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Studies on Polyphenol Content, Activities and Isozymes of Polyphenol Oxidase and Peroxidase During Air-Curing in Three Tobacco Types[1]

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Abstract. The change in polyphenol content in the primed leaves of burley, flue-cured, and Turkish tobaccos during air-curing was related to the activities and isozymes of polyphenol oxidase and peroxidase. The quantity of chlorogenic acid was rapidly reduced during the first week of curing. The decrease in rutin content during curing was less significant, especially when the concentration of chlorogenic acid was high in leaf tissues. This result was further confirmed by *in vitro* assays with partially purified tobacco polyphenol oxidase.

The polyphenol oxidase activity did not differ at any stage of curing in the 3 tobaccos. When the activity was measured by the oxidation of 3,4-dihydroxyphenylalanine it rose rapidly during the first day of curing and then decreased sharply so that in the fully cured leaf only 15 % activity remained. The increase in activity was not observed when chlorogenic acid was used as the substrate. A similar level of peroxidase activity was found in the 3 tobaccos before curing. Peroxidase activities increased rapidly during the first 24 hr of curing, declined thereafter, and remained highest in the flue-cured tobacco, less in the Turkish line, and least in the burley at the end of curing process.

By polyacrylamide gel block electrophoresis, 10 peroxidase isozyme bands, 2 cationic and 8 anionic, appeared identical in all 3 tobaccos. When catechol replaced benzidine-2 HCl as the electron donor, 1 cationic and 2 anionic peroxidase isozymes did not form. Of interest is that the same 10 peroxidase isozyme bands also exhibited polyphenol oxidase activities when treated with 3,4-dihydroxyphenylalanine or chlorogenic acid. Results suggest that in the crude tobacco leaf extract the peroxidase and polyphenol oxidase may associate as protein complexes, and peroxidase isozymes may differ in electron-donor requirements. Isozyme patterns for both oxidases at various curing intervals differed only quantitatively.

The enzymic conversion of chemical compounds in tobacco (*Nicotiana tabacum* L.) leaves during curing has been extensively reviewed by Frankenburg (6) and recently by Forsyth (5). It is known that yellowing and browning of tobacco leaves result from chlorophyll decomposition and phenolic oxidation, respectively. Phenolic decomposition involves the enzymic oxidation of the principal polyphenols chlorogenic acid and rutin to form brown pigments. During flue-curing, rutin was completely oxidized by the quinoids of chlorogenic acid: this oxidation may regenerate chlorogenic acid (11). In contrast, chlorogenic acid and rutin were not detected in air-cured leaves (16).

Polyphenol oxidase (PPO) and peroxidase are the major enzymes responsible for the oxidation of phenolic compounds. Multiple forms (isozymes) of these oxidases can be separated by electrophoretic techniques. Kerstetter and Keitt (7) reported 1 cationic and 5 anionic peroxidase isozymes in tobacco pith extracts. Seventeen anionic peroxidase isozyme bands were demonstrated in either healthy or virus-infected tobacco leaves, both young and senescent, by Novacky and Hampton (10). PPO isozymes have been studied in mushroom, potato, apple, *Neurospora crassa*, and other plant species but not in tobacco (3). Two groups of PPO, namely chlorogenic acid oxidase and catecholase, exist in tobacco leaves (2). Sisler and Evans (15) compared PPO from tobacco and a comparable preparation from mushroom and found that chlorogenic acid is a suitable substrate for the tobacco PPO. Reid (12) reported that rutin *per se* cannot be oxidized by tobacco PPO in the absence of chlorogenic acid, but it can be oxidized by peroxidase in the presence of H_2O_2. He also noted that scopolin and its aglycone scopoletin cannot be oxidized by these enzymes.

PPO and peroxidase activities rose rapidly during the first few days of flue-curing, then decreased sharply so that in the fully cured leaf no PPO activity and very little peroxidase activity remained

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(17). In the present study, such enzymic changes in relation to polyphenol decomposition in burley, flue-cured, and Turkish tobaccos during air-curing were investigated. It is hoped that the relationship between the soluble polyphenols and the related oxidases and their isozymes in tobacco leaves can be better understood, and the gained information may facilitate altering phenolic quantity in tobacco varieties.

Materials and Methods

Tobacco lines, Ky Iso 3 Burley 37, Ky Iso 6 F.C. 402 (flue-cured), and Ky Iso 7 Turkish, representing 3 types were employed in this study. Sixty plants of each line were grown for 6 weeks in the greenhouse and transplanted into the field in early June, 1967. The experimental design was a randomized block with 4 replications. The cultural practices were conventional burley methods. Four weeks after topping, 12 plants from each plot were sampled by harvesting the seventh or eighth leaf from the bottom of the stalk. The leaves were bulked into a plot sample and air-cured in an environmental chamber in darkness at 24° and 68% relative humidity. Leaf discs in 2-inch diameter were removed at each of the following curing intervals: at harvest, one-half, 1, 2, 4, 7, 10, and 17 days. Leaf discs collected on each curing interval represented all leaf areas. After treatment in liquid nitrogen, the leaf discs were freeze-dried and ground in a Wiley mill.

Extraction of leaf powders in the Soxhlet apparatus and quantitative determination of polyphenols by 2 dimensional paper chromatography and spectrophotometry have been previously described (14). For enzymic studies, the leaf powder was minced in a cold mortar in a 0.1 M tris-HCl buffer containing 17% (w/v) sucrose, 0.1% (w/v) ascorbic acid, and 0.1% (w/v) cysteine-HCl, pH 8. The extracts were centrifuged at 20,000*g* for 1 hr at 2°. The supernatant fluid was used for gel electrophoresis as well as for enzyme assays. Davis' (4) method of polyacrylamide gel electrophoresis was adapted to the Buchler starch gel vertical electrophoresis apparatus. A 2 cm layer of stacking gel was placed between 2 blocks of separating gel, a 20 × 12 cm block at the anionic end and a 4 × 12 cm block at the cationic end. Crude leaf extracts containing 100 to 150 μg of protein as determined by the method of Lowry *et al.* (8), were pipetted into the sample slots in the stacking gel. This 3-layer gel block (26 × 12 cm) was subjected to 50 mA current for 16 hr at 4°. By this procedure 10 samples can be tested under a similar experimental environment, and both anionic and cationic isozyme differences can be studied on the same gel block. After electrophoresis, peroxidase isozymes were visualized by 2 methods: (a) the gel was treated in a mixture of benzidine-2 HCl and H_2O_2 according to Ornstein (1), and (b) the gel block was submerged into 0.02 M catechol solution for 30 min followed by 0.1% (v/v) H_2O_2 until the bands were satisfactorily developed. Two different methods were employed to determine the PPO isozymes. In the first method, a 0.5% (w/v) solution of 3,4-dihydroxyphenylalanine was used as the substrate, and the second method involved treating the gel with 2 mM chlorogenic acid for 30 min and subsequently with a 0.5% (w/v) *p*-phenylenediamine solution.

The same crude leaf extracts were used for enzyme assays. The activity of PPO was determined by the standard Warburg manometric technique for oxygen uptake with 20 mM chlorogenic acid as the substrate at 32°, and by spectrophotometric measurement for absorbancy change employing 14.1 mM 3,4-dihydroxyphenylalanine as the substrate in 0.1 M phosphate buffer, pH 6, after the method of Constantinides and Bedford (3). Peroxidase activity was assayed by spectrophotometric measurement at 485 mμ of a colored complex produced by the mixture of, 1 ml each, 45 mM H_2O_2, 0.5% (w/v) *p*-phenylenediamine, and the enzyme preparation in 0.5 M phosphate buffer, pH 6.1 (9).

In order to determine the substrate specificity and competition, a partially purified PPO was prepared according to Clayton's method (2) with slight modifications. Fresh, deveined leaves (50–60 g) of Hicks, a flue-cured variety, were homogenized with 30 ml of 0.1 M phosphate buffer containing 0.1% (w/v) cysteine-HCl, pH 7.2. Leaf debris were homogenized again with 200 ml of the same buffer. Ammonium sulfate was added to the combined filtrates to make a final concentration of 20% (w/v). The precipitates were discarded by centrifugation and the supernatant fluid was made to 30% (w/v) $(NH_4)_2SO_4$ to yield precipitates which were dialyzed against 0.05 M phosphate buffer. This protein fraction recovered more than 65% of the total PPO activity. All purification procedures were conducted in an ice-bath or at 4°. Various concentrations of chlorogenic acid and rutin mixtures were assayed with 0.3 ml enzyme preparation (1 mg protein/ml) in Warburg flasks. After 30 min the reaction was stopped by plunging the flasks into boiling water. Aliquots of the reaction mixture were paper-chromatographed and the quantity of chlorogenic acid and rutin were determined as previously described.

Results and Discussion

Changes in Polyphenol Content During Air-curing. Chlorogenic acid was the predominant fraction of polyphenols in the mature and air-cured leaves. Based on variance analysis its quantity was significantly high in Ky Iso 6, intermediate in Ky Iso 7, and low in Ky Iso 3 at any stage of curing. Patterns of variation in phenolic quantity during curing appeared similar in all 3 lines. First, the chlorogenic acid content was rapidly reduced during

the first week of curing and further reduction became negligible thereafter. Although the loss of chlorogenic acid was 82 % in Ky Iso 3, 54 % in Ky Iso 7, and 43 % in Ky Iso 6, the reduction in terms of mg/g dry weight was 11.7, 13.2, and 14.6 for the respective lines. This suggests that the amount of chlorogenic acid decreased during curing is not proportional to its quantity in leaf tissues. Secondly, an inverse relationship between scopolin and scopoletin content before and after curing was apparent. This is expected since the hydrolysis of scopolin yields scopoletin during curing. Thirdly, the decrease in rutin content was much less than chlorogenic acid. A 3.2 % decrease in Ky Iso 6 as compared with 23 % in Ky Iso 3 suggests that a tobacco line high in chlorogenic acid content loses less rutin during curing.

Changes in PPO and Peroxidase Activity During Air-curing. Statistical analyses indicated that the 3 tobacco lines did not differ in the level of PPO activity either before or after curing. Clayton (2) observed a similar result when he compared the PPO activity in 2 tobacco varieties, Hicks and Virginia 21. Results obtained from 3 additional tobacco lines, namely, Ky Iso 1 Ky 16 (burley), Ky Iso 2 Ky 151 (dark fire-cured), and Ky Iso 4 Hicks (flue-cured), further support the present findings (Sheen and Calvert, unpublished data). Since the 6 Ky Iso lines of tobacco vary considerably in polyphenol content (14), one may conclude that polyphenol content and PPO activity in tobacco leaves are independent entities, and consequently, the PPO activity cannot be used as a criterion in evaluating varieties for their levels of soluble polyphenols. This

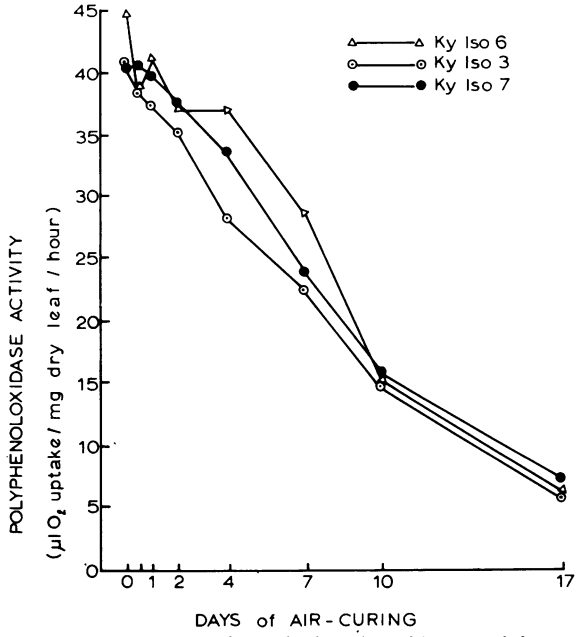

FIG. 1. Change in polyphenol oxidase activity as determined by oxygen uptake in 3 tobacco lines during air-curing.

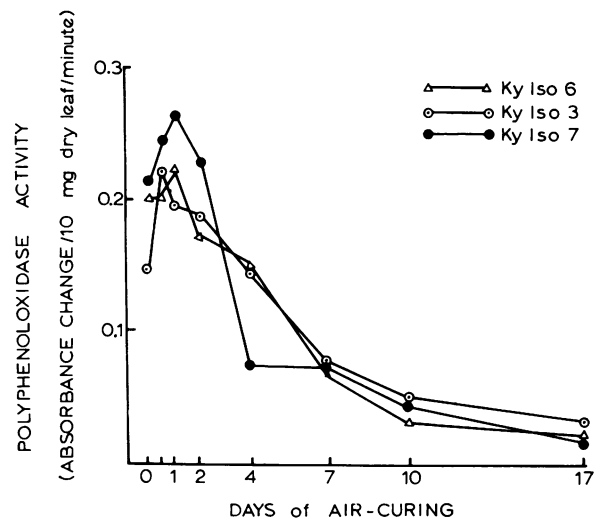

FIG. 2. Change in polyphenol oxidase activity as determined by browning effect in 3 tobacco lines during air-curing.

poor enzyme-substrate relation may be partially explainable due to the intracellular compartmentalization of PPO as observed in other plant systems (3).

When chlorogenic acid was used as the substrate, a steady decline in PPO reaction was noted as curing proceeded (fig 1). In contrast, the activity slightly increased at the beginning of curing when 3,4-dihydroxyphenylalanine was the substrate (fig 2). It has been suggested that crude PPO preparations from plants actually are mixtures of several PPO proteins which catalyze the oxidation of a wide variety of substrates, and each enzyme tends to catalyze the oxidation of 1 particular phenol or a particular type of phenolic compound more readily than others (18). Since 3,4-dihydroxyphenylalanine may be dehydrogenated by a number of PPO enzymes other than by chlorogenic acid oxidase, the increase

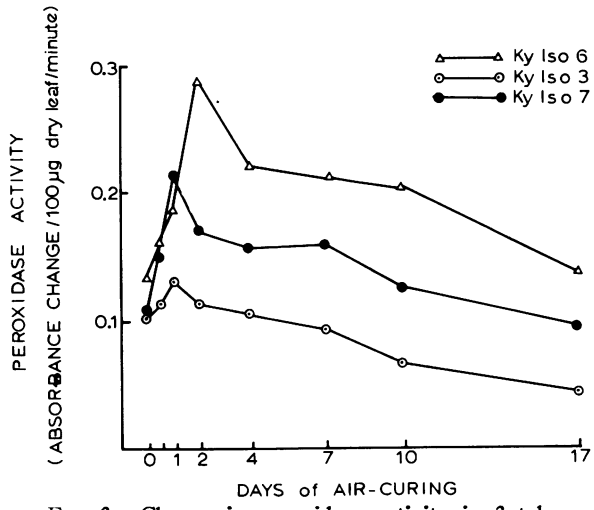

FIG. 3. Change in peroxidase activity in 3 tobacco lines during air-curing.

in PPO reaction may be attributed to an activation of these enzymes in the early phase of curing. However, an average loss of 85 % of the PPO activity at the end of curing was common for both substrates. This agrees with the findings reported by Zelitch and Zucker (19) who worked with Connecticut shade tobacco and used catechol as the substrate.

The 3 tobacco lines exhibited a similar level of peroxidase activity at the beginning of curing, and this was followed by an increase of approximately 100 % in Ky Iso 6 and Ky Iso 7, and 30 % in Ky Iso 3 (fig 3). A varietal difference became apparent after 48 hr and persisted to the termination of curing. It is interesting to note that the relative levels of peroxidase activity in the 3 lines are in the same order as their polyphenol contents. Although the role of peroxidase in polyphenol catabolism is still unknown, the past evidence suggested that it oxidizes rutin in the presence of H_2O_2 (12). However, a high peroxidase activity coupled with a small change of rutin quantity during air-curing in Ky Iso 6 does not indicate any direct relationship between peroxidase and rutin catabolism.

Isozyme Patterns of PPO and Peroxidase. The isozyme patterns of PPO and peroxidase for 8 curing intervals in a given tobacco line were compared on the same polyacrylamide gel block. Results from triplicate runs coincided. Since the zymograms of both oxidases appeared identical for all 3 tobaccos, those obtained from Ky Iso 7 will be used for illustration. When benzidine-2 HCl was used as the electron donor for peroxidase, 10 isozyme bands, 2 cationic (bands a and b) and 8 anionic (bands c to j) were visible (fig 4). Bands a, e, f, g, and i showed a strong enzymic activity, while bands d and h were weak. The activity of band j was detected only in case of an increased amount of leaf extract used for electrophoresis or a prolonged treatment of gel blocks in substrates. The present observation is in agreement with the findings reported by others (7, 10). With the substitution of catechol for benzidine-2 HCl peroxidase bands b, c, and d did not form (fig 5). This phenomenon was consistent in H_2O_2 concentrations ranging from 0.1 to 0.001 % (v/v), suggesting a difference in electron donor requirements for peroxidase isozymes. For PPO isozymes chlorogenic acid and 3,4-dihydroxyphenylalanine gave identical zymograms for the 3 tobacco types (fig 6). Of interest is the observation that the R_F values for the 10 PPO isozymes corre-

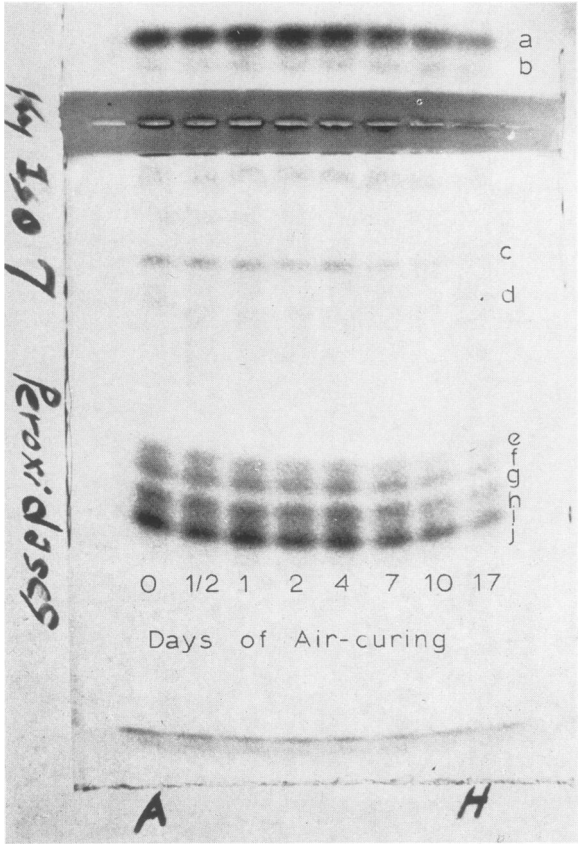

FIG. 4. Peroxidase zymogram developed with benzidine-2-HCl and H_2O_2 for 8 curing intervals in Ky Iso 7.

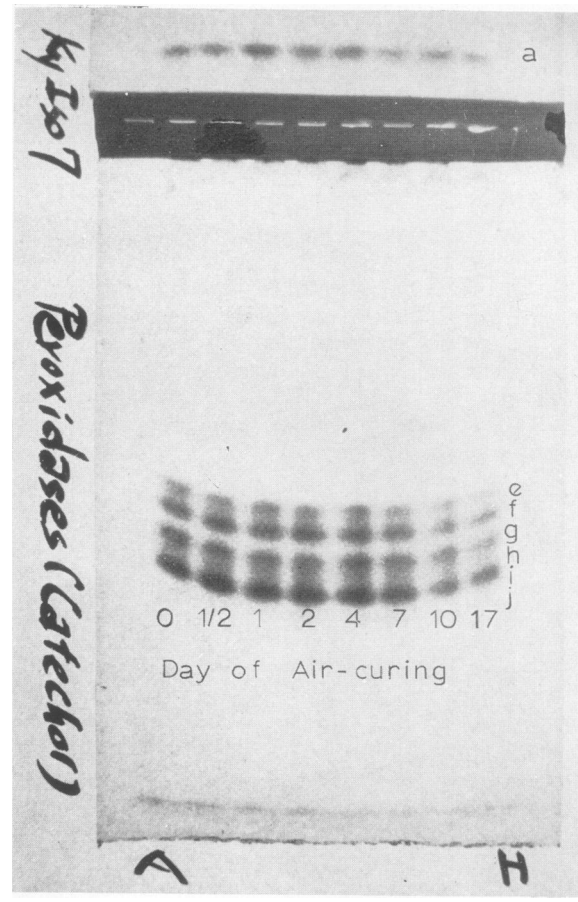

FIG. 5. Peroxidase zymogram developed with catechol for 8 curing intervals in Ky Iso 7.

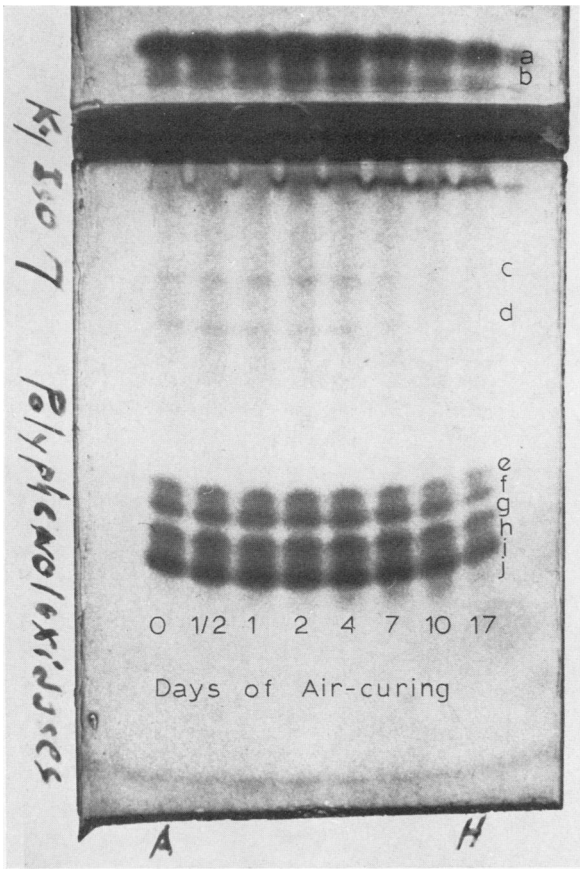

FIG. 6. Polyphenol oxidase zymogram developed with 3,4-dihydroxyphenylalanine for 8 curing intervals in Ky Iso 7.

spond to those for the peroxidase isozymes. This was confirmed by a comparison of the PPO and peroxidase zymograms obtained from the same gel block which was divided into halves and stained for 2 activities. Zymograms produced by the partially purified PPO further supported this coincidence. PPO has been found in particle-bound and soluble forms in leaf homogenates from many plant species including tobacco (3). Tobacco peroxidase was known to have been associated or aggregated with root indoleacetic acid oxidase (13). Therefore, one may reason that the soluble PPO may associate with peroxidase in the crude leaf extracts. However, the possibility of a single protein possessing 2 or more reactive sites, the impurity of substrate chemicals, and artifacts of extraction needs to be evaluated. The change in zymograms of both oxidases was quantitative during the course of air-curing. Generally, the trend of decreased activity agreed with the enzyme assay results.

Oxidation of Chlorogenic Acid and Rutin by Partially Purified Tobacco PPO. The partially purified PPO had high oxidative activity for chlorogenic acid but oxidized neither rutin nor catechol. Yet, catechol inhibited the oxidation of chlorogenic acid. This confirms Clayton's (2) findings for preparations of chlorogenic acid oxidase from tobacco. In the presence of 10 mM chlorogenic acid rutin neither inhibited nor stimulated PPO activity (table I). If the chlorogenic acid concentration was reduced to 5 mM or less, the initial velocity dropped accordingly. Furthermore, the browning intensity of the reaction mixture seems to be solely dependent upon the concentration of chlorogenic acid. Studies on the recovery of chlorogenic acid and rutin after 30 min incubation with PPO showed that rutin may be oxidized in the presence of chlorogenic acid but the loss of rutin was inversely correlated with the concentration of chlorogenic acid. This *in vitro* result paralleled the change in content of rutin during air-curing in the leaf of 3 tobacco lines. The mixtures of chlorogenic acid and rutin incubated with PPO yielded no quercetin (the aglycone of rutin), and spots having low R_F values and showing yellow color with ammonia fumes appeared on the chromatograms. Analyses of ultraviolet spectra for reaction mixtures revealed a decomposition of chlorogenic acid as evidenced by decreasing absorbancy at 328 mμ, whereas the absorbance maxima for rutin were unchanged. It may be speculated that the disappearance of free rutin possibly resulted from the formation of complexes between rutin and the quinoids of chlorogenic acid. Such complexes may or may not be formed *in vivo* since the quinoids of chlorogenic acid may react more readily with amino acids or proteins to form brown pigments. The contrasting observations on the loss of rutin during flue-curing reported by Penn and Weybrew (11) and during air-curing in the present study point out the importance of curing temperatures. The decrease in rutin content may be accelerated by high temperatures under which PPO, peroxidase, and other oxidases become more reactive.

Table I. *Quantity of Chlorogenic Acid and Rutin Oxidized and the Rate of Oxygen Uptake by Tobacco Leaf Polyphenol Oxidase*

Warburg flasks were charged with 300 μg of protein prepared by 30% (w/v) $(NH_4)_2SO_4$ precipitation as described in the text, 2 ml substrate in 0.1 M phosphate buffer, pH 7.0, and 0.2 ml concn KOH in the center wells of the flasks. The gas phase was air. The final volume was 2.5 ml. Initial velocities were averages of 3 5-min readings. Data are representative of 2 experiments.

Substrate concn		Quantity oxidized in 30-min reaction		
Chlorogenic Acid	Rutin	Chlorogenic Acid	Rutin	Initial velocity
mM	mM	%	%	μl O_2/min
10	0	97.4	...	14.4
0	1.5	...	0	0
100	1.5	62.3	0	15.4
50	1.5	78.0	19.6	14.3
10	1.5	96.3	92.4	14.1
5	1.5	97.6	95.7	10.6
1	1.5	98.9	100	5.0

Literature Cited

1. Canal Industrial Corporation Special Subject. 1963. Enzyme analysis. Canal Industrial Corporation, Bethesda, Maryland.
2. CLAYTON, R. A. 1959. Properties of tobacco polyphenol oxidase. Arch. Biochem. Biophys. 81: 404–17.
3. CONSTANTINIDES, S. M. AND C. L. BEDFORD. 1967. Multiple forms of polyphenol oxidase. J. Food Sci. 32: 446–50.
4. DAVIS, B. J. 1964. Disc electrophoresis, II. Method and application to serum human protein. N. Y. Acad. Sci. Ann. 121: 404–27.
5. FORSYTH, W. G. C. 1964. Physiological aspects of curing plant products. Ann. Rev. Plant Physiol. 15: 443–50.
6. FRANKENBURG, W. G. 1946. Chemical changes in the harvested tobacco leaf. Part II. Chemical and enzymatic conversion during the curing process. Advan. Enzymol. 6: 309–412.
7. KERSTETTER, R. E. AND G. W. KEITT, JR. 1967. Peroxidase isozyme pattern and aging in tobacco. Ass. S. Agr. Workers, Inc. Proc. 64: 278–79.
8. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–75.
9. LUCK, H. 1963. Peroxidase. In: Methods of Enzymatic Analysis. H. M. Bergmeyer, ed. Academic Press. p 895.
10. NOVACKY, A. AND R. E. HAMPTON. 1968. Peroxidase isozymes in virus-infected plants. Phytopathology 58: 301–05.
11. PENN, P. T. AND J. A. WEYBREW. 1958. Some factors affecting the content of the principal polyphenols in tobacco leaves. Tobacco Sci. 2: 68–72.
12. REID, W. W. 1959. Some aspects of polyphenols of tobacco. Tobacco Sci. 3: 103–08.
13. SEQUEIRA, L. AND L. MINEO. 1966. Partial purification and kinetics of indoleacetic acid oxidase from tobacco roots. Plant Physiol. 41: 1200–08.
14. SHEEN, S. J. AND J. CALVERT. 1969. Quantitative variation in polyphenol content in the green and air-cured leaves of tobacco (*Nicotiana tabacum* L.). Tobacco Sci. In press.
15. SISLER, E. C. AND H. J. EVANS. 1958. Direct spectrophotometric determination of chlorogenic acid oxidase activity. Biochim. Biophys. Acta 28: 638–39.
16. WEAVING, A. S. 1958. The polyphenols of flue-cured tobacco: Separation and identification of the major polyphenols. Tobacco Sci. 2: 1–8.
17. WESTON, T. J. 1968. Biochemical characteristics of tobacco leaves during flue-curing. Phytochemistry 7: 921–30.
18. YASANOBU, K. T. 1959. Mode of action of tyrosinase. Pigment Cell Biology. M. Gordon, ed. Academic Press, New York. p 583–607.
19. ZELITCH, I. AND M. ZUCKER. 1958. Changes in oxidative enzyme activity during the curing of Connecticut shade tobacco. Plant Physiol. 33: 151–55.