that no less than 23.9 per cent. of the total nitrogen of the leaf passed through one or both of these stages. Deamination of amino acids and synthesis of amides are therefore of great importance during the curing of the tobacco leaf.

An approximate idea of the proportion of the original protein nitrogen that shared in these reactions was secured from a study of the amino nitrogen in the extracts and of the amino nitrogen yielded by the leaf residues after these had been subjected to

[4] A full discussion together with the complete data of this investigation will be presented in *Bulletin* 324, Conn. Agr. Exp. Sta. (1931), in press.

[5] A. C. Chibnall, *Biochem. J.*, 18, 387, 395 (1924).

complete hydrolysis. The difference between the amounts of amino nitrogen produced by acid hydrolysis of the residues at two successive stages may be regarded as a measure of the amino nitrogen which gradually passed into solution as the katabolic reactions in the tissues proceeded. The residues from the fresh leaves yielded 104 gm of total amino nitrogen, those from the leaves that had been cured for 12 days yielded only 53 gm, consequently in this period 51 gm of potential amino nitrogen must have passed into a soluble form. But, instead of a corresponding increase of 51 gm of amino nitrogen in the extract from the 12-day cured leaves, this increase amounted to only 26 gm; 25 gm of amino nitrogen disappeared as such. It was found, however, that the accumulation of ammonia and amide nitrogen that occurred amounted together to approximately 28 gm, which corresponds satisfactorily to the observed deficit of amino nitrogen. It is clear, then, that at least half the potential amino nitrogen of the protein that underwent enzymatic hydrolysis in the first 12 days was further converted to ammonia and amide nitrogen.

The approximate constancy of the quantities of ammonia, amide and amino nitrogen in the extracts from the more fully cured specimens of leaves suggests some sort of an equilibrium condition in the relationships between these forms of nitrogen. In the period from 12 to 51 days the sum of the three forms diminished by only 5 gm. The unassigned or "rest" nitrogen likewise diminished by about 5 gm in spite of the fact that, during this interval, some 23 gm of nitrogen, originally present in the form of coagulable protein, were converted to a soluble form. Extensive changes took place in the forms in which the nitrogen is combined in the leaves and the data suggest that, although many side reactions doubtless occurred, the *essential* sequence of reactions was, protein nitrogen → amino acid nitrogen → ammonia nitrogen → amide nitrogen. Whether the nitrogen that escaped from the tissues, presumably in the form of ammonia, was derived from subsequent hydrolysis of the amides or directly from the deamination of amino acids was not ascertained. Probably both sources were available since the presence in the leaves of enzymes that hydrolyze amides was suggested by the extensive decrease in amide nitrogen that ensued during fermentation of the fifth lot of leaves.

Perhaps the most significant result of these studies is the demonstration of the rapidity of the katabolic reactions during the early stages of curing. Three quarters of the loss of water and of soluble carbohydrate and more than half of the loss of organic solids and of ether soluble constituents occurred during the first 12 days; more than three quarters of the quantity of protein digested underwent this process in the same period. It is obvious that far-reaching chemical changes set in very shortly after leaves are detached from the plant.

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POLLEN-STATISTICS: A NEW RESEARCH METHOD IN PALEO-ECOLOGY

THE number and importance of the papers on ecology in American botanical literature clearly testify to the popularity of ecology among American botanists. Yet the domain of Paleo-ecology has been almost untouched, although researches along such lines would, in many cases, lead to a better understanding of actual ecological problems. In Europe no other branch of Paleo-ecology has been more popular during the last years than pollen-statistics, or the method of tracing the history of the forests from the occurrence of the fossil tree pollen grains in peats and sediments, which was elaborated some twenty years ago by the Swedes G. Lagerheim and L. von Post. There are signs that an activity in this field similar to that in Europe is not far off in this country, and it has been considered appropriate, therefore, to give here a brief description of the working methods, together with some practical hints which might prove useful to beginners.

THE FIELD WORK

The field work is chiefly confined to the summer. During the winter, however, when the lakes are frozen over, samples of bottom sediments could be obtained by means of borings through the ice. Several types of boring rods are in use. The more obsolete ones are being supplanted more and more by the Hiller peat auger, manufactured by the Beus and Mattson Company of Mora, Sweden. This company offers the auger in two different models; a smaller one with extension rods of 100 cm each, which is kept in a leather case and carried by a strap over the shoulder, and a bigger one with extension rods of 150 cm each. The approximate cost of the two models is about twenty-five and forty-five dollars, respectively. If the field work is carried out with the help of an assistant, preference should be given to the heavier auger, which is more reliable than the smaller one. The field apparatus also includes a spade, a big knife, forceps, glass tubes in which to keep the samples (about 7.5 cm long and 1.3 cm inside diameter and corked at both ends), diopter compass, and a geodetical set for taking levels and distances.

At the boring place a big sod of the surface material is first removed with the spade. From the walls of the sod the first samples are taken, say, from 2, 5, 10, 15 and 20 cm below the surface. Then the auger