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BIOCHEMICAL CHARACTERISTICS OF TOBACCO LEAVES DURING FLUE-CURING

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Abstract—During normal flue-curing of ripe tobacco leaves, polyphenol oxidase and peroxidase activities rose rapidly during the first few days of curing, and then decreased sharply so that in the fully cured leaf no polyphenol oxidase activity and very little peroxidase activity remained. The ascorbic acid content decreased steadily during the cure, leading to a transient rise in dehydro-ascorbic acid, although very little of either remained in the cured leaves. The chlorogenic acid content rose during the first one or two days of curing, and later fell slightly. Detached tobacco leaves were fed with water, with ascorbic acid or with chlorogenic acid and flue-cured with either a normal or an extended yellowing period. There was very little browning in normal cures, and the colour of the leaves was found to be unrelated to either the normal or additional levels of ascorbic acid or chlorogenic acid. With extended yellowing, however, there was a greater production of pigment, which was related to the chlorogenic acid content of the leaves but which was unaffected by ascorbic acid. There was a great excess of enzyme available for the oxidation of the chlorogenic acid present. Ascorbic acid was probably of little or no significance in preventing oxidation of chlorogenic acid, but in both normal and extended cures, the ascorbic acid content was related to the lustre of the leaves.

INTRODUCTION

WHEN leaves are detached from the parent plant they rapidly degrade protein and chlorophyll due to the cutting off of the supply of hormones from the roots. These senescence changes of leaves can be prevented by the rooting of the leaf or by the application of hormones.[1] Kinetin prevents senescence in attached and detached tobacco leaves,[2] but the protein hydrolysis of detached leaves is not halted by increased nutrient supply and it occurs both in the light and in the dark.[3,4] After detachment, there is a transitory rise in the rate of respiration of leaves, and increases in the activities of some enzymes,[5–7] but leaves in the light continue to photosynthesize so that carbohydrates and amino acids are conserved, while leaves in the dark hydrolyse starch, rapidly become starved and respire sugars and amino acids.[8] During flue-curing, leaves behave as detached leaves in the dark.

Tobacco leaves are harvested when technically ripe, and are already senescent at this stage since they have lost up to 40 per cent of their chlorophyll and their protein and nitrogen contents are decreasing.[9] During the yellowing stage of the first few days of curing, the green colour of the leaf is lost and there is an increase in the hydrolytic activity of the leaf cells in which metabolism continues in a semi-orderly manner. At a later stage the biochemical

[1] D. J. OSBORNE, *J. Sci. Food. Agri.* **16**, 1 (1965).
[2] R. WOLLGHIEM, *Flora Jena* **156**, 291 (1965).
[3] K. MOTHES and L. ENGELBRECHT, *Flora Jena* **143**, 428 (1956).
[4] H. B. VICKERY, G. W. PUCHER, A. J. WAKEMAN and C. S. LEAVENWORTH, *Bull. Conn. Agric. Expt. Sta.* **496** (1946).
[5] W. O. JAMES, *Plant Respiration*, p. 40. Clarendon Press, Oxford (1953).
[6] H. O. ASKEW, *New Zealand J. Sci.* **34**, 17 (1953).
[7] J. UVARDY, M. HORVARTH, K. KISBAN, L. DEZSI and G. L. FARKAS, *Experientia* **20**, 214 (1964).
[8] S. RANJAN and M. M. LALORAYA, *Plant Physiol.* **35**, 714 (1960).
[9] J. M. MOSELEY, W. G. WOLTZ, J. M. CARR and J. A. WEYBREW, *Tobacco Sci.* **7**, 67 (1963).

mechanisms become less and less orderly, resulting in autolysis. During air-curing extensive autolysis occurs so that sugars and amino acids are respired and brown oxidized-phenolic pigments form, but in flue-curing the temperature of the leaves is raised after a few days' curing and enzymes are inactivated so that autolysis and browning are not extensive.[10,11]

The brown pigments found in air-cured leaves contain phenolic material, especially chlorogenic acid and rutin, often associated with protein and protein-iron complexes.[12–15] Some of these types of pigments are found in flue-cured tobacco but in much smaller amounts.[13,15,16] The normal yellow colour of good quality flue-cured tobacco is due to xanthophylls and carotenes, and the preservation of this colour is a sign that the reactions in which polyphenols are converted to pigments have been suppressed. In the preparation of commercial tobacco many leaves are brown or dark brown when cured, and in these leaves the suppression of those reactions has been incomplete.[17]

The pigment-forming reactions during air-curing depend upon the oxidation of chlorogenic acid and rutin, both of which can be oxidized by polyphenol oxidase or by peroxidase.[11,18,19] The production of brown pigments during air-curing is proportional to the content of chlorogenic acid in the fresh leaf, but may not be related to the level of polyphenol oxidase content.[20,21] Data is lacking concerning pigment production in flue-curing, but the process, when it occurs, is believed to be similar to that in air-curing and it has been suggested that ascorbic acid prevents browning in the initial stages of flue-curing by preventing the formation of quinones.[22] Furthermore, it is not known what effect artificially increased levels of ascorbic acid and chlorogenic acid might have on pigment production in normal cures and in cures in which the yellowing period is extended. The extension of yellowing may lead to the production of phenolic pigments as happens during air-curing, since the leaves will be able to autolyse, whereas in normal cures the increase in temperature and the drying of the leaf prevents this. To investigate this aspect, ripe leaves were fed with ascorbic acid and chlorogenic acid, and flue-cured with a normal yellowing period of 2·5 days, or with an extended yellowing period of 4 days. The behaviour of ascorbic acid, chlorogenic acid, polyphenol oxidase, and peroxidase were investigated during flue-curing with normal and extended yellowing stages to determine their relation to the colour of the cured leaf.

RESULTS AND DISCUSSIONS

A number of leaves were assayed for their chlorogenic acid, ascorbic acid, dehydroascorbic acid, chlorophyll, polyphenol oxidase, and peroxidase contents during several cures and the behaviour of these parameters, which was similar in all leaves, is summarized in Figs. 1 and 2.

[10] W. G. FRANKENBURG, *Tobacco N.Y.* **142**, 10 (1956).
[11] S. BABLER, Ph.D. Thesis, Consolidated Tech. Univ. Zurich (1957).
[12] J. S. JACOBSON, *Arch. Biochem. Biophys.* **93**, 580 (1961).
[13] Y. KABURAKI, Y. OBI, H. OHE and Y. SATO, *Sci. Papers, Central Res. Inst. Japan. Monopoly Corp.* **106**, 41 (1964).
[14] H. E. WRIGHT, JR., W. BURTOMAN and E. C. BERRY, JR., *Arch. Biochem. Biophys.* **86**, 94 (1960).
[15] H. E. WRIGHT, JR., W. BURTOMAN and E. C. BERRY, JR., *Phytochem.* **3**, 525 (1964).
[16] V. C. RUNECKLES, *Arch. Biochem. Biophys.* **102**, 354 (1963).
[17] W. G. FRANKENBURG, *Advan. Enzymol.* **6**, 309 (1946).
[18] E. A. H. ROBERTS, *Chem. & Ind.* 1354 (1957).
[19] E. C. SISLER and H. J. EVANS, *Biochem. Biophys. Acta.* **28**, 638 (1958).
[20] P. G. ASMAEV and V. K. YUNOSHEV, *Tabak USSR* **18**, 43 (1957).
[21] M. ZUCKER and H. T. STINSON, JR., *Tobacco Sci.* **4**, 229 (1960).
[22] K. ISHITOYA, *Coresta Inf. Bull.* **1**, 99 (1961).

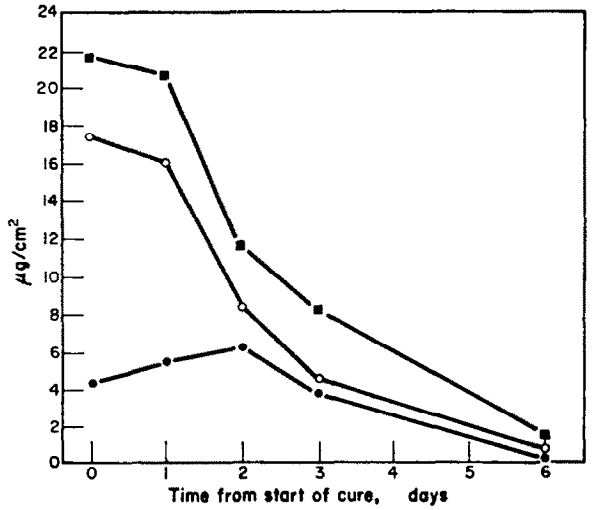

FIG. 1. THE BEHAVIOUR OF ASCORBIC ACID IN TOBACCO LEAVES DURING FLUE-CURING.
■ Total ascorbic acid; ○ ascorbic acid; ● dehydro-ascorbic acid.

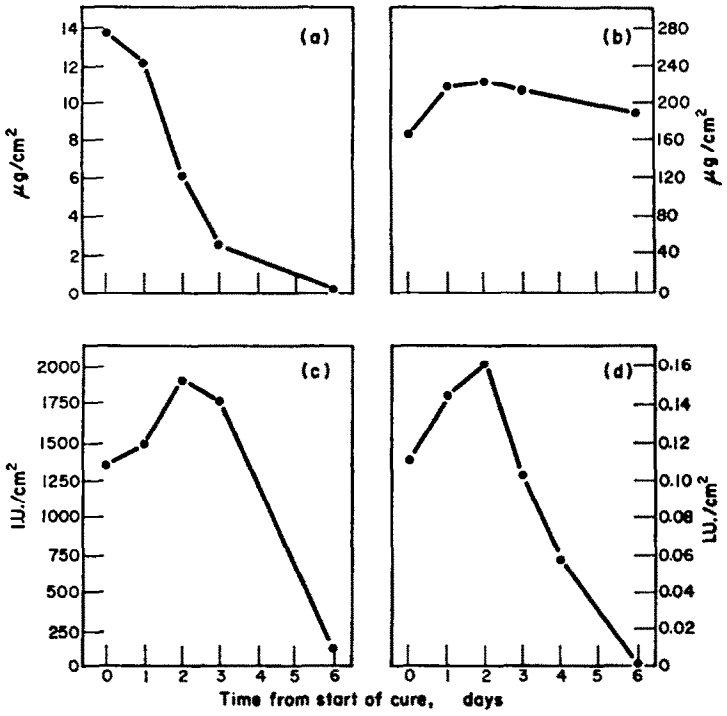

FIG. 2. BIOCHEMICAL BEHAVIOUR OF TOBACCO LEAVES DURING FLUE-CURING.
(*a*) chlorophyll content; (*b*) chlorogenic acid content; (*c*) peroxidase activity; (*d*) polyphenol oxidase activity.

The leaves began to yellow on the plant prior to ripening and this loss of chlorophyll continued during curing, indicating that protein hydrolysis and senescence were occurring. In a typical cure, the chlorogenic acid content rose in the initial phase of yellowing, reached a maximum at 1 to 2 days after the start of the cure, and subsequently declined. The loss between the maximum value and that at the end of the cure was between 31 and 55 $\mu g/cm^2$ (mean value 45 $\mu g/cm^2$). The loss of chlorogenic acid did not lead to the production of appreciable amounts of brown pigment since all the leaves were of a light lemon colour, and their aqueous extracts were only slightly coloured, having an absorptivity of 0·131–0·181 at 450 nm, compared to 0·480 for a light-orange coloured leaf obtained from commercial sources.

TABLE 1. CHLOROGENIC ACID CONTENT IN RELATION TO COLOUR OF CURED TOBACCO

	Chlorogenic acid content ($\mu g/cm^2$)				
	Fresh leaf	Maximum value reached in cure	Cured leaf	Loss*	Absorptivity at 450 nm/10 cm^2
	145	245	200	45	0·151
	175	248	195	53	0·154
	220	267	236	31	0·171
	180	225	176	49	0·163
	174	230	175	55	0·181
	170	231	197	34	0·150
	105	152	121	31	0·170
	121	168	118	50	0·151
	198	276	225	51	0·171
	163	200	171	29	0·168
	215	279	222	57	0·131
	187	248	201	47	0·175
Mean	171	231	186	45	0·161

* Loss = difference between maximum value and cured leaf content.

Total ascorbic acid and ascorbic acid decreased rapidly during curing, which was reflected in a transitory rise in the dehydro-ascorbic acid content, and at the end of yellowing there was only between 1 and 7 $\mu g/cm^2$ of ascorbic acid in the leaves.

The activities of polyphenol oxidase and peroxidase increased at the beginning of the cure, reached a maximum at about 48 hr after the commencement of the cure, and subsequently declined, and by the end of the cure very little or no activity remained.

The amounts of chlorogenic acid in freshly harvested and cured leaves and its rate of increase and decrease during the cure were not related to the colour of the cured leaves or to the colour of their aqueous extracts (Table 1). The pale yellow colour of the extracts was due mainly to xanthophylls and carotenes extractable with hot 80 per cent acetone or ethanol and containing none, or very little, of the brown phenolic pigments. There appears, therefore, to have been a loss of chlorogenic acid during curing without browning of the leaves.

A rise in the activities of polyphenol oxidase and peroxidase, common in leaves after detachment, is also a response to infection, and in both cases the reaction seems to be due to

an increase in hydrolytic activity in the cells which releases enzymes from bound forms,[7,22–27] although some recent work indicates that increases in enzyme activity in detached or injured non-senescent leaves can be due to *de novo* synthesis.[28–30] Infection is often associated with both the accumulation of phenolic material at the infection site, and with localized increases in ascorbic acid oxidase which leads to a reduction in ascorbic acid and an increase in dehydro-ascorbic acid.[25,31] Thus the behaviour of tobacco leaves during flue-curing is similar to that of both diseased and detached leaves.

The activity of the enzymes in rising to a peak at about 48 hr of curing and then declining was not obviously related to the temperature of the curing chamber, as was the case with α-amylase.[32] The temperature of the chamber was 35–37° by the 48th hr from the start of the cure and remained constant until about the 64th hr when it was rapidly raised and reached 46° by the 72nd hr and 60–70° by the 96th hr, at which time considerable enzyme activity still remained. The rapid drying of the leaves at the higher temperature renders them too brittle to be effectively sampled until they have been wetted back at the end of the cure.

It is believed that the redox potential of the leaf cells during the yellowing phase is low enough to prevent quinone and dehydro-ascorbic acid accumulation,[11,33,34] and this prevents browning reactions from occurring. At the end of the yellowing phase the cells have lost vitality and the redox potential rises sufficiently to allow the accumulation of quinones and the commencement of browning. Browning is prevented by the rise in temperature and the drying of the leaves, which inactivates enzymes and restricts the mobility of their substrates. From the results (Fig. 2d) it is seen that even after 3 days of curing when the leaves were still hydrated there was still about 0·1 I.U. of polyphenol oxidase activity extractable per cm^2 of leaf using the assay conditions described. This activity would be sufficient to oxidize about 35 μg of chlorogenic acid per cm^2 per min at 25°, and yet the total loss of chlorogenic acid between its maximum value and that at the end of the cure was only on average 45 μg during a total time of about 100 hr. Clearly the amount of enzyme activity that is extractable is out of all proportion to the amount of oxidation of chlorogenic acid occurring in the leaf, and there is a very great potential for polyphenol oxidation and brown pigment formation to occur if yellowing were continued long enough to enable the redox potential to rise sufficiently to permit quinone accumulation. After 3 days of normal curing the dehydration of the leaf prevented browning and this effect would be independent of any redox potential effect. The total of 17·7 μg/cm^2 of ascorbic acid present in the fresh leaf would be of no potential value in preventing the oxidation of chlorogenic acid at the rate at which it could occur if polyphenol oxidase were fully active. The average total of 8·2 μg/cm^2 of ascorbic acid present after 48 hr of curing could not, by itself, significantly alter the loss of chlorogenic acid.

With normal curing after feeding treatments, there was no production of brown phenolic

[23] T. G. Nye and R. E. Hampton, *Phytochem.* **5**, 1187 (1966).
[24] G. L. Farkas, L. Dezsi, M. Horvarth, K. Kisban and J. Uvardy, *Phytopath. Z.* **49**, 343 (1964).
[25] H. Suseno and R. E. Hampton, *Phytochem.* **5**, 819 (1966).
[26] L. Lovrekovich, Z. Klement and G. L. Farkas, *Science* **145**, 165 (1964).
[27] G. L. Farkas and L. Lovrekovich, *Phytopath. Z.* **55**, 519 (1965).
[28] H. Hydo and I. Uritani, *Plant Cell Physiol. Tokyo* **7**, 137 (1966).
[29] T. Takeo, *Agri. Biol. Chem.* **30**, 1211 (1966).
[30] G. Bagi and G. L. Farkas, *Phytochem.* **6**, 161 (1967).
[31] I. Uritani, *Symp. Biochem. of Plant Phenolic Substances*, p. 98, Colorado State Univ. (1961).
[32] R. Spencer and T. J. Weston, *Tobacco Sci.* **10**, 94 (1966).
[33] L. W. Mapson, *Ann. Rev. Plant. Physiol.* **9**, 119 (1958).
[34] H. S. Mason, *Advan. Enzymol.* **16**, 105 (1955).

pigment and the colour of the cured leaves was light lemon and neither the colour nor the absorptivities of pigment extracts were related to the chlorogenic acid or ascorbic acid contents of the leaves, although those leaves fed with ascorbic acid had a much better lustre (Table 2).

TABLE 2. THE EFFECT OF ADDED ASCORBIC ACID AND CHLOROGENIC ACID ON COLOUR PRODUCTION DURING FLUE-CURING WITH A NORMAL YELLOWING TIME

Feeding* treatment	AA content in fresh leaf (μg/cm^2)	CA content (μg/cm^2)		Absorptivity at 450 nm/10 cm^2	Cured leaf colour
		Fresh leaf	Cured leaf		
Water	32	158	165	0·15	Light lemon
	29	180	176	0·16	Mid-lemon
	20	184	241	0·14	Light lemon
	25	131	197	0·16	Light lemon
	51	155	174	0·18	Light lemon
	31	135	163	0·20	Light lemon
AA	50	165	178	0·15	Light lemon
	121	200	181	0·18	Light lemon
	85	145	163	0·15	Light lemon
CA	34	220	236	0·17	Light lemon
	20	234	281	0·17	Light lemon
	21	193	269	0·15	Light lemon

* AA = ascorbic acid; CA = chlorogenic acid.

In the extended yellowing cure after feeding treatments there was substantial browning of the leaves, producing dark lemon and orange colours, and these pigments could not be extracted with hot 80 per cent acetone or ethanol. The loss of chlorogenic acid, represented by the reduction between its maximum value during curing and that at the end of the cure, was greater than in normal curing, and the colour of the leaves and the optical densities of their extracts were related to the chlorogenic acid contents of the leaves (Table 3). Pigment production was shown to be proportional to the chlorogenic acid content (*a*) in the fresh leaf, (*b*) in the cured leaf, (*c*) at its maximum value, and also proportional to (*d*) the total loss of chlorogenic acid between its maximum value and the level at the end of the cure. The best relationships were with the maximum value and with the amount of chlorogenic acid lost.

Regression equations and correlation coefficients (r) were calculated for these associations and were shown to be:

$$A = 0{\cdot}00132B + 0{\cdot}153;\ r = 0{\cdot}864\dagger$$
$$A = 0{\cdot}002C + 0{\cdot}091;\ r = 0{\cdot}817*$$
$$A = 0{\cdot}0013D + 0{\cdot}071;\ r = 0{\cdot}897\dagger$$
$$A = 0{\cdot}0029E + 0{\cdot}097;\ r = 0{\cdot}919\dagger$$

where: A = Absorptivity of leaf extracts; B = Chlorogenic acid content in fresh leaf; C = Chlorogenic acid content in cured leaf; D = Chlorogenic acid at its maximum value; E = Loss of chlorogenic acid from its maximum value to its value at the end of cure; † Significant at 0·1 per cent level of probability; * Significant at 1 per cent level of probability.

TABLE 3. THE EFFECT OF ADDED ASCORBIC ACID AND CHLOROGENIC ACID ON SOME LEAF COMPONENTS DURING FLUE CURING WITH EXTENDED YELLOWING

	Time from start of cure (hr)					Cured leaf			
	0		48	72					
Feeding* treatment	AA (μg/cm^2)	CA (μg/cm^2)	CA (μg/cm^2)	CA (μg/cm^2)	Polyphenol oxidase (I.U./cm^2)	CA (μg/cm^2)	CA loss†	Absorptivity at 450 nm/10 cm^2	Leaf colour
Water	31	181	261‡	231	0·040	159	102	0·41	Dark lemon
	29	130	210	175	0·038	123	87	0·34	Dark lemon
	34	123	204	183	0·024	117	87	0·35	Dark lemon
	33	130	182	176	0·029	110	72	0·32	Light orange
	27	97	161	140	0·030	99	62	0·25	Mid-lemon
	39	110	172	150	0·031	105	65	0·28	Dark lemon
	32	105	167	149	0·034	104	63	0·31	Mid-orange
AA	63	160	214	201	0·031	134	80	0·34	Dark lemon
	72	136	210	202	0·021	119	91	0·34	Dark lemon
CA	31	168	219	192	0·035	122	97	0·39	Mid-orange
	34	179	238	203	0·038	144	94	0·36	Mid-orange
	40	153	228	186	0·028	136	92	0·35	Light orange

* AA = ascorbic acid; CA = chlorogenic acid; † CA loss = difference between maximum value and value at end of cure.
‡ CA at 48 hr was maximum level measured during the cure.

The chlorogenic acid content of the fresh leaf was a good index of the maximum amount of chlorogenic acid produced during curing and it gave a good indication of the browning potential of the leaf, although this potential was only realized during conditions of extended yellowing. In commercial curing, large numbers of leaves are cured together and, due to variations in field treatment and ripeness, many leaves undergo excess yellowing so that a wider range of colours is obtained.

Ascorbic acid did not affect the colour of the cured leaves, nor did it affect pigment production, although in both types of cure the leaves with additional ascorbic acid had very much better lustre. Polyphenol oxidase activity was also unrelated to pigment production in both types of cure.

These findings are in agreement with the current hypothesis that when browning pigments are formed in cured tobacco they are derived from polyphenols, especially chlorogenic acid, and that they are formed only during autolysis.[10,11,13,15–17] There was no basis to suppose that ascorbic acid acted to inhibit pigment formation and to prevent oxidation of chlorogenic acid.[22]

EXPERIMENTAL

Sampling Procedure

Nicotiana tabacum (var. Hicks Broadleaf) plants were grown in a greenhouse in sand culture with a nutrient drip-feed system.[32] Leaves were harvested when technically ripe and flue-cured in cabinets by conventional procedures. Samples of tissue were taken from the leaves using cork-borers of either 6 or 16 mm dia. As leaves shrink during flue-curing, samples of a constant size will include a greater proportion of the leaf as the cure progresses, and parameters measured on a unit area basis will show a spurious inflation during the cure. To overcome this, the areas to be sampled during a cure were marked on the fresh leaf lamina with Indian ink using a cork-borer. When samples were required during a cure these marked areas were punched out with an over-size cork-borer and trimmed with scissors to the ink line. All assays were therefore comparable on a fresh-leaf area basis, and variations of parameters reflected variations in the whole leaf.

Preparation of Enzyme Powders

Leaf discs were rapidly homogenized with a few drops of water at 0–4° in a glass tubular pestle and mortar to form a paste. Proteins were precipitated by the addition of a cold mixture of ethanol and diethyl ether (4/1, v/v, 10 ml). The mixture was centrifuged after standing for 10 min at 0–4°, the supernatant discarded, and the residue washed with diethyl ether and dried under reduced pressure.[35] The residue was a light brown powder which contained enzymic material.

Assay of Polyphenol Oxidase

Since chlorogenic acid is the major polyphenol in tobacco, the polyphenol oxidase assay was carried out using this compound as the substrate and using a modification of the methods of Sisler and Evans.[19] Each sample of powder was ground with phosphate buffer (0·2 M, pH 6·0, 10 ml), shaken for 10 min and centrifuged to yield a clear supernatant which was used as the crude enzyme solution. Enzyme solution (3·5 ml) was added to an aqueous chlorogenic acid solution (0·4 per cent w/v, 0·6 ml) and incubated at 25°. Aliquots of the incubated solutions (0·3 ml) were taken from the reaction mixture at minute intervals, diluted with water (10 ml) and the absorptivity at 325 nm measured. For a limited period of time the reduction in absorptivity was linear and this phase was taken as a measure of the initial rate.[19] From observations of chemically oxidized chlorogenic acid and by comparison with other methods of assay the absorptivity of the quinone formed was estimated to be about 25 per cent of that of the parent chlorogenic acid. Under the stated conditions, one I.U. of activity was represented by a reduction of 0·103 absorptivity units.

The enzyme reaction had a pH optimum of 6·0, and with reaction mixtures having 0·03–0·05 I.U. of activity, maximum initial velocity was achieved at substrate concentrations above $12{\cdot}5 \times 10^{-4}$ M and a concentration of 16×10^{-4} M was used in the assays. K_m was estimated as $6{\cdot}0 \times 10^{-4}$ M.

A disc of tissue was taken from each side of a leaf and these were combined to form one sample. If samples were restricted to the middle half of the leaf the variation between samples taken from any one leaf was no more than ±4 per cent from the mean.

[35] H. V. BERGMEYER, *Methods of Enzymatic Analysis*, pp. 895 and 905. Academic Press, New York (1963).

Assay of Peroxidase

Each sample of enzyme powder was ground with phosphate buffer (0·5 M, pH 6·1, 10 ml), shaken for 10 min, centrifuged, and the supernatant made up to 200 ml with buffer. Peroxidase activity was assayed from the rate of increase in absorptivity at 485 nm in reaction mixtures containing H_2O_2 (0·03 M, 1·5 ml), *p*-phenylene diamine (1 per cent w/v, 0·5 ml), and enzyme solution (2 ml) at 21°,[35] using an appropriate blank in which buffer replaced the enzyme solution.

The pH optimum of the reaction was found to be 6·4 and mixtures were prepared with pH 6·1 buffer which, on the addition of *p*-phenylene diamine, became pH 6·4. The ratio of H_2O_2 to *p*-phenylene diamine was approximately equimolar, which was essential in order to keep these components mutually saturated. During calibration tests, the non-enzymic dehydrogenation of one μmole *p*-phenylene diamine gave an increase in absorptivity of 0·00045 units under the conditions of assay used. For enzyme preparations of up to 120 I.U. activity per ml, maximum initial velocity was achieved using 0·5 ml of *p*-phenylene diamine solution. A disc of tissue was taken from each side of a leaf and these were combined to form one sample. Variations between samples taken from any one leaf were no more than ± 5 per cent from the mean if samples were restricted to the middle half of the leaf.

Estimation of Ascorbic Acid

Dehydro-ascorbic acid was estimated by a modification of the method of Shaffert and Kingsley[36] by which dehydro-ascorbic acid osazone was produced in strong acid conditions using thiourea to prevent autoxidation of ascorbic acid. Total ascorbic acid was estimated after the conversion of ascorbic acid to dehydro-ascorbic acid, using Norit carbon, and ascorbic acid values were obtained from the difference of these two values.

2,4-dinitrophenyl hydrazine (1·5 g) was added to methanol (100 ml) and boiled for 2–3 min. Conc. HCl (1 ml) was added and the solution cooled. Further HCl (29 ml) was added, the solution stood overnight and filtered.

Fresh leaf discs were homogenized in a solution of oxalic acid (0·5 per cent w/v, 10 ml) and centrifuged to remove particulate matter. Aliquots of the supernatant (1 ml) were mixed with thiourea (saturated solution in 50 per cent aqueous ethanol, 0·1 ml) and 2,4-dinitrophenyl hydrazine (1 ml), and heated at 75° for 90 min. The remainder of the supernatant was mixed with Norit carbon (approximately 0·25 g), shaken for 1 min and filtered. Aliquots of the filtrate (1 ml) were mixed with thiourea and 2,4-dinitro phenyl hydrazine and heated as before. The osazones formed were collected by centrifuging and, after decanting off the supernatant, the precipitate was dissolved in acetone (1 ml). The osazones were separated by ascending paper chromatography on Whatman No. 1 paper using $CHCl_3$/chlorobenzene (3/1 v/v) as solvent.[37] The red dehydro-ascorbic acid osazone (R_f 0·41) was extracted into glacial acetic acid (5 ml) and the absorptivity of the resulting solution measured at 506 nm.

There was considerable variation in the concentration of ascorbic acid over the area of a leaf (± 10 per cent from the mean) and to minimize this variation discs were taken from the tip, middle and butt areas of each side of a leaf and the six discs pooled to make one sample.

Estimation of Chlorogenic Acid

Leaf discs were homogenized in 50 per cent aqueous ethanol (v/v, approximately 10 ml), boiled briefly to inactivate enzymes, and made up to 10 ml. Aliquots (0·2–0·5 ml) were separated by two-dimensional paper chromatography using methyl ethyl ketone/propionic acid/water (2/1/1, v/v/v) in the first direction and 5 per cent aqueous ethanol in the second. Papers were pre-washed with 50 per cent aqueous ethanol to remove impurities. Several fluorescent spots, including chlorogenic acid, rutin, and scopoletin, were observed on the developed chromatogram under u.v. light. The chlorogenic acid spot was extracted in 50 per cent ethanol (10 ml) and its absorptivity measured at 330 nm using appropriate blanks. Variation between samples taken from any one leaf was about ± 5 per cent from the mean, and to minimize this a disc was taken from each of the four quadrants of the leaf and these were pooled to form one sample.

Estimation of Pigments

Discs were taken from each of the four quadrants of a fresh leaf, pooled and refluxed for 15 min in hot 80 per cent aqueous ethanol solution (10 ml) to extract the chlorophyll. The chlorophyll concentration was determined from the absorptivity of the extract at 435 and 670 nm.

The brown pigments of cured leaves were extracted by soaking leaf discs in water (10 ml per cm^2) for 18 hr. The extracts were clarified by centrifuging and their absorptivities measured at 450 nm. Leaf colour was estimated subjectively on a scale light lemon, mid-lemon, dark lemon, light orange, mid-orange and dark orange.

[36] R. R. Shaffert and G. R. Kingsley, *J. Biol. Chem.* **212**, 59 (1955).
[37] C. A. Patschky, *Angew. Chem.* **62**, 50 (1950).

Feeding of Additional Levels of Chlorogenic and Ascorbic Acids

The petioles of harvested leaves, all at the same stage of ripeness, were cleanly cut with a razor and placed in beakers containing water (for the controls), ascorbic acid (10 per cent w/v), or chlorogenic acid (1 per cent w/v), for 4 hr whilst the air around the leaves was gently agitated using electric hair-dryers. Preliminary assays showed that the ascorbic acid entered the leaves as the reduced and not as the oxidized form, and that both added ascorbic and chlorogenic acids were fairly uniformly distributed within the leaves. The leaves were cured with yellowing periods of either 2·5 or 4 days.

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