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CHANGES IN OXIDATIVE ENZYME ACTIVITY DURING THE CURING OF CONNECTICUT SHADE TOBACCO[1]

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The activities of enzymes initially present are believed to be responsible for the major chemical changes that occur during the curing of tobacco leaves (3, 10). Accordingly, the stability of specific enzymes under curing conditions could markedly influence the final composition of the cured leaf. Vickery and Meiss (10) and others (3, 4) have shown that the major changes in composition occur during the first 8 to 12 days of air curing. Respiration, which causes a loss of about 16 % of the total organic solids, also ceases by the end of this period (10). However, most previous studies of enzymes have dealt with non-oxidative reactions, and it has been observed that the activities examined persist even beyond 12 days (1, 2, 3, 8). Such results seem surprising since at least 50 % of the proteins are broken down during curing (4, 10). It would appear that proteins of widely different degrees of stability are present and it has recently been suggested (8) that many of the enzymatically active proteins are included in the stable group.

Since oxidations are fundamental to the curing process, the activities of some soluble oxidative enzymes which vary greatly in their mechanism of action have been investigated. A marked decline, and in some cases a complete loss, of enzymic activity as well as a disappearance of oxygen uptake by leaf tissue has been observed. These results correlate well with earlier investigations on the chemical changes that occur during curing.

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Methods

Sampling and Curing: Leaves were picked by the statistical method (9) from tobacco plants of the shade-grown variety Connecticut 49. Five samples were collected from 10 plants, the 16th to the 20th leaf being taken. All leaves were mature according to commercial standards. Each sample contained 10 leaves. One sample was used to represent the initial time, and the other four were strung on laths and air cured in a chamber maintained at 32° C and 65 % relative humidity with an air velocity of 20 to 25 feet per minute for 2, 6, 9 and 13 days respectively. At the end of each time interval, a sample was withdrawn and weighed, and 1-cm discs to be used for the determination of respiratory activity were punched from identical positions in each leaf. The leaf discs were floated in a small volume of water in Warburg vessels which contained KOH in the center wells, and the rate of oxygen uptake was measured in the dark at 30° C.

Preparation of Extract Containing Soluble Enzymes: After removal of the discs, the leaves were washed with tap water and ground in a chilled plate mill. Sufficient water was added to the cured samples during the grinding procedure to compensate for the water lost during curing. Each extract was filtered through cheesecloth, and collected in the cold in a container to which activated carbon (Darco G-60) had been added to make the final concentration 1 g per 100 g of initial fresh weight of the sample. All subsequent steps were carried out at a temperature between 0 and 5° C. The pH of the homogenate (about 5.5) was raised to about 6.5 by the dropwise addition of 2.5 *N* KOH. The volume, in milliliters, at this stage was approximately the same as the initial fresh weight of the sample in grams. The extract was centrifuged for 10 minutes at 20,000 × G and the supernatant fluid was dialyzed for 20 hours against several changes of distilled water. Turbidity developed during the dialysis, and the insoluble protein was removed by centrifugation for 10 minutes at 20,000 × G. The volume of the supernatant fluid was then measured and samples were used for the enzyme assays. Each assay was carried out at two levels, one twice as high as the other. In order to determine the amount of soluble protein present, a sample of the dialyzed extract was treated with sufficient ethanol in the cold to make the final concentration 75 % by volume. Nitrogen was determined in the precipitate produced.

Malic Dehydrogenase: The assay used was based upon the reaction shown in equation 1 (6).

$$\text{DPNH} + \text{H}^+ + \text{oxalacetate} \rightleftharpoons \text{L-malate} + \text{DPN}^+ \quad (1)$$

To a cuvette of 1 cm light path containing 100 micromoles of tris(hydroxymethyl)aminomethane hydrochloride (TRIS) buffer at pH 7.5, were added 0.3 micromole of reduced diphosphopyridine nucleotide (DPNH), enzyme extract, and water to make the final volume 3.0 ml. The blank cell contained all of the components except DPNH. At zero time, one micromole of oxalacetate was added to each cuvette, and the rate of disappearance of DPNH was measured at 340 mμ in the Beckman model DU spectrophotometer. One unit of enzyme is defined as the amount necessary to cause a decrease in absorbance of 0.01 at 25° C in one minute under the conditions described.

Polyphenol Oxidase: The method was based upon measurement of the rate of formation of dark-colored polymeric compounds by determination of non-specific absorption at 400 mμ (equation 2). To

$$\text{Catechol} + \tfrac{1}{2}\,\text{O}_2 \rightarrow \text{brown pigment} \quad (2)$$

a cuvette containing 100 micromoles of potassium phosphate buffer at pH 6.0, enzyme extract and sufficient water to make the final volume 3.0 ml, were added 20 micromoles of catechol. The rate of "browning" in the interval between 15 and 45 seconds after addition of the substrate was measured in a Beckman model B spectrophotometer. One enzyme unit is defined as the amount required to cause an increase in absorbance of 0.01 in one minute at 25° C. The activity was found to be proportional to enzyme concentration over a 10-fold range.

Diaphorase: The assay was based on measurement in Thunberg tubes of the rate of reduction of 2,6-dichlorophenolindophenol (equation 3) in the

$$\text{DPNH} + \text{H}^+ + \text{dye} \rightarrow \text{reduced dye} + \text{DPN}^+ \quad (3)$$

Coleman model 8 colorimeter at 590 mμ (5). The components of the assay mixture were 100 micromoles of TRIS buffer at pH 7.5, 0.15 micromole of 2,6-dichlorophenolindophenol, enzyme extract, and water to make the final volume 6.0 ml. The side arm of the Thunberg tube contained 0.15 micromole of DPNH. The tubes were evacuated with a water pump, and after a suitable period to permit temperature equilibration, DPNH was tipped in to start the reaction. One unit is the amount of enzyme needed to cause a decrease in absorbance of 0.01 in one minute at 25° C. This is equivalent to about 0.0025 micromole of DPNH oxidized.

Glycolic Acid Oxidase: This activity was determined by the manometric method (11) (equation 4). The Warburg vessels contained 100 micromoles

$$\text{Glycolate} + \text{O}_2 \rightarrow \text{glyoxylate} + \text{H}_2\text{O}_2 \quad (4)$$

of TRIS buffer at pH 8.3, excess riboflavin phosphate, enzyme extract, and water to make the final volume 2.0 ml. Ten micromoles of potassium glycolate were placed in the side arm and KOH was present in the center well. After equilibration at 30° C, the substrate was tipped in and the rate of oxygen uptake was measured for two successive 10-minute periods beginning five minutes after the addition of the glycolate. One unit is defined as the amount of enzyme required to cause an uptake of 1 μl of oxygen in 10 minutes.

Glutamic Dehydrogenase: The oxidation of glutamate was determined by following the rate of formation of DPNH (7) (equation 5). The cuvettes

L-Glutamate + $DPN^+ + H_2O$
$\rightleftharpoons DPNH + H^+ + \alpha$-ketoglutarate + NH_3 (5)

contained 50 micromoles of TRIS buffer at pH 8.8, 0.9 micromole of disphosphopyridine nucleotide (DPN^+), enzyme extract, and water to make the final volume 3.0 ml. Blank cells contained all components except DPN^+. There was no DPNH formation in the absence of substrate. At zero time, 30 micromoles of potassium L-glutamate were added to each cuvette, and the rate of DPNH formation was measured at 340 mμ in the Beckman model DU spectrophotometer for five minutes. One unit is the amount of enzyme required to cause an increase in absorbance of 0.01 in one minute at 25° C.

Results and Discussion

The average initial fresh weight of the five samples was 262 g (table I) with a coefficient of variation of 0.95 %. The sampling error was thus satisfactorily small. The fresh weight had diminished to less than one half of the initial weight by the 6th day, which coincided with the beginning of the yellow stage. The brown stage had begun by the 9th day, and the leaves of the last sample on the 13th day were completely brown. During the curing, the leaves lost 80 % of their fresh weight; hence the solute concentration was increased about 5-fold in the 13-day period.

As shown in table I, about two thirds of the total soluble protein N disappeared during curing. The figure for the zero time sample is probably too low because of less efficient extraction. The overall decrease agrees well with the losses in total soluble protein that can be calculated from the data of Pogell et al (8). Since the loss of total protein during curing represents about one half of that present initially (4, 10), a disproportionately large change must have occurred in the soluble protein fraction which is known to include many oxidative enzymes.

The oxygen uptake by the leaf discs diminished slowly for the first two days, but decreased rapidly thereafter, and was barely detectable by the 9th day. At the end of the yellow stage, when browning begins, it seems safe to assume that death of the plant cells had occurred. This time also corresponds to the point after which little further loss of total organic solids occurs (10).

Table I

Changes in Composition and Respiration Rate During Curing

Curing time	Fresh weight	Total soluble protein N	Oxygen uptake
days	*g*	*mg*	*μl/15 discs × hr*
0	262	101	60.1
2	198	121	52.3
6	125	101	20.6
9	78	40.9	2.8
13	50	36.8	0.0

Table II

Oxidative Enzyme Activity of Initial Sample

Enzyme activity	Volume of extract assayed	Arbitrary units
	ml	*per ml* *
Malic dehydrogenase	0.002	2,800
	0.004	2,930
Polyphenol oxidase	0.10	76.0
	0.20	84.5
Diaphorase	0.005	1,140
	0.01	1,090
Glycolic acid oxidase	0.10	221
	0.20	194
Glutamic dehydrogenase	0.05	34.4
	0.10	35.4

* To convert arbitrary units per ml to total units per sample, multiply by 290, the final volume of the extract in ml.

The sensitivity of the enzyme assays and the activities of the initial sample are shown in table II. The reproducibility of the assays at two levels of enzyme concentration supports the validity of the activity measurements.

The changes in oxidative enzyme activity are plotted in figure 1, the assay at zero time being taken as 100 %. Malic dehydrogenase and polyphenol oxidase are quite stable for the first two days, but activity is lost rapidly thereafter so that by the 13th day only 23 and 13 %, respectively, remain. Diaphorase decreases in a more regular fashion, and only 13 % of the initial activity was found at the 13th day of curing. Glycolic acid oxidase and glutamic dehydrogenase are much less stable than the others, 1 and 4 % of the initial activity, respectively, remaining on the 9th day, and no activity being detectable by the 13th day with the sensitive assays used.

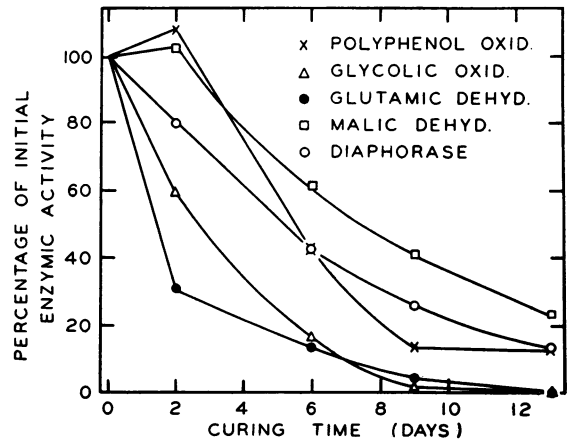

Fig. 1. Relative changes in activity of polyphenol oxidase, glycolic acid oxidase, glutamic dehydrogenase, malic dehydrogenase, and diaphorase during curing.

TABLE III

SPECIFIC ACTIVITY OF OXIDATIVE ENZYMES DURING CURING *

CURING TIME IN DAYS	MALIC DEHYDROGENASE	POLYPHENOL OXIDASE	DIAPHORASE	GLYCOLIC ACID OXIDASE	GLUTAMIC DEHYDROGENASE
0	8,220	230	3,200	596	100
2	7,090	207	2,140	294	21.2
6	5,020	97.8	1,370	92.7	13.8
9	8,360	74.8	2,050	21.6	10.6
13	5,220	79.9	1,150	0.0	0.0

* Expressed as arbitrary units per mg of protein N.

Since much of the soluble protein disappears during curing (table I), it is of interest to compare the various enzymic activities in terms of protein N. It is clear from table III, that malic dehydrogenase activity decreased at about the same rate as the soluble protein during curing, since the specific activity did not change appreciably. Diaphorase, polyphenol oxidase, glutamic dehydrogenase, and glycolic acid oxidase activities, however, decreased more rapidly than the soluble protein, a result which emphasizes the unstable nature of these oxidative enzymes.

The technique used for the extraction of soluble protein is necessarily arbitrary, and the possibility existed that the losses in enzymic activity observed were caused by an unsatisfactory extraction procedure or by a change in the solubility of the enzyme rather than by an alteration in the enzyme protein. Glycolic acid oxidase can be conveniently assayed in the crude homogenate, and a comparison was therefore made of the activity recovered in the final enzyme extract with that originally present (table IV). There was essentially no difference in the recoveries compared with the 40-fold decrease in activity of glycolic acid oxidase between the 2nd and the 9th day of curing; hence it can be concluded that the loss in activity of glycolic acid oxidase was not attributable to an inefficient extraction procedure or to a change in its solubility. An inactivation of the enzyme must have occurred.

At least some oxidative enzymes do not survive air curing, and a considerable variation in stability is exhibited by different enzymes. The overall similarity of the loss in oxidative enzyme activity to the loss in the rate of oxygen uptake (table I) suggests that respiration ceases during curing because inactivation of oxidative enzymes accompanies the breakdown of protein and dehydration of the tissues. The loss both in respiration and enzymic activities shown confirms results obtained from a similar experiment carried out one year earlier.

TABLE IV

RECOVERY OF GLYCOLIC ACID OXIDASE ACTIVITY IN ENZYME EXTRACT

CURING TIME	TOTAL GLYCOLIC ACID OXIDASE ACTIVITY		RECOVERY
	HOMOGENATE	ENZYME EXTRACT	
days	*arbitrary units*		*%*
2	63,600	35,600	56
6	11,700	9,360	80
9	1,170	883	75

It seems clear that many of the soluble proteins involved in respiration are altered during curing. The present studies show that some of the critical enzymes remain functional for limited periods of time only and lead to the conclusion that the rate of enzyme inactivation controls the final composition of the cured leaf. This is an inference which may be helpful in developing improved conditions for the process.

SUMMARY

The rate of oxygen uptake by intact tobacco leaf tissue and the activities of malic dehydrogenase, polyphenol oxidase, L-glutamic acid dehydrogenase, diaphorase, and glycolic acid oxidase in extracts have been studied at intervals during the process of air curing. Both the enzymic activities and the respiration rate decreased rapidly during the first 13 days, and it is suggested that the major oxidative changes involved come to a halt as a result of inactivation of the enzymes. Many of the soluble proteins including certain enzymes undergo alteration during curing. These observations throw light upon some of the results described in previous chemical investigations of the curing process.

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OBSERVATIONS ON THE INCORPORATION OF C^{14} INTO TARTARIC ACID AND THE LABELING PATTERN OF D-GLUCOSE FROM AN EXCISED GRAPE LEAF ADMINISTERED L-ASCORBIC ACID-6-C^{14} [1,2]

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Vickery and Palmer (12) have speculated on the possible precursor relationship of D-glucose to (+)-tartaric acid in plants. C-2 and C-3 of D-glucose have the same configuration as C-2 and C-3 of (+)-tartaric acid. They proposed a metabolic path in which C-5 and C-6 were lost, presumably via an intermediate such as 5-keto-gluconic acid.

Hough and Jones (9) have stated that hexuronic acids, L-ascorbic acid, and (+)-tartaric acid commonly occur together in nature. Conceivably, L-ascorbic acid might be the precursor of (+)-tartaric acid through loss of C-1 and C-2 of the former and subsequent oxidations of the terminal carbons of the four carbon fragment to carboxyl groups. C-4 and C-5 of L-ascorbic acid have the same configuration as (+)-tartaric acid.

We have attempted to test the latter possibility. L-Ascorbic acid-6-C^{14} [4] (3 mg containing 4.7 μc of C^{14}) was fed through the cut stem to a single grape leaf (Mission variety, second leaf from the tip of an actively growing vine) in 0.3 ml of distilled water. The leaf was illuminated by a pair of 10-watt daylight fluorescent bulbs at a distance of 12 cm. Practically all of the radioactive solution was taken up in five hours. After 8 hours, the leaf was placed in a closed 350-ml container in the dark for an additional 17 hours. Finally, the accumulated respiratory CO_2 was aspirated into a gas trap of N NaOH. Approximately 5 % of the administered label was lost as CO_2 during the dark period.

The soluble constituents of the leaf were separated and recovered as described in another paper (11). Most of the activity remained in the particle-free extract. Very little activity was removed during passage through a cationic exchange resin (Dowex 50, H^+). About one half of the activity remained on the anionic exchange column (Dowex 1, formate). Most of this activity was eluted with a 3 N formic acid gradient (10) in four peaks. The 1st peak coincided with the first traces of acid through the column and might be due to inadequate washing of the column after loading. The 2nd, a very narrow sharp peak, came in the region characteristic of ascorbic acid. Two peaks of lesser activity followed, the last corresponding to malic acid on a paper chromatogram (10). The tartaric acid peak, located by its acid titration curve and by its ammonium metavanadate reaction, had such low activity as to be undetectable by the solid sample counting procedure employed. After three recrystallizations, the potassium acid tartrate from this peak had a specific activity of 24 cpm per mg of carbon (gas-phase counter, 80 % efficient). This amount of C^{14} was too low to be of any significance as concerns the possible metabolic conversion of ascorbic acid to tartaric acid in the grape leaf. The fact that some activity did enter the tartrate molecule from C-6 of L-ascorbic acid suggested that this label was derived, indirectly perhaps, from the sugar pool of the leaf.

In order to explore the latter possibility, the neutral effluent of the Dowex 1 column was concentrated in vacuo to a thick sirup and chromatographed on paper using a solvent composed of ethyl acetate, pyridine, and water (8 : 2 : 1) (13). About 50 % of the C^{14} remained very close to the origin after development. Another 30 % was found in the sucrose. About 5 % each was found in the glucose, fructose, and xylose bands. The glucose region, which was free of other reducing sugars and showed a single darkened area in the corresponding radioautogram, was cut out and eluted. About 0.7 mg of glucose was recovered. It had a specific activity of 100,000 cpm per mg of carbon as determined by wet combustion of an aliquot after dilution with unlabeled D-glucose.

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