144. INVESTIGATIONS INTO THE CHEMISTRY OF THE FLUE-CURING OF TOBACCO

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IN connexion with an attempt to grow in Assam tobacco suitable for the manufacture of cigarettes, the opportunity was taken of starting investigations upon the chemical changes that occur in flue-curing. This appears to be the process best suited for the type of leaf grown.

The curing was carried out in substantially the same way as recommended by Brown [1933]. The curing barn is filled with leaf, preferably of the same texture and ripeness, and the barn is then sealed. The door and all ventilators are closed, so that no moisture can escape. The furnace is lit and the temperature of the barn maintained at 85–90° F. At this temperature metabolic processes will be taking place almost at their maximum rate and the sealing of the barn ensures that the loss of moisture will be slight. The temperature is maintained at about 90° F. until the leaf begins to yellow at the edges, after which the temperature is slowly raised, 5° at a time, still keeping the barn sealed. During this period the yellow colour spreads over the leaf and by the time the temperature has reached 110° F. the whole leaf should be yellow, with only a slight greenish tinge. The full yellow colour should be developed by the time the temperature has reached 115° F. The time taken to reach this stage will depend upon the nature of the leaf. In our experiments it took from 36 to 48 hr. Meanwhile it is necessary to reduce the humidity in the barn somewhat. If the humidity remains high with the temperature above 110° F. the leaf will assume a reddish brown colour. This defect is generally referred to as sponging, and it is believed that we can now account for this, as will be shown later in this paper. On the other hand if the leaf is allowed to dry out too rapidly the cells are killed prematurely and the chemical changes characteristic of curing are arrested. The leaf then remains green in patches.

To fix the bright lemon-yellow colour it is necessary to strike a mean between these two courses. The humidity in the barn is reduced by opening the ventilators gradually. Fig. 1 shows the stages at which humidity was increased in one curing. The ventilators were quarter opened when the temperature reached 105° and ventilation was then gradually increased up to 120° F. when the leaf began to curl. At 135–140°, when the ventilators are fully open, the blade of the leaf is practically dried out, but the midrib still remains moist. This is dried out by increasing the temperature in stages to 160°, during which period the ventilation is again reduced. This temperature also serves largely to inactivate the enzymes so that when the cured leaf is allowed to take up moisture again (for greater ease of handling without damage) further chemical changes in the tissue are arrested.

The chemical changes that occur during flue-curing do not appear to have been fully investigated. Vickery *et al.* [1933] have studied the curing to the brown stage of Connecticut shade-grown tobacco, and other investigations on

miscellaneous curing processes have been summarized by Bodnar & Barta [1935]. These changes bear a strong resemblance to those found to occur during water culture in the dark of the tobacco leaf.

These latter changes have been studied in great detail by Vickery *et al.* [1937], and Chibnall [1939] has summarized these findings. An outline of the conclusions is given below.

Fig. 1. *A*, top and bottom ventilators $\frac{1}{2}$ open. *B* top and bottom ventilators $\frac{1}{2}$ open. *C*, top and bottom ventilators fully open. *D*, bottom ventilator $\frac{1}{2}$ closed. *E*, bottom ventilator fully closed. *F*, top ventilator $\frac{1}{2}$ closed. *G*, top ventilator fully closed.

Tobacco leaves were detached from the plant early in the morning when carbohydrate content was at a minimum. As water culture was in the dark no further production of carbohydrate by photosynthesis occurred. The first 20 hr. of water culture Chibnall regards as the 'carbohydrate phase', for the carbohydrates are here the main source of energy. The organic acids are also concerned to some extent in the metabolic changes, while protein is hydrolysed to amino-acid and thence oxidized to NH_3 , the latter condensing with fumaric or oxaloacetic acids to produce asparagine.

In the second phase, lasting from 20 to 70 hr., carbohydrates play a minor part, while significant changes take place in the organic acids. Protein decomposition and asparagine formation continue.

In the third and final phase lasting from the 70th hr. onwards the supply of organic acids has become exhausted and the leaf tissue now derives its energy almost entirely from protein. The leaves are yellowing, and disintegration of the chloroplasts and breakdown of the cytoplasm are increasing. In this 'protein phase' the amino-acids are eventually no longer able to function both as respiratory substrates and NH_3 acceptors (asparagine formation) and the resultant accumulation of NH_3 is probably the chief cause of the death of the cell.

The three phases might be expected to be characterized by R.Q. of 1, >1 , and <1 , but owing to overlapping of organic acid and protein phases the rise in R.Q. during the second phase may be obscured.

EXPERIMENTAL

Methods

The respiratory activity of tobacco leaf was measured essentially as described by Deb & Roberts [1940] for tea, using the Barcroft-Warburg apparatus [Dixon, 1934]. 300 mg. tobacco leaf in circles of area 1 cm.² were weighed into each of four or five Warburg vessels and covered with 3 ml. *M*/15 phosphate buffer at pH 5.8. The inner cup contained a small roll of filter paper soaked with KOH. A second identical series of vessels was set up but in this case the KOH was omitted. After equilibration of temperature the manometric levels were read at suitable intervals over a period of 1–3 hr. O₂ uptakes are obtained directly from the vessels containing KOH. In the vessels without KOH, O₂ will be consumed and CO₂ given out, so that the net change in the manometric level will be considerably smaller. After making allowance for the difference in solubility of O₂ and CO₂ in water the CO₂ output can be calculated from the change in manometric levels of the vessels without KOH, and the observed O₂ uptake.

For tea the above method has proved accurate provided that care is taken in the sampling of the tissue, and although cumbersome, has the advantage over the Dickens & Šimer method of affording a measure of R.Q. at frequent intervals over a long period. In the Dickens & Šimer method the mean R.Q. over a given period may be determined with greater accuracy but no information is afforded as to the variations of the R.Q. within the experimental period.

CO₂ retention. The above method of estimating R.Q. has been of little use in working with animal tissues owing to the retention of CO₂ by phosphate buffers and protein. For plant tissues this CO₂ retention is of smaller importance. Turner [1938] finds no difference between the R.Q. of carrot tissue determined by this method and that of Dickens & Šimer, and my own observations with tea leaf show that CO₂ retention both at the beginning and end of a long period of shaking is entirely negligible.

With tobacco leaf CO₂ retention is appreciable. The amount of bound CO₂ in the tissue may be determined manometrically by tipping 0.5 ml. 2*N* H₂SO₄ from a side arm into the main compartment of the Warburg vessel which contains the tissue suspended in the buffer solution, thus liberating bound CO₂.

The following example, in which the R.Q. of leaf in the closing stages of curing is being determined, illustrates the magnitude of the retention effect. After 2 hr. shaking at 36°, the O₂ uptake per 100 mg. of tissue (moisture content 54.35 %) amounted to 194 μl. while the CO₂ output was 139 μl. This gives an average R.Q. of 0.72; uncorrected for CO₂ retention.

The CO₂ liberated on adding acid to the original tissue amounted to 48 μl. per 100 mg. tissue, while the amount set free after 2 hr. shaking was only 20 μl. It follows that 28 μl. (the difference between the above figures) of the bound CO₂ in the original tissue was set free in the course of 2 hr shaking, and this figure must be subtracted from the observed CO₂ output to give the amount of CO₂ produced in the course of metabolism. The CO₂ produced in the 2 hr. shaking thus amounts not to 139 μl. but 111 μl. and the R.Q. must be reduced from 0.72 to 0.57.

The correction is relatively small for fresh uncured leaf but increases as the process continues. As the leaf dries out, its CO₂ retention appears to increase. The evolution of the bound CO₂ during the shaking proceeds at a fairly uniform rate. For this reason the R.Q. values recorded over successive intervals of time, although too high, have considerable value in indicating the nature of the chemical changes taking place at different periods during the shaking.

O₂ tension. The R.Q. of young tobacco leaves has been measured on many occasions and does not depart significantly from 1.0. On the other hand the R.Q., as measured by the above method, of leaf ripe for curing varies from 1.3 to 1.7. It was at first thought that this implied a predominantly organic acid type of metabolism in such leaves but subsequent analyses showed that the leaf contained appreciable amounts of reducing sugars.

The ripe leaf is considerably thicker than the young leaves, for which an R.Q. of 1.0 was obtained, and the possibility arose that the high R.Q. was due to the inability of O₂ to diffuse into the interior of the tissue and to maintain an O₂ tension sufficient to ensure aerobic oxidation of the carbohydrates. In other words some anaerobic fermentation may also be taking place within the tissue with the result that the R.Q. would exceed unity.

The correctness of this view was shown by carrying out the determinations in an atmosphere of O₂ when the R.Q. value was found to be unity.

Table 1. *Apparent R.Q. in air and O₂*

Atmosphere	O ₂ uptake/1 hr./ 100 mg. tissue	CO ₂ output/1 hr./ 100 mg. tissue	R.Q.
Air	40	50	1.25
O ₂	76	77	1.01

Results

Ripe tobacco leaf was only available for a comparatively short period and in limited amounts. The results obtained therefore are of a preliminary nature only but it is believed that the methods developed are suitable for a thorough investigation of the flue-curing process and it is hoped at a later date to carry out such an investigation. Meanwhile the preliminary results are presented in the hope that they may be of some assistance to other workers in the field.

We had neither the resources nor the amount of material to carry out full analyses such as were undertaken by Vickery and Pucher. Samples of leaf were taken at suitable intervals during the whole curing process, but these samples were not sufficiently large to be representative for chemical analysis. From the results obtained a decrease in total organic solids is apparent as the result of curing, but the sampling error was too high to enable an accurate estimate of this loss to be made.

For the purposes of the experiment six leaves were selected for each sample. Disks of 1 cm.² were punched in the blades of these leaves, avoiding the mid-rib. In the dried-out leaf it was impossible to draw such samples and the tissue was cut by scissors into squares of approximately 1 cm.² The samples were then thoroughly bulked and subsamples weighed out into the Warburg vessels. The agreement between replicates was a guarantee of the accuracy of this second sampling but of course there is no certainty that the original sample was truly representative. However, as the leaves were chosen to approximate to the mean degree of curing and as the quantity we are interested in is a ratio and not an absolute quantity it is possible that smaller samples may suffice than if an analysis were to be carried out.

As the barn is kept sealed for the first 24 hr. no sample during that time could be taken. Table 2 gives a typical result for leaf after it has been in the curing barn for 40 hr.

Table 2. *40 hr. cured leaf*

hr. ...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2
O ₂	50	43	41	37
CO ₂	48	40	36	33
R.Q.	0.96	0.93	0.88	0.89

Uptakes and outputs are expressed in $\mu\text{l.}$ per 100 mg. tissue (moisture content 70.5%). No correction is made in this case for CO_2 retention but with leaf at this stage the effect is small and would only reduce the R.Q. by two or three units in the second decimal place.

The rate for the first half hour is equivalent to $3.4 \mu\text{l. O}_2$ per mg. dry wt. per hr. against $3.7 \mu\text{l. O}_2$ for the original uncured leaf. There is appreciable loss in solid matter during 40 hr. possibly amounting to 10 % of the total so that the actual fall in respiratory activity is rather greater than would appear from the above figures.

There can be no doubt from the above result, confirmed as it has been on several occasions, that protein is being metabolized as a respiratory substrate at this stage of the process although carbohydrate, organic acids, or both, still account for the greater part of the respiration.

In general a decrease both in the R.Q. and in the respiratory coefficient (Q_{O_2}) is at first observed as curing proceeds, but when the temperature of the barn exceeds 120°F. a fresh series of effects is noted when samples are investigated manometrically.

The average R.Q. over a period of 2 hr. is well below 0.7, the value for protein oxidation, even without making the correction for CO_2 retention, which is quite appreciable in such cases. The initial rate of O_2 uptake is high, but this falls rapidly in contrast to the slow decrease observed for fresh leaf or for samples withdrawn at lower temperature in the barn. As the rate of O_2 uptake falls the R.Q. rises and eventually after about 2 hr. may reach 0.8. The Q_{O_2} value is then only some 10 % of the original value for the uncured leaf. In general the longer the leaf has been in the barn at temperatures over 120°F. the lower is the R.Q. for the first half hour of shaking. The leaf when placed in the Warburg vessel loses its bright lemon-yellow colour and takes on a brown tint while the reaction medium instead of remaining colourless becomes reddish brown. This solution gives all the reactions for a polyphenol.

A typical series of figures for O_2 and CO_2 in such a case is given in Table 3.

Table 3

hr. ...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	2-2 $\frac{1}{2}$
O_2	150	55	33	20	24
CO_2	47	26	18	16	21
R.Q.	0.31	0.47	0.55	0.80	0.88

Note the rapid fall in the rate of O_2 uptake in successive half hour periods and the corresponding rise in the R.Q.

Reddening of partly cured tobacco leaf after moistening. Although this very characteristic effect is always observed in tobacco leaf after the temperature of the barn has reached 120°F. , there is no reason to believe that it forms part of the normal curing process. Properly flue-cured tobacco always has a bright lemon-yellow colour whereas this chemical change in the Warburg vessel is associated with the development of a dark brown colour. Again, judging by the figures in Table 3, it takes about $1\frac{1}{2}$ hr. to complete the reaction associated with the low R.Q. after which the chemical changes in the leaf are probably mainly those of protein degradation and oxidation. On the other hand, leaf may be left in the curing barn for another 48 hr. after 120°F. is reached and still be lemon-yellow, yet it will still undergo this low R.Q. change when suspended in water.

The most probable explanation of this effect is that at c. 120° F. the cytoplasm of the tobacco leaf undergoes a rapid change in its permeability so that the polyphenols may diffuse into it. In the curing when this point is reached the tissue is dried out to such an extent that diffusion of the vacuole contents into the cytoplasm is prevented, but if the tissue be moistened, as it is in the Warburg vessel, free penetration of the cytoplasm by the polyphenols may now take place with consequent oxidation to brownish red pigments.

The evidence in favour of this view will now be considered. A sudden increase in the permeability of the protoplasmic membrane of plant tissue at a moderately elevated temperature has been established in at least two other cases and there is every reason to believe that this is a general phenomenon. Thus Sen [1928] has shown that the electrical resistance of the *Mimosa pudica pulvinus* decreases slowly with increasing temperature over the range 30–50°, but at 55° there is a very sharp drop in the resistance. Sen interprets this as due to the complete permeability of the protoplasm to vacuole solvents at this temperature so that the tissue as a whole will have a lower electrical resistance.

A second example of this is provided by the effect of heat on tea leaf. If the leaf is maintained at a temperature of 120° F. under conditions where moisture loss is slow, it gradually reddens and eventually the whole leaf assumes a uniform reddish brown colour analogous to that produced in tea-fermentation. Microscopic examination reveals that in the reddened leaf the polyphenols have penetrated into the cytoplasm where they have been oxidized to form the pigments characteristic of fermented tea.

It appears that at temperatures of 50–60° the protoplasmic membranes lose their powers of semi-permeability and the cytoplasm becomes freely permeable to all solutes of the vacuole. If the contents of the vacuole include oxidizable substrates, such as polyphenols, these substances will then become oxidized by the oxidase system of the tissue and pigmentation follows, hence the reddening of both tea and tobacco leaf under these conditions.

The oxidation of polyphenols during the reddening of tobacco leaf is indicated by the low R.Q. recorded during this phase. Such oxidations in general involve no CO₂ formation so that the R.Q. is nil. With simultaneous protein and polyphenol oxidation the R.Q. may be anything between zero and 0.7 and this is in fact observed. The part played by polyphenols in this stage is also indicated by the appearance in the suspending liquid of polyphenol oxidation products.

The tobacco leaf is known to contain rutin, the rhamnoglucoside of quercetin, and it has been shown by Neuberg & Kobel [1935] that this substance is transformed by tobacco enzymes into a soluble brown pigment. The question arises as to whether the amount of rutin, or other polyphenols, in the tobacco leaf is sufficient to account for the low R.Q. phase observed in the experiments on partially cured tobacco.

It is possible to arrive at an approximate measure of the polyphenol content of green leaf tissue by a manometric method. If the tissue is ground to a fine paste, its respiratory powers are almost completely suppressed [Roberts, 1941] and unless it contains some oxidizable substrate such as a polyphenol a suspension of this paste in water will consume O₂ at only a very low rate. On the other hand if polyphenols are present there will be a more or less vigorous uptake of O₂ until oxidation of these substances is complete. The oxidized polyphenols will in turn promote a secondary oxidation of carbohydrate and in consequence some CO₂ will be produced, and the oxidized polyphenol will be again reduced. The efficiency of the oxidized polyphenol as a H-acceptor in such processes is not high as much of it undergoes further changes, probably

by polymerisation, into pigments incapable of oxidizing carbohydrates. Eventually the whole of the polyphenol is converted into these pigments and carbohydrate oxidation ceases. The reactions involved are analogous to those in the 'fermentation' of tea [Roberts, 1941] and may be summarized.

The O_2 uptakes and CO_2 outputs recorded for a suspension of tobacco leaf are plotted in Fig. 2. Assuming that the oxidation of carbohydrate follows the course $\text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 \rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O}$, the amount of O_2 consumed in carbohydrate oxidation in this case equals $22\mu\text{l.}$ per 100 mg. so that the amount of O_2 required for complete oxidation of the polyphenols must be $68-22\mu\text{l.}$ In other words the polyphenol content of the fresh tobacco leaf is equivalent to $460\mu\text{l. O}_2$ per g. Assuming that only 1 atom O is required for the oxidation of rutin this is equivalent to 2.3 % rutin (or 1.2 % quercetin) on the fresh weight of the tissue. If 2 atoms O are consumed in the oxidation of the polyphenol these percentages will be correspondingly lower.

Fig. 2. O_2 uptake and CO_2 output of a suspension in water of finely ground tobacco leaf.

The polyphenol content of plant tissues may also be determined by another manometric method. The tobacco leaf is extracted thoroughly with boiling water and an aliquot of the infusion (2 ml.) placed in the main compartment of a Warburg vessel. After temperature equilibrium is reached 1 ml. 2*N* NaOH, is tipped in from a side arm and the O_2 uptake measured until the autoxidation has come to a standstill. Assuming that 5 atoms O are consumed per mol. polyphenol [Harrison & Roberts, 1939] the percentage polyphenol in the tobacco leaf, expressed as rutin, may be calculated from the uptake and the mol. wt. of rutin. On several samples of leaf the rutin content averaged 1.8 %, in fairly good agreement with the figure obtained by the earlier method, in view of the assumptions made.

We may now deal with the low R.Q. phase of the partly cured tobacco. In the example already quoted in Table 3 the O_2 uptake and CO_2 outputs after $2\frac{1}{2}$ hr. amount to 282 and $128\mu\text{l.}$ per 100 mg. tissue respectively. The CO_2 output must be corrected for the decrease in CO_2 retention of the tissue during this

period, and this reduces the figure for the true CO_2 output to $100\mu\text{l}$. This amount of CO_2 is produced by true respiratory processes and the R.Q. of the processes leading to CO_2 production at this stage of the curing may be assumed to be 0.8 without any serious error. This is equivalent to an uptake of $125\mu\text{l}$. O_2 and leaves the consumption of $157\mu\text{l}$. O_2 per 100 mg. tissue to be accounted for, possibly in terms of polyphenol oxidation.

In the original green leaf the O_2 equivalent of the polyphenols was $460\mu\text{l}$. O_2 per g. fresh leaf, or $2000\mu\text{l}$. O_2 per g. dry wt. For the partly cured leaf of moisture content 17%, the O_2 equivalent unaccounted for is $1570\mu\text{l}$. per g. or $1890\mu\text{l}$. per g. dry wt. No account is here taken of the loss of dry wt. during the curing process but this loss is unlikely to exceed 15% of the original dry matter of the leaf. Considering the assumptions made, it will be seen that the hypothesis that the low R.Q. phase is due to simultaneous oxidation of polyphenols and protein in the curing leaf is fully borne out by the correspondence between the polyphenol contents found in the fresh leaf and deduced in the partly cured leaf from a study of the R.Q. values.

DISCUSSION

From the foregoing results it is possible to form an idea of the changes that take place in flue-curing. During the yellowing stage there is a steady fall both in the rate of O_2 consumption by the tissue and in the R.Q., corresponding to a gradual exhaustion of respiratory substrates and an increasing call on the protein matter for metabolic purposes.

The quality of the fully cured tobacco may well depend upon the extent to which these changes take place and an investigation on these lines might give much insight into the factors necessary for high quality leaf.

The cause of red leaf is now clear. If the leaf is still moist when the critical temperature is reached at which the semi-permeability of the protoplasmic membranes breaks down, polyphenols diffuse into the cytoplasm where they are oxidized to form brownish red pigments. To avoid this the leaf must be sufficiently dried out by the time the critical temperature is reached to prevent diffusion of the vacuole contents into the cytoplasm. It must be the aim of further researches to determine how far the leaf must be dried to prevent this diffusion.

It seems probable that in the fixing stage chemical changes do not take place to any great extent owing to the dryness of the tissue. Enzymes, however, are still active and if the tissue is moistened, as when suspended in water in Warburg vessels, both protein and polyphenol oxidation can take place. Only after the temperature has been maintained at 160°F . for some time, during which period the mid-rib is dried out, is it safe to allow the tissue to pick up moisture, for by then the enzymes are sufficiently destroyed and further marked chemical changes in the leaf are unlikely.

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