Phytochemistry, 1971, Vol. 10, pp. 2705 to 2711. Pergamon Press. Printed in England.

ROLE OF PHENYLALANINE IN THE BIOSYNTHESIS OF FLAVONOIDS AND CINNAMIC ACIDS IN STRAWBERRY LEAF DISKS

L. L. CREASY

Department of Pomology, Cornell University, Ithaca, New York, U.S.A.

(*Received* 19 *January* 1971)

Abstract—Alteration of the turnover of phenylalanine ammonia-lyase and cinnamic-4-hydroxylase in leaf disks actively synthesizing phenolics was accomplished by inhibition and by withholding substrate. Measurement of the result of these manipulations on the accumulation of cinnamic acids and flavonoids suggested that the ammonia-lyases are obligatory enzymes in phenolic synthesis but are not necessarily rate limiting.

INTRODUCTION

THE REACTIONS converting the aromatic amino acids phenylalanine or tyrosine to the corresponding cinnamic acids catalyzed by the appropriate ammonia-lyase, phenylalanine ammonia-lyase (PAL)[1] or tyrosine ammonia-lyase (TAL),[2] are generally considered the initial steps in the biosynthesis of a large variety of plant phenolics.[3] Because of the position of these reactions at the beginning of a complex synthetic pathway, they have received considerable attention as possible controlling reactions,[4–7] since initial steps are the likely places for overall pathway regulation.[8] Due to its generally higher activity in many plants,[5] PAL has been studied most frequently. Factors which are known to influence the accumulation of phenolic end-products have been shown to similarly influence the activity of PAL. Some particular factors studied include phytochrome control in dark grown seedlings,[9, 10] feedback repression[11, 12] or inhibition[13] and changes in the activity of PAL controlled by temperature through a PAL decay system.[14, 15] Any control of ammonia-lyases at the first step of phenolic biosynthesis, would equally control the synthesis of all the end-products and it is therefore likely that additional regulatory mechanisms function at each major branch point in the biosynthesis of the numerous phenolic types.[8] Evidence of the existence of isozymes of PAL has been obtained.[16–18]

[1] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[2] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
[3] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, Academic Press, London (1967).
[4] M. ZUCKER, *Plant Physiol.* **40**, 779 (1965).
[5] M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, *Can. J. Bot.* **44**, 341 (1966).
[6] L. L. CREASY, *Phytochem.* **7**, 441 (1968).
[7] H. SHERF and M. H. ZENK, *Z. Pflanzenphysiol.* **57**, 401 (1967).
[8] F. GIBSON and J. PITTARD, *Bacteriol. Rev.* **32**, 465 (1968).
[9] H. SMITH and T. H. ATTRIDGE, *Phytochem.* **9**, 487 (1970).
[10] F. DURST and H. MOHR, *Naturwissenschaften* **53**, 707 (1966).
[11] G. ENGELSMA, *Planta* **82**, 355 (1968).
[12] T. MINAMIKAWA and I. URITANI, *J. Biochem.* **58**, 53 (1965).
[13] M. ZUCKER, K. R. HANSON and E. SONDHEIMER, In *Phenolic Compounds and Metobolic Regulation* (edited by B. J. FINKLE and V. C. RUNECKLES), p. 69, Appleton-Century-Crofts, New York (1967).
[14] G. ENGELSMA, *Planta* **90**, 133 (1970).
[15] G. ENGELSMA, *Planta* **91**, 246 (1970).
[16] T. MINAMIKAWA and I. URITANI, *J. Biochem.* **57**, 678 (1965).
[17] E. A. HAVIR and K. R. HANSON, *Biochem.* **7**, 1896 (1968).
[18] S. I. AHMED and T. SWAIN, *Phytochem.* **10**, (in press) (1970).

Although it has been tacitly agreed that ammonia-lyases are involved in the synthesis of phenylpropanoids and their derivatives, information has recently been advanced to point out the lack of conviction concerning the obligate role of the ammonia-lyases.[19] The experiments which are described below provide some assurance of the role of phenylalanine in the synthesis of flavonoids in strawberry leaf disks.

RESULTS

The goal was to influence the turnover number[20] of some enzymes purportedly in the biosynthesis of flavonoids without disturbing the quantity of enzymes present. To accomplish this we supplied a substrate, a competitive inhibitor, or withheld substrate, and determined the effect of the treatment on the synthesis of a number of phenolics known to accumulate in this tissue.[21, 22] Table 1 gives the results of supplying water, sucrose, phenylalanine or tyrosine to leaf disks of strawberry exposed to light and supplied with ample carbon dioxide.[23] Sucrose is not an effective stimulator of phenolic synthesis in green tissues when carbon dioxide is well supplied and had little effect on the synthesis of cinnamic

TABLE 1. THE EFFECT OF SOME METABOLITES ON THE ACCUMULATION OF CINNAMIC ACIDS AND FLAVONOIDS IN STRAWBERRY LEAF DISKS. FLUORESCENT LIGHT (2·5 mW/cm²), AIR STREAM, 25°, 22 hr, ACETONE POWDER YIELD = 1·6 mg/cm²

	Content in nmoles/cm²				
		Compound fed			
Component	$t = 0$	Water	Sucrose*	L-Phenylalanine*	L-Tyrosine*
Cinnamic acid	0·26	0·22	0·04	8·2	0·22
p-Coumaric acid	7·9	45·0	40·5	58·5	26·7
Caffeic acid	3·3	9·0	9·0	12·2	9·1
Catechin	7	29	17	25	18
Biflavan†	1	24	22	23	15
Flavolan‡	6	16	15	21	13
Flavonols§	11	21	19	18	15
Net change in total cinnamic acid units‖	—	+133	+109	+153	+77
PAL¶	—	222	218	197	216

* $2·6 \times 10^{-3}$ M, 0·67 ml/cm².
† Ref. 22.
‡ Ref. 23.
§ Ref. 21.
‖ Counting 2 units/mole of biflavan.
¶ Median rate (in nmoles/cm²/hr at 25°) × (22 hr).

[19] T. SWAIN and C. A. WILLIAMS, *Phytochem.* **10, 2115** (1970).
[20] J. S. FRUTON and S. SIMMONDS, *General Biochemistry*, p. 211, Wiley, New York (1953).
[21] L. L. CREASY, E. C. MAXIE and V. L. SINGLETON, *Proc. Am. Soc. Hort. Sci.* **85,** 325 (1964).
[22] L. L. CREASY and T. SWAIN, *Nature, Lond.* **208,** 151 (1965).
[23] L. L. CREASY, *Phytochem.* **7,** 1743 (1968).

acids or flavonoids. Phenylalanine, a substrate for the ammonia-lyase stimulated the accumulation of all the cinnamic acids measured but had little effect on the flavonoids measured. There was no stimulation in the PAL activity and in fact a small decrease usually resulted. If the supply of cinnamic acids was limiting flavonoid biosynthesis in this tissue, the significant increases in their availability would have resulted in some stimulation in flavonoid synthesis. Since this did not occur, it was concluded that some other mechanism was rate limiting. Tyrosine has been reported to be a competitive inhibitor of phenylalanine ammonia-lyase *in vitro*[1] as well as a substrate for ammonia-lyase activity.[2] Assays of this plant material for the deamination of tyrosine show only very small rates of activity, i.e. less than 5 per cent of the activity toward phenylalanine. Depending on the supply of phenylalanine and tyrosine in the plant, we might expect externally supplied tyrosine to function as a competitive inhibitor *in vivo*. The results in Table 1 support this view. Tyrosine inhibited the accumulation of both cinnamic acids and flavonoids, presumably the inhibition of PAL created a limiting supply of cinnamic acids for flavonoid synthesis. The degree of inhibition was about the same for both cinnamic acid and flavonoid accumulation. Tyrosine did not have any effect on the amount of PAL in the tissue. The median rate of PAL reported is probably a high estimate of the average rate because of a lag in the build up of carbohydrates and increase in PAL.

The results of the second experiment are given in Table 2 and show that the effects of phenylalanine or tyrosine can be tempered by the presence of the other. These disks showed

TABLE 2. ACCUMULATION OF CINNAMIC AND *p*-COUMARIC ACIDS IN STRAWBERRY LEAF DISKS SUPPLIED WITH AROMATIC AMINO ACIDS. 2·5 mW/cm² FLUORESCENT LIGHT, AIR STREAM, 25°, 21 hr

Treatment	Content, nmoles/cm²	
	Cinnamic acid	*p*-Coumaric acid
$t = 0$	1·4	6·7
H_2O	1·5	16·7
10^{-3}M Phenylalanine	5·7	44·0
10^{-3} Tyrosine	1·0	16·3
10^{+3}M Phenylalanine + 10^{-3} Tyrosine	4·9	32·0
$2{\cdot}6 \times 10^{-3}$ M Phenylalanine	12·0	53·1
$2{\cdot}6 \times 10^{-3}$ M Tyrosine	1·5	21·0
$2{\cdot}6 \times 10^{-3}$ M Phenylalanine + $2{\cdot}6 \times 10^{-3}$ M Tyrosine	10·0	36·0

some increase in the accumulation of *p*-coumaric acid in response to tyrosine suggesting possibly some activity of TAL. The stimulation of the accumulation of both cinnamic acid and *p*-coumaric acid by phenylalanine at two concentrations could be significantly inhibited by the addition of tyrosine. Although there are many possible problems involved in comparing the results with these two amino acids with respect to their rates of uptake, accumulation in the plant or fate in the cell, the results do support the idea that phenylalanine in this tissue is absolutely involved in both the synthesis of cinnamic acids and flavonoids. Since significant increases in the accumulation of cinnamic acids result from phenylalanine treatment with no changes in the amount of PAL present, it is unlikely that PAL is normally

the rate limiting step in cinnamic acid biosynthesis. The data are more consistent with the idea that the supply of phenylalanine is normally rate limiting. Reducing cinnamic acid synthesis however creates a condition where cinnamic acids are rate limiting for flavonoid synthesis.

The second enzyme which was investigated was the cinnamic 4-hydroxylase (CH) catalyzing the conversion of cinnamic acid to *p*-coumaric acid.[24, 25] This enzyme requires molecular oxygen for activity so it was reasoned that by making leaf disks anaerobic the turnover number of the hydroxylase would be lower. Not wishing to become involved with the effect of long term anaerobic conditions on plant metabolism, initial experiments reported in Fig. 1 were carried out using small amounts of radioactive phenylalanine for short

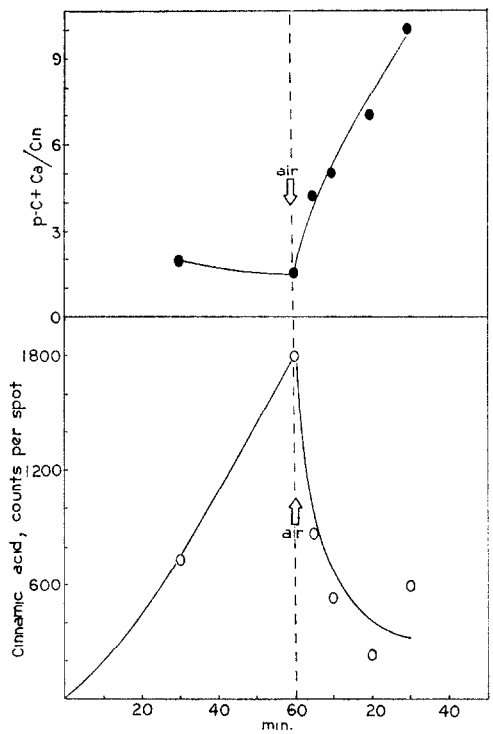

FIG. 1. THE EFFECT OF OXYGEN ON THE ACCUMULATION OF ^{14}C-CINNAMIC ACID RESULTING FROM FEEDING ^{14}C-PHENYLALANINE TO LEAF DISKS IN THE DARK AT 25°.

The leaf disks were pretreated with light for several days. Air was introduced by releasing the vacuum and then opening the dishes. ○—○ Cinnamic acid, ●—● the activity of *p*-coumaric acid plus caffeic acid divided by the activity of cinnamic acid.

periods. The disks were pretreated with light and carbon dioxide for several days and then transferred to anaerobic conditions in the dark and supplied with 1·6 nmoles of phenylalanine-U-^{14}C/cm^2. The time of sampling was measured after supply of the phenylalanine. After 1 hr of anaerobic conditions, air was introduced and the frequency of sampling increased. The cinnamic acid increased rapidly in the tissue while oxygen was withheld presumably because of the lack of turnover of the hydroxylase enzyme. Upon introduction

[24] D. W. RUSSEL and E. E. CONN, *Arch. Biochem. Biophys.* **122**, 256 (1967).
[25] N. AMRHEIN and M. H. ZENK, *Naturwissenschaften* **55**, 394 (1968).

of air the amount of radioactive cinnamic acid decreased rapidly even though the disks were still in contact with radioactive phenylalanine. Measuring the radioactivity of *p*-coumaric acid and caffeic acid and determining the ratio between the total of the hydroxylated and non-hydroxylated cinnamic acids revealed that the ratio stayed rather constant and low in the absence of oxygen and increased rapidly on the introduction of air. This increase was due to a rapid disappearance of cinnamic acid and an increase in the activity of *p*-coumaric acid. Since the activity of PAL in samples of these leaf disks was 35 nmoles cinnamic acid hr/cm^2 at 25°, the phenylalanine seemed to be taken up at a rate faster than it could be metabolized (since the radioactivity of phenylalanine increased throughout the experiment) and the initial rate of change in the ratio of hydroxylated cinnamic acids to non-hydroxylated cinnamic acid upon introduction of air was so great, the turnover of the hydroxylase must be several times that of the ammonia-lyase.

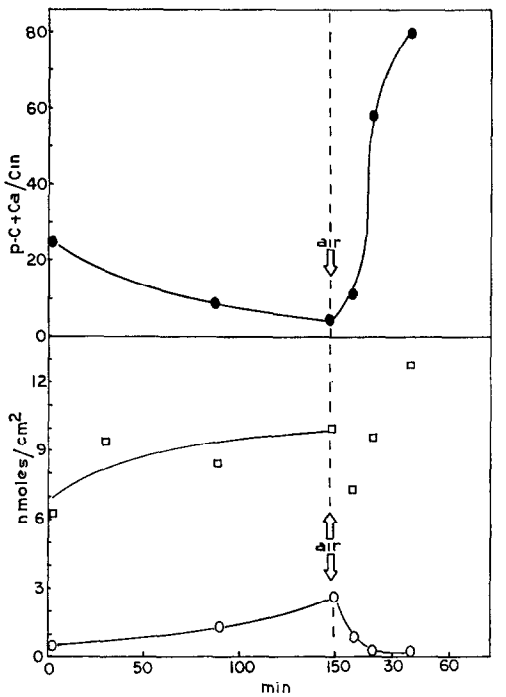

FIG. 2. THE EFFECT OF OXYGEN ON THE CONTENT OF CINNAMIC AND *p*-COUMARIC ACIDS AND ON THE RATIO OF HYDROXYLATED TO NON-HYDROXYLATED CINNAMIC ACIDS IN LEAF DISKS IN THE DARK AT 25°. The disks were pretreated for 24 hr at 22° in the light (2·5 mW/cm^2). ○—○ Cinnamic acid, □—□ *p*-coumaric acid, ●—● total amount of *p*-coumaric and caffeic acid divided by the amount of cinnamic acid.

The experiment reported in Fig. 2 shows that the changes occurring upon initiation of anaerobic conditions are sufficient to measure chemically. The accumulation rate of cinnamic acid was only about 1 nmole/cm^2/hr which is less than the PAL activity expected in these tissues. The initial rate of disappearance of cinnamic acid in air is over 12 nmoles/cm^2/hr. This would suggest that these tissues which were supplied only with water, carbon dioxide and light for a 24-hr pretreatment had built up considerably greater capacity to hydroxylate

cinnamic acid than capacity to form cinnamic acid and the hydroxylase is not a controlling step in cinnamic acid biosynthesis.

The leaf disks in the next experiment, reported in Fig. 3, were pretreated for 24 hr with light, carbon dioxide and 10^{-2} M L-phenylalanine. This resulted in a large accumulation of both cinnamic acid and *p*-coumaric acid at the beginning of the dark anaerobic conditions.

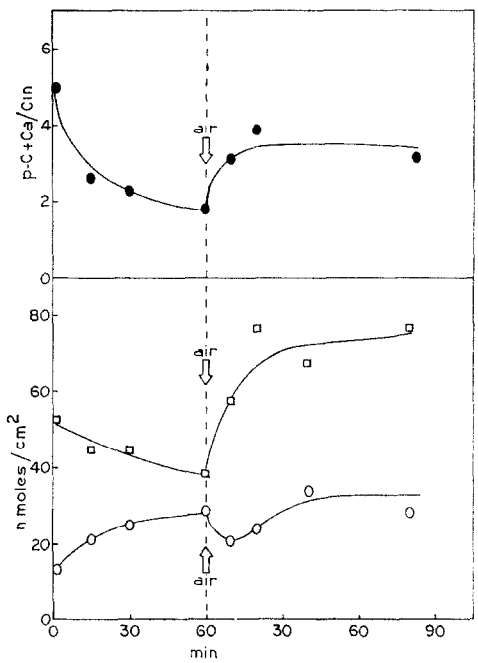

FIG. 3. THE EFFECT OF OXYGEN ON THE CONTENT OF CINNAMIC AND *p*-COUMARIC ACIDS AND ON THE RATIO OF HYDROXYLATED TO NON-HYDROXYLATED CINNAMIC ACIDS IN LEAF DISKS IN THE DARK AT 25°. The disks were pretreated for 24 hr at 22° with 10^{-2} M L-phenylalanine in the light (2·5 mW/cm²). ○—○ Cinnamic acid, □—□ *p*-coumaric acid, ●—● amount of p-coumric and caffeic acid divided by the amount of cinnamic acid.

The initial accumulation rate (anaerobic) of cinnamic acid was 35 nmoles/cm²/hr although this decreased considerably as the high levels of cinnamic acid were accumulated. Upon the introduction of air, there was a transient drop in the level of cinnamic acid followed by an increase. Under these conditions of high amounts of phenylalanine, the activity of the hydroxylase is not in great excess and cannot hydroxylate cinnamic acid at a rate greater than cinnamic can be produced. The transient changes are initiated by temporary effects of the high levels of cinnamic acid accumulated during the brief anaerobic treatment. This tends to support the idea that phenylalanine is rate limiting normally but PAL or CH can become rate limiting under conditions of high phenylalanine.

The ratios of hydroxylated cinnamic acids to cinnamic acid for these two experiments are also given in Figs. 2 and 3. The presence of a large excess of phenylalanine reduces the ratio considerably because of the proportionately greater stimulation of cinnamic acid accumulation than *p*-coumaric and caffeic acid accumulation. The tendency for a decrease in

the ratio during anaerobic conditions is consistent with the fact that cinnamic acid increases. The increase in ratio is an expression of the introduction of oxygen into cinnamic acid.

These experiments indicated that the cinnamic hydroxylase of strawberry leaf disks is not a rate limiting step in the biosynthesis of cinnamic acids. The changes in flavonoids during these brief anaerobic conditions are too small to be useful in interpreting the regulatory role of the hydroxylase in flavonoid biosynthesis. The results do support the idea that ammonia-lyases are obigatory enzymes in the biosynthesis of cinnamic acids in strawberry leaves.

EXPERIMENTAL

Plant material. Strawberry plants (*Fragaria vesca* var. Alpine) were grown in the greenhouse at Ithaca, New York. Leaf disks (1 cm dia.) were cut from the lamina of freshly cut leaves and floated on solutions with the lower epidermis upward.

PAL-assay. Acetone powders were prepared from frozen leaf disks and the assay carried out as previously described.[6]

Flavonoid measurement. The measurement of catechin, biflavan and flavolan were carried out as previously described.[26] Flavonols were measured in aqueous samples cleared of chlorophyll, by determining the difference spectra of the samples with and without added aluminium chloride. These were compared to spectra of authentic flavonols of known concentration.

Cinnamic acid measurement. At the time of sampling, leaf disks for acid measurement were frozen in liquid N_2. When the analysis was begun, the tubes were transferred to a N_2 atmosphere and N NaOH was added. The samples were left to hydrolyze overnight and then were neutralized with HCl and finally buffered to pH 5 with citrate. An aliquot was passed through a column of Polyclar AT (GAF Corp.) followed by washing with several column volumes of water. The combined effluents were acidified with HCl and extracted with Et_2O. The extract was dried and taken up in MeOH of which an aliquot was applied to silica gel TLC plates and developed in toluene–ethyl formate–formic acid (5:4:1 v/v). The spots corresponding to *p*-coumaric and caffeic acids were scraped from the plate and eluted from the powder. The spectra of these samples were determined vs. appropriate blanks and were compared with standards given similar treatment. A second aliquot of the Et_2O extract was applied to cellulose TLC plates and developed with *n*-BuOH–2 N NH_4OH (1:1, use upper layer). The spot corresponding to cinnamic acid was scraped from these plates and treated as already described for the other acids.

Radioisotope measurements. Phenylalanine-U-^{14}C of high specific activity (383 mc/m mole) was purchased from New England Nuclear Corp., Boston, Massachusetts. The phenylalanine was supplied to the leaf disks at 1·6 nmoles/cm^2 (0·62 μc/cm^2) in a volume of 0·04 ml/cm^2. The samples were handled exactly like those for measurement of cinnamic acids. When the acids had been separated on the TLC plates, the radioactive spots were scanned with a Berthold Radiochromatogram Scanner and the activity of each spot determined by integration of the spot with the same instrument.

Anaerobic conditions. The reduction in total pressure of O_2 was accomplished by the holding of the leaf disks at aspirator vacuum in specially designed dishes. The approximate drop in pressure of O_2 from this technique would be from 160 mm Hg to about 6 mm Hg. Additional reductions in O_2 were effected by the inclusion of a proprietary O_2 absorber (Seez-O_2) or by an initial flushing of the dish with N_2 before reducing the pressure.

[26] L. L. CREASY and T. SWAIN, *Phytochem.* **5**, 501 (1966).