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THE ROLE OF PHENYLALANINE IN FLAVONOID BIOSYNTHESIS

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Abstract—The incorporation of ^{14}C-labelled L-phenylalanine and sucrose into caffeic acid in sunflower discs has been determined under a number of conditions which affect the net rate of synthesis of the latter compound. The results indicate that L-phenylalanine may not be an obligate precursor in the biosynthesis of phenylpropanoid compounds, including flavonoids, in higher plants.

INTRODUCTION

IT IS generally agreed that L-phenylalanine, or perhaps in certain plants L-tyrosine, is an obligatory intermediate in the biosynthetic transformations which lead from shikimic acid to the hydroxycinnamic acids, the coumarins, and the phenylpropanoid moieties of the various classes of flavonoids and the lignins. The evidence for this opinion has been ably reviewed by Neish,[1] Grisebach[2,3] and others[4] and rests on the following well-documented facts. ^{14}C-labelled L- (or DL)-phenylalanine is incorporated, usually in relatively good radioactive yield (low dilution), into a wide variety of phenylpropanoid compounds (we include here the flavonoids in this definition) in a variety of diverse plant taxa.[1-4] Phenylalanine ammonia-lyase (PAL), which irreversibly catalyses the conversion of L-phenylalanine into *trans*-cinnamic acid, is widely distributed in the plant kingdom and has been shown to increase in amount under circumstances where the overall quantity of phenylpropanoid compounds is also enhanced.[5-13] Finally, the majority of the enzymes involved in individual steps in the conversion of shikimate to L-phenylalanine have been demonstrated in plants,[14] along with enzymes catalysing the hydroxylation of the aromatic ring to yield the commonly occurring 4-hydroxy-[15,16] and 3,4-dihydroxy-[17] cinnamic acids, *p*-coumaric and caffeic

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acids. The incorporation of these latter compounds into various flavonoids and lignins has been demonstrated, although there is still some question as to whether the final oxygenation pattern of ring B of the flavonoids is determined at the cinnamate[18] or at a C_{15} intermediate stage.[19]

Nevertheless, the results of certain simple experiments that we have carried out on the biosynthesis of caffeic acid conjugates in sunflower leaves (*Helianthus annuus* L.) and potato tubers (*Solanum tuberosum* cv. Majestic) have led us to examine the above evidence in more detail. As a result, we concluded that phenylalanine might not be an obligate precursor of the phenylpropanoid moiety in flavonoids under all circumstances. The evidence on which this conclusion is based is presented below.

RESULTS

Our investigations were aimed at determining the way in which individual enzyme-catalysed steps in the aromatic pathway to caffeic acid were controlled in higher plants. Recent research has shown that the pathway to aromatic amino acids in micro-organisms and in higher fungi is subject to varying types of end-product or feedback inhibition.[20,21] In some organisms multiple forms of certain of these enzymes exist[22] and in other groups of the enzymes occur as inseparable protein aggregates.[23]

We first examined the effect of various presumed precursors and light on the net rate of synthesis[24] of the caffeic acid conjugates (mainly chlorogenic acid) in discs of sunflower leaves and potato tubers floating on water. As expected, we found that both light and sucrose greatly enhanced the synthesis of caffeic acid in either tissue (Figs. 1 and 2). The effect of white light on chlorogenic acid biosynthesis in potato tubers has been correlated to changes in the level of PAL;[7] several other enzyme activities are also enhanced but the nature of the photoreceptor is unknown. It is of interest, therefore, to note that we found differences between the potato and the sunflower discs with two inhibitors of protein synthesis. Whereas cycloheximide ($3{\cdot}5 \times 10^{-5}$ M) expectably[25] inhibited chlorogenic acid formation strongly in both tuber and leaf, chlorotetracycline (aureomycin) at 5×10^{-5} M completely abolished biosynthesis of the acid only in the potato and had no effect in the sunflower discs. We also found differences with DCMU (3-(3′,4′-dichlorophenyl)-1,1dimethylurea). At 10^{-4} M, this herbicide inhibited the amount of chlorogenic acid formed in the tuber discs by 20–25% in both light and dark, 10^{-5} M concentrations having no effect. In contrast, inhibition of chlorogenic acid formation by DCMU in sunflower leaf discs only occurred in the light, being 47 and 19% with 10^{-4} and 10^{-5} M DCMU respectively. This latter result was expected, since DCMU is known to inhibit photosystem II in the chloroplasts.[26] These differences in the behaviour of potato tuber and sunflower leaf disc tissue warrant further investigation. One other interesting feature which emerged was that the relative rates of synthesis of chlorogenic acid in tuber discs from three different cultivars of potato were approximately correlated with their initial content of the acid. Thus, Pentland Falcon (420 μg/g fr. wt. of chlorogenic acid), Majestic (106 μg/g) and Maris Page (30 μg/g) had rates of synthesis of chlorogenic acid on 0·15 M sucrose solution of 3·6, 1·8 and 0·2 μg/g/hr respectively. Since there was no shortage of elementary

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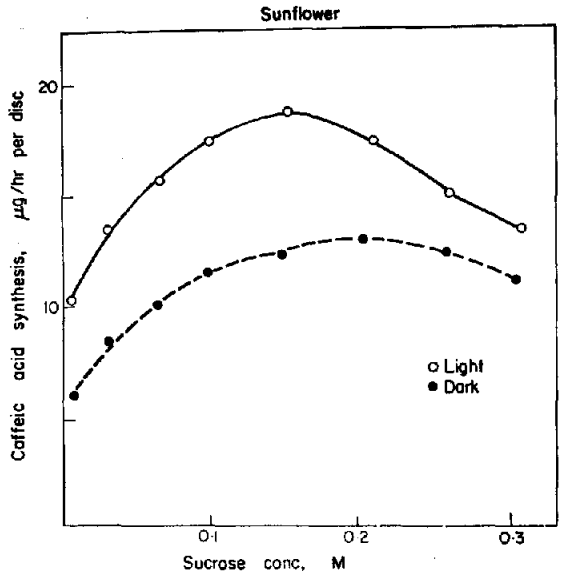

FIG. 1. THE EFFECT OF LIGHT AND SUCROSE ON THE SYNTHESIS OF CAFFEIC ACID IN SUNFLOWER LEAF DISCS.

Abscissa: Sucrose concentration (M).
Ordinate: Chlorogenic acid, μg/disc/hr.
○——○ Light, ●——● Dark.

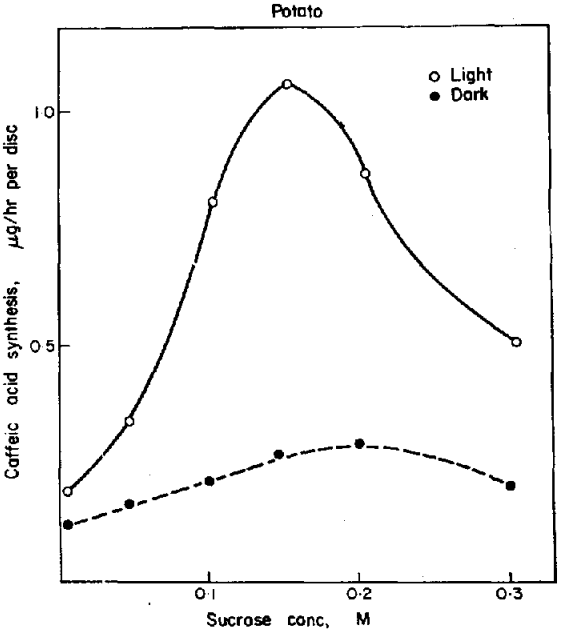

FIG. 2. THE EFFECT OF LIGHT AND SUCROSE ON THE SYNTHESIS OF CAFFEIC ACID IN POTATO TUBER DISC.

Abscissa: Sucrose concentration (M).
Ordinate: Chlorogenic acid, μg/disc/hr.

substrate (sucrose) in this experiment it would appear that the net rate of synthesis of the cinnamic acid in the tuber is, as would be expected,[27] under tight control in each cultivar. The effect of other precursors on the rate of synthesis of caffeic acid in potato and sunflower is shown in Table 1. It can be seen that quinic, shikimic and cinnamic acids all stimulate the

TABLE 1. EFFECT OF VARIOUS PRECURSORS ON THE RATE OF SYNTHESIS OF CAFFEIC ACID IN SUNFLOWER AND POTATO

Precursor added	% Increase*	
	Sunflower	Potato
Sucrose, 0·15 M light	110	310
dark	120	110
Quinic acid, 10 mM	35	95
Shikimic acid, 1 mM	95	320
Phenylalanine, 0·5 mM	−5	−70
+ 0·15 M sucrose	55	
Cinnamic acid, 1 mM	90	130
p-Coumaric acid, 0·5 mM	−12	
+ 0·15 M sucrose	−5	

* Of the rate of synthesis of caffeic acid (μg/g/hr) over the appropriate control. All experiments were carried out in the light (600 lx) for 48 hr unless otherwise stated.

synthesis of caffeic acid in both tissues. L-Phenylalanine and *p*-coumaric acid, on the other hand, gave a slight depression of synthesis in the sunflower leaf on water, and phenylalanine strongly depressed caffeic acid formation in the potato. The addition of the amino acid to leaf discs floating on 0·15 M sucrose increased caffeic acid synthesis, but *p*-coumaric acid, under these circumstances, again showed a slight depression.

We decided next to measure the rate of incorporation of L-phenylalanine-U-^{14}C into caffeic acid in sunflower leaf discs under several varied conditions which altered the net rate of synthesis of the cinnamic acid in the tissue. We assumed that after the steady-state conditions for synthesis had been established in the discs, the amount of radioactivity incorporated from an applied labelled obligate precursor should be a constant fraction of the product being synthesized and thus result in the compound having constant specific activity. We further argued that under two conditions where the net rate of synthesis of caffeic acid was widely different, the incorporation of any obligate precursor should be comparably different. On the basis of the results given above, such differences in synthesis could be accomplished in several ways; for example by exposing the discs either to light or to dark, either to 0·15 M sucrose or to water, and so on.

We first tested these ideas using sucrose-U-^{14}C as the precursor, adding it to discs floating either on unlabelled 0·15 M sucrose or on water. We had previously shown that the net rate of synthesis of caffeic acid under either of these conditions was roughly constant over 96 hr. The results are shown in Fig. 3. At the end of this period, 50–70% of the initial sucrose U-^{14}C was still available in solution.

It can be seen (Fig. 3) that the total activity incorporated from the labelled sugar into caffeic acid parallels almost exactly the net amount of the product synthesized under the two

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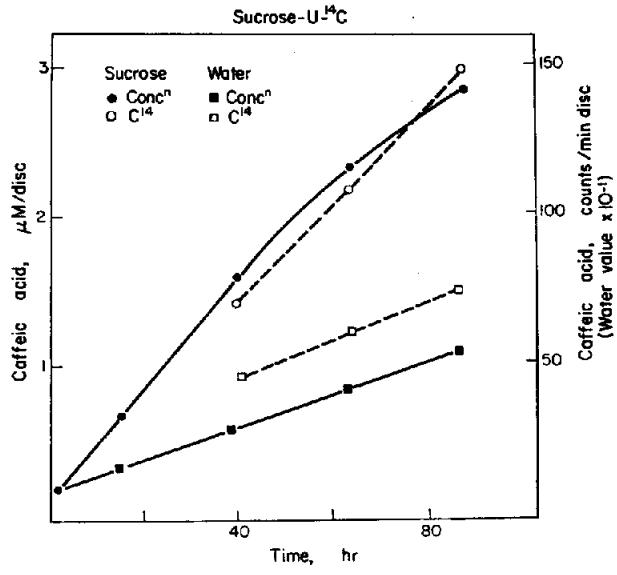

FIG. 3. THE INCORPORATION OF SUCROSE-U-^{14}C INTO, AND RATE OF SYNTHESIS OF CAFFEIC ACID IN SUNFLOWER LEAF DISCS.

Abscissa: Time (hr).
Ordinate: Left, caffeic acid, μM/disc.
Right, activity in caffeic acid, cpm/disc.
Concentration on ●——●sucrose and ■——■ water.
^{14}C incorporation on ○ - - - ○ sucrose and □ - - - □ water.

different conditions. The specific activity of the caffeic acid isolated from discs thus remained roughly constant with time. On 0·15 M sucrose, it was 44, 48 and 55 counts/min/μM after 40, 64 and 88 hr, while, on water, the specific activity was expectedly higher (since there was no unlabelled sucrose-diluting incorporation) and was 760, 650 and 710 counts/min/μM at the same time intervals. Furthermore, the activity in the caffeic acid as a percentage of that in the whole methanol extract remained constant with time, being 3·03 ± 0·09% on sucrose and 5·12 ± 0·3% on water over the whole time period.

Application of the same technique to L-phenylalanine-U-^{14}C, however, gave equivocal results. The results of one of the experiments where L-phenylalanine was added to leaf discs on either water or 0·15 M sucrose is shown in Fig. 4. In neither case is there a correspondence between synthesis and incorporation of labelled precursor. This experiment was stopped after 47 hr because the L-phenylalanine-U-^{14}C in the sucrose solutions had fallen below 30% of the initial value. It should be noted that the total amount of activity in the methanol extract of the discs increased greatly with time under both sets of conditions, so there is no doubt that the labelled amino acid was getting into the cells. This is reflected by the fall with time in the activity in the caffeic acid as a percentage of that in the whole extract: on sucrose, this fell from 2·5 to 1·0% from 29 to 47 hr and on water from 1·3 to 0·9% over the same period. Two other sets of experiments were carried out with L-phenylalanine-U-^{14}C, both involving the variation in the synthesis of caffeic acid in leaf discs in light and dark. In one of these, relative incorporation into the cinnamic acid was abnormally high, reaching 26% of the total activity in the soluble fraction in the light after 72 hr. Furthermore, the amount of L-phenylalanine taken up was only 30% of that observed previously, and more of the amino

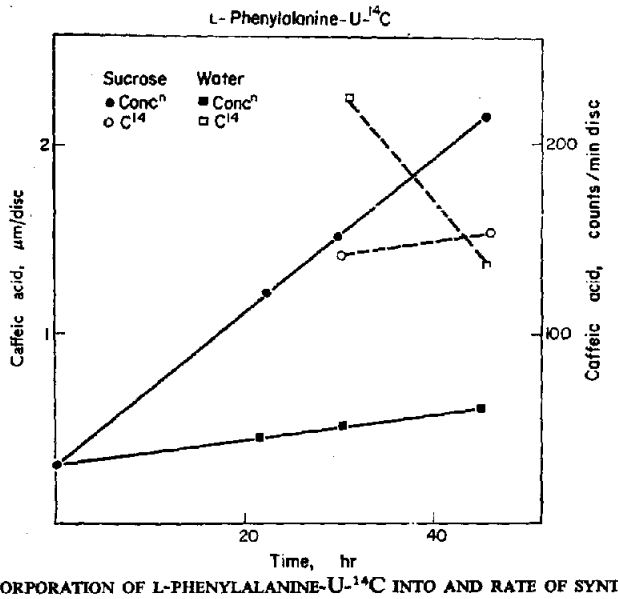

FIG. 4. THE INCORPORATION OF L-PHENYLALANINE-U-^{14}C INTO AND RATE OF SYNTHESIS OF CAFFEIC ACID IN SUNFLOWER LEAF DISCS.

Abscissa: Time (hr).
Ordinate: Left, caffeic acid, μM/disc.
Right, activity in caffeic acid, cpm/disc.
Concentration in ●——● Sucrose and ■——■ water.
^{14}C incorporation on o - - - o sucrose and □ - - - □ water.

acid was incorporated into the insoluble residue. As a result the specific activity of the caffeic acid, in the light-treated discs, actually increased with time. In the second set of light experiments, the total amount of activity in the soluble fraction was of the same order as that in sucrose experiments with phenylalanine (Fig. 4). In both light and dark, however, there was a roughly 20% fall in the specific activity of caffeic acid between 50 and 74 hr.

DISCUSSION

Our own data are capable of several different interpretations. It is possible that there are major differences between sucrose and phenylalanine in the time required to reach a steady state in either their uptake, their entry into the active pool, or in the control of various rates of their metabolism. The feedback control of caffeic acid biosynthesis and turnover may also be subject to greater variations at the post-aromatic level than at the elementary precursor level. Thus we do not claim that these results unambiguously determine whether phenylalanine is an obligate precursor of caffeic acid and related flavonoid compounds or not. Nevertheless, we feel that they do indicate that the role of phenylalanine in flavonoid biosynthesis needs to be re-examined more critically.

This belief is reinforced by our examination of some of the earlier data. We first looked at the reported relative incorporation of shikimic acid and phenylalanine (or cinnamic acid) into various hydroxylated phenylpropanoid compounds (e.g. piceid,[28] quercetin,[29] delphinidin[30] and tea catechins)[31] and found, surprisingly, that the alicyclic acid was often the better

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precursor as judged by dilution values. One would expect that, all things being equal, the nearer a precursor is to the final product, the better would be the incorporation. One cannot be certain that uptake, transport and pool sizes will be equivalent for different compounds under test, but great differences in incorporation as found for delphinidin[30] and the tea catechins[31] indicate that other explanations may be required. In this case it seems probable, as we have suggested earlier,[32,33] that there may be routes to the ubiquitously hydroxylated phenylpropanoid moieties in flavonoids which do not involve the non-hydroxylated compounds, phenylalanine and cinnamic acid. Indeed, both Grisebach and Barz,[3] and Zaprometov and Bukhlaeva[31] have recently supported our suggestion.

We next examined the reported relationship between the increase in PAL activity and phenolic biosynthesis occasioned by light in several different plant species.[8-11,13,33,34] Unfortunately, it is not readily possible to directly compare the results from these various experiments, as they are not all expressed on the same basis (Table 2). However, it can be

TABLE 2. THE EFFECT OF LIGHT ON THE ACTIVITY OF PAL AND THE RATE OF SYNTHESIS OF PHENOLIC COMPOUNDS IN SEVERAL PLANT TISSUES

Tissue	Unit	Time (hr)	Activity or rate of synthesis* (μM/hr/unit) PAL L	PAL D	Phenolics† L	Phenolics† D	Ref.
Pea buds	g fr. wt.	10	0·24	0·04	0·023	0	9, 34
Gherkins seedlings	hypocotyl	6	42·0	6·2	5·64	0·37	13
Buckwheat seedlings	g fr. wt.	12	4·2‡	0·75‡	0·076	0·004	10
Strawberry leaf	g fr. wt.	65	3·0		3·2		8
Mustard seedlings	1000 shoots	24	6·0	0·72	6·7		11, 35

* Calculated from figures and graphs given in the references quoted. The accuracy of these calculations cannot, therefore, be guaranteed to more than ±10. L is light and D dark.

† Total phenols where given: Quercetin and kaempferol glycosides in the pea; hydroxycinnamic acids in gherkin; leucocyanidin, rutin and chlorogenic acid in buckwheat; leucoanthocyanins (+) catechin, cinnamic acids and cyanidin glycosides in strawberry.

‡ Assuming 25 mg protein/g fr. wt.

seen from our calculated values that in the first three examples given the activity of PAL in the dark-grown tissue is sufficient to account for the increase in the rate of synthesis in the light. In the other two examples, strawberry and mustard, the activity of PAL even after light-induced stimulation is not quite sufficient to account for the rate of synthesis of the phenolic compounds in the light. Again, there are many difficulties in interpreting such data, including the fact that in most cases the estimation of enzyme activity was carried out using acetone powders and so variable amounts of the activity may have been lost. Nevertheless, it appears that although there is a reasonably good qualitative correspondence between increases in the activity of PAL and the rate of synthesis of soluble phenolic compounds in the tissues on exposure to light, the two phenomena may not be directly related. The deamination of phenylalanine can lead equally to the formation of lignin[6] as to the flavonoids and both pathways will

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need to be under control in the cells. We have shown that in mung bean there are two distinct forms of PAL and the activity of these forms is affected differently by light.[36] We suggested that these forms of PAL were perhaps allosteric,[7] one being responsible for the lignin pathways and one for the flavonoid pathway. If this is the case, it is not surprising that there will be wide variations between the total activity of all forms of PAL and the rate of synthesis of the soluble phenylpropanoid compounds.

The above evidence, like our own experiments, casts some doubt on the place of phenylalanine as an obligate precursor[37] of cinnamic acids and flavonoids in all plants under all circumstances. It is possible that in many cases it is merely a readily available reserve substance which is called into play under conditions where rapid synthesis of lignin and flavonoids are called for; for example, when dark-grown plants are illuminated. At other times, perhaps presently unknown pathways from shikimate to the flavonoids are more important. We are only just beginning to understand the intimate relationships involved in the control of the biosynthesis of secondary plant products; however, and it is certain that many further experiments will be required to elucidate the exact role of phenylalanine in the biosynthesis of phenylpropanoid compounds.

EXPERIMENTAL

Materials

Phenylalanine-U-^{14}C and sucrose-U-^{14}C were obtained from the U.K.A.E.A., Amersham, Bucks. Potato tubers of various cultivars were obtained from the same experimental plots grown in the field of the Plant Breeding Institute, Cambridge. Sunflower leaves were taken from 6-week-old plants grown in 16-hr days under 2000 lx at 25° in a growth chamber.

Other compounds were commercial samples which, where necessary, were recrystallized until chromatographically pure.

Methods

Radioactivity was measured with an end-window geiger counter fitted with a co-incidence corrector (background 2·4 counts/min). Plant materials were extracted with MeOH and the MeOH extracts, after concentration, were hydrolysed with 0·1 N NaOH *in vacuo* under N_2 for 1 hr, acidified with N H_2SO_4 and the caffeic acid produced extracted with Et_2O. The Et_2O extract was separated chromatographically on paper in BAW (6:1:2), eluted and the caffeic acid determined spectroscopically. In radioactive experiments, the compound was further purified to constant specific activity in 2% HOAc and benzene–HOAc–H_2O (6:7:3, v/v/v).

Feeding Experiments

Discs (1 cm dia., 10–12) of either potato tuber (1 mm thick) or sunflower leaf were supported by a porous plastic plate at the surface of the various solutions (5 ml) given in Table 1, in petri dishes (5 cm dia.). It was found that evaporation over 96 hr was minimal. Radioactive compounds were added to the dishes with adequate stirring, as appropriate.

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