

Correlation between Chlorophyll and Chlorogenic Acid Content in Tobacco Leaves¹

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ABSTRACT

A positive correlation ($r = 0.75$, $P < 0.01$) was obtained between chlorophyll and chlorogenic acid content in the seedling leaves of burley and dark tobaccos. The dark tobaccos contained significantly higher concentrations of both constituents than the burleys. Such a correlation also occurred in a cytoplasmic mutant of chlorophyll-variegated tobacco when the green and yellow laminae were compared. In addition, the activity of phenylalanine ammonia-lyase and polyphenol-oxidase was higher in the green lamina than in the yellow tissue, which coincided with quantitative distribution of chlorogenic acid. Chlorophyll deficiency induced by streptomycin in tobacco seedlings resulted in a progressive decrease in chlorogenic acid content. However, an interruption of streptomycin treatment provoked accumulation of the two compounds. Dark-grown seedlings showed an increase in the content of chlorophyll and chlorogenic acid upon illumination. Incorporation of L-phenylalanine- $U^{14}C$ into chlorogenic acid during leaf greening was drastically reduced owing to the presence of phenylpyruvate; the latter compound is a possible by-product of chlorophyll biosynthesis. This phenomenon was also evident with light-grown leaves. Results suggest that in addition to phenylalanine ammonia-lyase as a key enzyme regulating chlorogenic acid biosynthesis, an alternative pathway involving the conversion of phenylpyruvate to cinnamate may be functional in tobacco leaves. This pathway may bear importance as to higher chlorogenic acid content in dark tobaccos than in burleys.

Dark and burley tobaccos (*Nicotiana tabacum* L.) represent two major types of cultivars for cigarette manufacture. They are visually distinguishable by leaf color: deep green for dark tobacco and light green for burley. This color difference reflects the amount of chlorophyll and is controlled by two pairs of nuclear genes (5). Dark green color is the dominant phenotype.

In studies of leaf chemical constituents in tobacco cultivars, an association of high polyphenol content with dark tobaccos and low with burleys has appeared in the literature (18, 23, 26). This quantitative contrast persists throughout growth

stages and curing processes (23). Chlorogenic acid (5-*O*-caffeoylquinic acid)² is the predominant phenolic compound in tobacco leaf (25, 26). Biosynthesis of chlorogenic acid takes place in the leaf (30) and begins with the deamination by PAL³ of L-phenylalanine to cinnamic acid which subsequently undergoes hydroxylation and the formation of caffeoyl ester with quinic acid (2, 9, 16). Recently, Harper *et al.* (10) have suggested dual pathways of phenolic biosynthesis that may not involve PAL.

I have correlated the content of chlorogenic acid with that of chlorophyll among tobacco cultivars and within single genotypes. Biochemical bases of the correlation have been investigated in experiments including (a) studies of leaf chemical constituents and PAL, PPO, and PRO activities in a chlorophyll-variegated mutant of tobacco; (b) the bleaching effect of streptomycin on chlorogenic acid accumulation; and (c) the incorporation of precursors into chlorogenic acid in leaves of dark- and light-grown seedlings. Results will be discussed in relation to the quantitative difference of chlorogenic acid in dark and burley tobaccos.

MATERIALS AND METHODS

Plant Materials. Twenty-five tobacco cultivars consisting of 8 burley and 17 dark tobaccos were cultured in plastic tubes (3 × 12 cm) containing vermiculite with a full strength of Hoagland's nutrient solution in growth chambers. Seedlings were placed about 30 cm under cool-white fluorescent lights which provided 1600 ft-c for a 16-hr photoperiod at 28 C. At the five- to six-leaf stage, the apical buds were removed to induce physiological aging of the leaf. One week later, leaves from 20 to 25 plants of each entry were harvested and bulked as one sample. All samples were freeze-dried and ground to pass through a 40-mesh screen. The ground powders were stored in amber bottles at -80 C. The same growth conditions and methods of sample preparation were followed for other experiments unless otherwise described.

A cytoplasmic chlorophyll-variegated mutant of tobacco, whose inheritance and morphological characteristics are identical to that described by Burk *et al.* (4), was used to study the relation between chlorophyll quantity and polyphenol accumulation. Eighteen plants in 25-cm pots were randomly arranged on greenhouse benches into three replications. The greenhouse conditions were maintained at 27 C during the day and 18 C at night with fluorescent and incandescent lights to

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² In accordance with the recent change in the stereospecific numbering of quinic acid, C₈ is now C₅ and vice versa. IUPAC and IUB, *J. Biol. Chem.* 243: 5809-5819, 1968.

³ Abbreviations: PAL: L-phenylalanine ammonia-lyase; PPO: polyphenoloxidase; PRO: peroxidase.

provide a 16-hr photoperiod. At flowering, leaves from each replication were dissected into green and yellow sections for chemical analyses.

Chlorophyll and Polyphenol Determination. Freeze-dried leaf powders were extracted with 85% acetone. The acetone extracts were read at 644 nm and 663 nm for determination of chlorophylls *a* and *b* by the method of Arnon (1). Polyphenol quantity was determined by paper chromatography and spectrophotometry according to the procedures previously described (23).

PAL, PPO and PRO Preparation and Assay. Phenylalanine ammonia-lyase prepared from acetone powder at subzero temperatures was assayed with L-phenylalanine for cinnamate production in terms of increase in absorbancy at 279 nm according to the method of O'Neal and Keller (17). Polyphenoloxidase and PRO were extracted from leaf powders with 0.1 M tris-HCl buffer, pH 8, and assayed by spectrophotometric methods and polyacrylamide gel slab electrophoresis. Details of the enzyme assay and electrophoretic procedures have been reported elsewhere (22, 24).

Streptomycin Bleaching Experiment. Burley 21 seedlings at the five- to six-leaf stage were cultured in a full strength of Hoagland's nutrient solution with or without streptomycin (2 mg/ml) in a growth chamber. Leaves from 10 plants were collected at each of 0, 1, 2, 4, 6, and 8 days during the course of the treatment. After 4 days, a portion of streptomycin-treated seedlings was transferred back to streptomycin-free nutrient solution to study the recovery of chlorophyll synthesis and chlorogenic acid accumulation.

Incorporation of Precursors into Chlorogenic Acid in Seedling Leaves. Burley 21 seedlings at the four- to five-leaf stage in vermiculite were irrigated with Hoagland's nutrient solution devoid of KNO_3 and $\text{Ca}(\text{NO}_3)_2$ to induce chlorophyll deficiency and chlorogenic acid accumulation (25, 27). The deletion of $\text{Ca}(\text{NO}_3)_2$ was, however, compensated with an equal molar concentration of calcium in the form of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$. When chlorosis appeared, seedlings were transferred to darkness for 96 hr. The dark-grown leaves, approximately 5 cm in length, were detached and subjected to greening under 1600 ft-c light intensity at 28 C in 0.01 M phosphate buffer, pH 7. The buffer solution contained 25 mM KCl and 25 mM quinic acid, neomycin (25 $\mu\text{g}/\text{ml}$), L-phenylalanine- U^{14}C (4.44×10^{-5} cpm/ml) according to Zucker and Levy's method (31), with or without cycloheximide (0.5 mg/ml) or phenylpyruvate (0.5 mg/ml). The same medium was used in a separate experiment to study the effect of phenylpyruvate on the incorporation of L-phenylalanine- U^{14}C into chlorogenic acid in green leaves. The leaves, 7 to 8 cm in length, came from Burley 21 seedlings cultured in complete nutrient solution with a 16-hr photoperiod.

For the above experiments, eight leaves were taken at 0-, 2-, 3-, 4-, 6-, 8-, and 11-hr intervals. Changes of chlorophyll, chlorogenic acid, and L-phenylalanine content as well as the specific radioactivity of the latter two compounds were studied. L-Phenylalanine was detected on paper chromatogram with ninhydrin spray and quantitated spectrophotometrically at 540 nm according to Giri *et al.* (8). The radioactivity of L-phenylalanine and chlorogenic acid in eluates of paper chromatograms was determined in a Packard automatic Tri-Carb liquid scintillation spectrometer (model 4322) at 6 C.

RESULTS AND DISCUSSION

Correlation between Chlorophyll and Chlorogenic Acid Content. The mean quantities of chlorophyll in burley and dark tobaccos were 9.83 and 15.22 mg/g dry weight, respectively (Table I). These values differed significantly at the 1%

Table I. *Chlorophyll and Chlorogenic Acid Content in the Seedling Leaves of Burley and Dark Tobaccos*

Cultivar	Tobacco Type	Chlorophyll	Chlorogenic Acid
		<i>mg/g dry wt</i>	
Burley 21	Burley	9.59	6.00
Burley 37	Burley	9.41	7.48
Kentucky 10	Burley	11.40	7.62
Kentucky 12	Burley	9.02	7.73
Kentucky 16	Burley	10.04	7.68
T.I. 97	Burley	8.03	7.30
T.I. 1018	Burley	11.19	8.03
T.I. 1349	Burley	9.89	7.97
Hicks	Dark	17.10	16.73
Kentucky 151	Dark	17.86	15.82
F.C. 604	Dark	15.94	11.35
T.I. 223	Dark	16.83	9.05
T.I. 290	Dark	13.19	8.76
T.I. 451	Dark	13.33	8.66
T.I. 456	Dark	15.55	9.15
T.I. 508	Dark	15.38	9.97
T.I. 583	Dark	14.30	8.03
T.I. 998	Dark	14.56	8.66
T.I. 1308	Dark	14.02	11.91
T.I. 1310	Dark	13.75	8.20
T.I. 1318	Dark	14.22	14.47
T.I. 1335	Dark	15.80	14.86
T.I. 1388	Dark	14.08	13.48
T.I. 1352	Dark	16.41	14.22
T.I. 1362	Dark	15.89	14.45

level of probability. The difference in chlorogenic acid content between the sample means of 7.48 mg/g dry weight for the burleys and 11.63 mg/g dry weight for the dark tobaccos was also statistically significant, which confirms the previous results that burleys are low in polyphenol content (23). Based on 25 entries, a positive correlation coefficient $r = 0.75$ ($P < 0.01$) was obtained for chlorophyll and chlorogenic acid content.

It is a general belief that chlorophyll content does not influence the rate of CO_2 assimilation when light intensity and CO_2 concentration are not limiting. For example, green and yellow (*aurea*) varieties of *Ulmus* sp. and *Sambucus nigra* could assimilate CO_2 at about the same rate in spite of the yellow leaves having one-tenth of the normal chlorophyll content (11). To extend this generalization, the difference in polyphenol (chlorogenic acid) content in the leaf of burley and dark tobaccos may not be attributed to the efficiency of carbon dioxide assimilation because of difference in chlorophyll content. The positive correlation between the two leaf constituents, therefore, requires a different explanation.

Leaf Chemical Constituents in the Chlorophyll-variegated Mutant. There was 8-fold more chlorophyll in the green laminae than in the yellow tissues (Table II). Chlorophyll *a/b* ratios were 3.4:1 and 2.6:1 for the green and yellow sections, respectively. These results are in agreement with other reports (12, 32) based on similar chlorophyll-variegated mutants of tobacco. Both lamina tissues showed an identical pattern of polyphenols, which consists of chlorogenic acid and the isomer neochlorogenic acid (3-*O*-caffeoylquinic acid), rutin, and scopolin in detectable quantities. Since isomeric conversion may occur during extraction and chromatographic separation, chlorogenic acid content in this experiment includes neochlorogenic acid. Chlorogenic acid and scopolin content in the green laminae were twice that of the chlorophyll-deficient tissues.

Table II. *Quantity of Chemical Constituents and Enzyme Activity in Chlorophyll-variegated Tobacco Laminae*

Chemical Constituent and Enzyme	Green Lamina ¹	Yellow Lamina
Chlorophyll <i>a</i> (mg/g dry wt)	9.25**	1.04
Chlorophyll <i>b</i> (mg/g dry wt)	2.76**	0.40
Chlorogenic acid (mg/g dry wt)	8.49**	4.13
Rutin (mg/g dry wt)	0.25	0.29
Scopolin (mg/g dry wt)	0.38*	0.18
Total polyphenols (mg/g dry wt)	9.12**	4.60
PAL (μ moles cinnamate/mg protein·hr)	5.29**	2.16
PPO ($\Delta A/100$ mg dry wt·min)	2.74**	1.79
PRO ($\Delta A/mg$ dry wt·min)	6.33**	4.49

¹ * and ** indicate significant difference between the sample means in each row at the 5% and 1% level of probability, respectively, as determined by a *t* test.

However, rutin quantity showed no difference between these laminae. This may be attributed to the small amounts of light of the short wavelengths passing through the glass in the greenhouse. Zucker and Stinson (32) have investigated a mature variegated leaf of Connecticut shade tobacco and reported a greater concentration of chlorogenic acid and PPO in green tissue extracts than that of the white tissues. The present results support their findings. In addition, the green tissue extracts exhibited higher PAL and PRO activity than the chlorophyll-deficient tissue. Polyacrylamide gel slab electrophoresis of lamina extracts revealed 6 PPO and 15 PRO isozyme bands which are identical to those illustrated in a recent paper (22). Differences in chlorophyll content gave rise to variation in banding intensity only.

Although cytoplasmic chlorophyll mutants offer advantages in minimizing nuclear gene effect, the difference in photosynthetic capability and related metabolism between lamina tissues varying in chlorophyll content cannot be overlooked. Chloroplasts from yellow sections of a cytoplasmic chlorophyll mutant of tobacco exhibited essentially no photosystem II reaction, but they had a normal rate of photosystem I activity (12). The possible impact of photosystem II on metabolic processes including polyphenol biosynthesis and degradation can be expected. Diffusion of photosynthetic products from adjacent green laminae may partially overcome the physiological deficiency of the yellow sections.

Chloroplasts isolated from an array of plant species are capable of converting monohydric to *o*-dihydric phenols by the activity of cresolase, an enzyme of the PPO type as reported by Sato *et al.* (20). Their method was followed to detect cresolase activity with tobacco chloroplasts purified by the method of Whitfield and Spencer (28). Results were negative in all cases. Since chlorogenic acid possesses hydroxyl groups at the *ortho*-position, its low content in the yellow tissue is not attributed to deficiency of cresolase activity in chloroplasts. The observed positive correlation between PPO activity and chlorogenic acid content may reflect an active turnover of chlorogenic acid involving catecholase which, as a type of PPO, catalyzes the oxidation of *o*-dihydric phenols to the corresponding quinones (6). A large metabolic pool of chlorogenic acid may thus induce an active catecholase synthesis. Hyodo and Uritani (13) have found in sweet potato tissues that an increase in polyphenol content might be involved in the enhancement of catecholase activity.

Effect of Streptomycin on Chlorophyll and Chlorogenic Acid Content. Chlorophyll level in plants may be lowered by environmental stress and metabolic inhibition. Streptomycin

was reported to suppress chlorophyll formation, but it enhanced anthocyanin synthesis in mustard seedling leaves (21). In tobacco seedling leaves, streptomycin caused a steady decrease in chlorophyll content after 2 days (Fig. 1). A slight accumulation of chlorogenic acid occurred during the initial 24 hr, and thereafter its concentration decreased at the same pace as did chlorophyll. Controls showed a slight decrease in chlorophyll and chlorogenic acid content during the experiment. This is possibly associated with an increase of dry matter in the leaf. The interruption of streptomycin treatment resulted in a gradual recovery of chlorophyll synthesis and a concomitant accumulation of chlorogenic acid.

Changes of Chlorophyll and Chlorogenic Acid Content during Greening of Tobacco Leaves. Upon illumination, the dark-grown tobacco leaves increased in chlorophyll production for 6 hr, after which there followed a rapid degradation (Fig. 2). Chlorophyll degradation is usually linked with the degradation of protein (19). Nitrogen stress in the present experiment may hasten the occurrence of these senescent phenomena. Illumination also stimulated an accumulation of chlorogenic acid during the first 2 hr. Chlorogenic acid in this experiment excluded neochlorogenic acid, which constitutes less than 10% of total chlorogenic acids. The absence of net chlorogenic acid synthesis after the initial hours indicates a rapid turnover of this compound. This was substantiated by a steady increase of ¹⁴C incorporation up to 8 hr. When phenylpyruvate was added to the incubation medium, the incorporation of L-phenylalanine-U-¹⁴C into chlorogenic acid was drastically reduced. This phenomenon was repeated when phenylpyruvate was added after 4 hr of illumination.

In the presence of cycloheximide, chlorophyll content increased slightly at the beginning of illumination and then declined slowly for the remaining period of the experiment (Fig. 3). Quantitative changes of chlorogenic acid appeared not to be affected by cycloheximide. The incorporation of L-phenylalanine-U-¹⁴C, however, was drastically decreased. Since cycloheximide directly inhibits protein synthesis at a ribosomal level (14), its effect may be manifested not only by the biosynthesis of chlorophyll and chlorogenic acid but also by the catabolism of these leaf constituents.

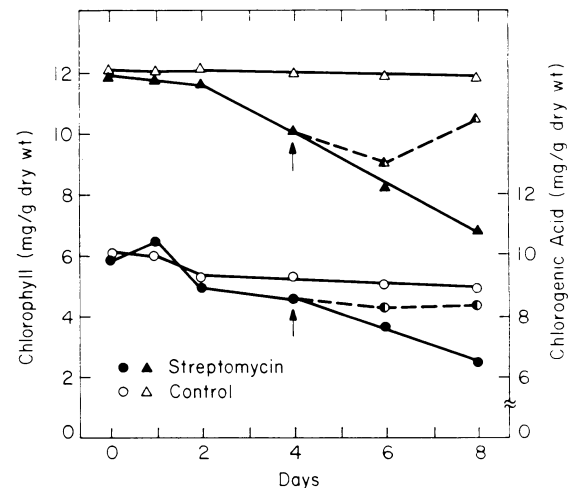


FIG. 1. Effect of streptomycin on chlorophyll (Δ , \blacktriangle) and chlorogenic acid (\circ , \bullet) content in tobacco seedling leaves. Arrow indicates the time at which a portion of streptomycin-treated seedlings was transferred to the streptomycin-free nutrient solution, and the subsequent effect on the change of chlorophyll and chlorogenic acid content is shown by dotted lines. Each point indicates the mean of two determinations.

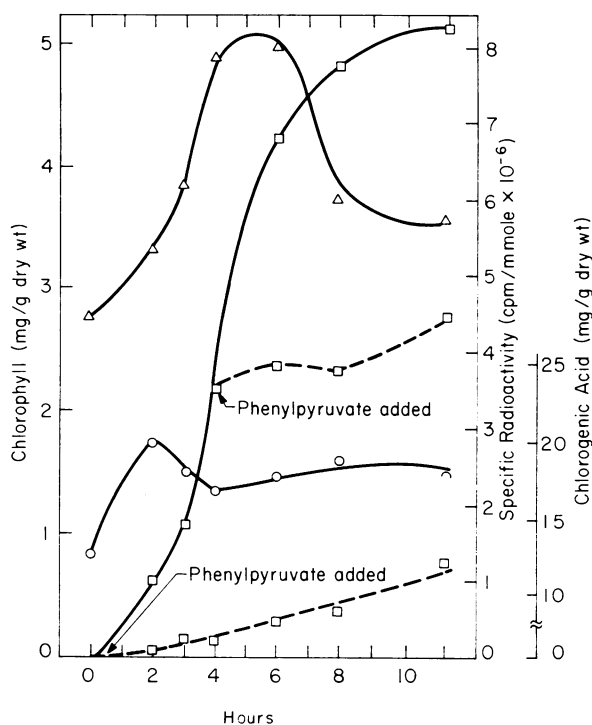


FIG. 2. Incorporation of L-phenylalanine-U-¹⁴C into chlorogenic acid (○) during leaf greening. Excised leaves of tobacco seedlings conditioned in nitrogen stress and darkness were cultured in Petri dishes with a buffer solution containing 500 cpm L-phenylalanine-U-¹⁴C/mg fresh weight. Buffer preparation and experimental conditions are detailed under "Materials and Methods." Increase of the specific radioactivity (□) of chlorogenic acid in the phenylpyruvate treatments is shown by dotted lines. Chlorophyll content (Δ) is the sum of chlorophylls *a* and *b*. Each point represents the mean of two experiments.

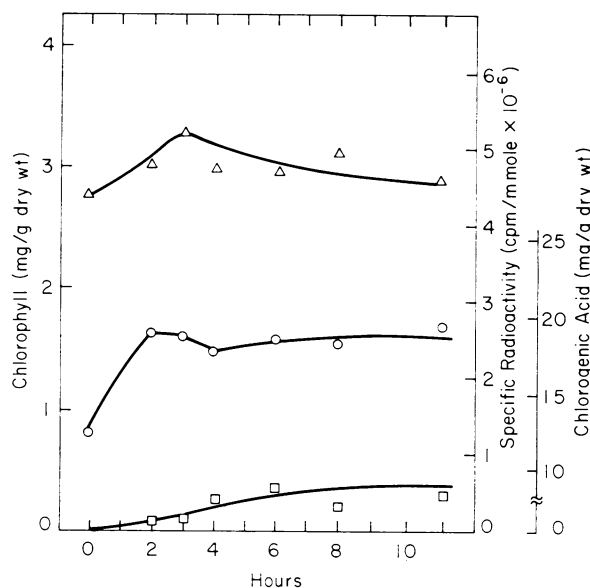


FIG. 3. Effect of cycloheximide on chlorophyll (Δ) and chlorogenic acid (○) content and specific radioactivity (□) of chlorogenic acid during leaf greening. Experimental materials and procedures and the control experiments (without cycloheximide) were identical to that described in Figure 2. Each point represents the mean of two experiments.

Effect of Phenylpyruvate on the Incorporation of L-Phenylalanine-U-¹⁴C into Chlorogenic Acid in Tobacco Leaves. In contrast to those subjected to nitrogen stress and darkness, normal green leaves of Burley 21 seedlings contained 12 to 13 mg chlorophyll/g dry weight. This quantity decreased about 20 to 30% in the excised leaves at the end of 11-hr incubation. Chlorogenic acid content remained relatively constant and at comparable levels among and within the treatments (Table III). The specific radioactivity of chlorogenic acid in the control was twice that in the leaf-greening experiments, suggesting a greater turnover rate of this compound in normal green leaves than in the nitrogen-deficient tissues. Phenylpyruvate showed a dosage effect on the incorporation of L-phenylalanine-U-¹⁴C: the more phenylpyruvate added, the less was the specific radioactivity of chlorogenic acid. Inhibition of chlorogenic acid metabolism by cycloheximide was again evident.

Biological conversion of phenylpyruvate to cinnamate as a possible step in polyphenol biosynthesis has been proposed by Neish (16). Although the isolation of PAL in plant tissues favors the direct deamination of L-phenylalanine to cinnamate (15), which is often controlled by photosynthetic reactions (29),

Table III. Effects of Phenylpyruvate and Cycloheximide on the Quantity and Specific Radioactivity of Chlorogenic Acid and L-Phenylalanine in Tobacco Leaves

Leaves were excised from tobacco seedlings grown in complete nutrients and light. The procedure of feeding L-phenylalanine-U-¹⁴C was identical to that of the leaf-greening experiments.

Treatment	Chlorogenic acid		L-Phenylalanine	
	Quantity	Specific radioactivity	Quantity	Specific radioactivity
	mg/g dry wt	cpm/mmole × 10 ⁻⁶	mg/g dry wt	cpm/mmole × 10 ⁻⁶
0 hr	8.9	—	0.5	—
Control				
2 hr	8.5	2.7	0.7	56.7
3 hr	9.7	6.8	0.8	75.8
4 hr	10.1	9.2	0.6	87.8
6 hr	9.7	15.2	1.1	98.0
8 hr	9.5	16.0	1.5	103.6
11 hr	8.5	17.5	1.5	115.4
Phenylpyruvate (0.5 mg/ml)				
2 hr	8.7	0.5	1.1	72.6
3 hr	9.8	0.8	1.8	85.1
4 hr	9.4	1.1	1.0	86.2
6 hr	10.3	2.9	1.5	136.7
8 hr	9.9	3.0	2.0	165.5
11 hr	8.7	4.2	3.6	102.6
Phenylpyruvate (0.1 mg/ml)				
2 hr	8.7	1.3	0.8	71.3
3 hr	10.1	2.7	0.9	75.5
4 hr	10.5	3.7	0.9	85.7
6 hr	9.8	4.7	1.4	92.6
8 hr	10.2	7.1	1.7	103.6
11 hr	8.5	7.8	2.0	106.5
Cycloheximide (0.5 mg/ml)				
2 hr	8.5	0.5	0.8	68.2
3 hr	9.8	0.8	0.9	76.3
4 hr	9.8	1.0	1.0	102.5
6 hr	10.4	1.5	1.0	114.8
8 hr	8.9	2.3	1.3	153.6
11 hr	7.1	2.9	1.8	151.1

these processes are by no means mutually exclusive with the phenylpyruvate pathway. Questions may be raised as to the conversion of phenylpyruvate into phenylalanine catalyzed by transaminase. This direction of transamination seems to be insignificant in the leaf-greening experiments in view of the fact that the specific radioactivity of L-phenylalanine isolated from paper chromatograms was comparable for the control and phenylpyruvate treatments at any given sampling interval. Normal green tissues, however, increased the pool size of L-phenylalanine owing to the addition of phenylpyruvate. Nevertheless, the increase in pool size did not lower the specific radioactivity of this amino acid except the 11-hr samples. Possibilities exist that difference in uptake and transport of L-phenylalanine and phenylpyruvate across membranes and localized conversion of phenylpyruvate to L-phenylalanine and subsequently to cinnamate in cell compartments may hinder a swift equilibrium of L-phenylalanine pool in the cell.

Parallelism in quantitative changes of chlorophyll and chlorogenic acid due to chloroplast mutation, streptomycin, and cycloheximide treatments and during the greening of seedling leaves points to the possible relationship between chlorophyll and chlorogenic acid metabolism. There are two currently recognized hypotheses on chlorophyll biosynthesis (3, 7). One involves δ -aminolevulinic acid synthetase which catalyzes the formation of δ -aminolevulinic acid by condensation of succinyl-CoA and glycine. The other hypothesis suggests the formation of δ -aminolevulinic acid through transamination of L-alanine, L-glutamic acid, and L-phenylalanine to γ , δ -dioxo-valeric acid. When L-phenylalanine is the amino donor, phenylpyruvate will be the by-product. If the latter metabolic pathway is functional in tobacco leaves, the more chlorophyll synthesized in the leaf, the higher will be the phenylpyruvate production. This may lead to increasing the pool size of cinnamate and, consequently, an accumulation of chlorogenic acid. As a result, dark tobaccos which contain more chlorophyll than burleys would also have greater amounts of chlorogenic acid. This may explain in part the positive correlation between the quantities of chlorophyll and chlorogenic acid in tobacco leaves.

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