Kinetin-Mediated Stimulation of Accumulation of Buckwheat Flavonoids in the Dark

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A short treatment of excised buckwheat cotyledons with a solution of kinetin lead to an up to 9-fold stimulation of anthocyanin biosynthesis, to an about 50 percent increase in the accumulation of rutin, and to an about 30 percent increase, on the average, in the accumulation of C-glycosylflavones in the treated material during its posttreatment incubation in the dark. When the treated cotyledons were incubated in a solution of L-phenylalanine anthocyanin accumulation in the dark practically attained the same high level as it was observed in the illuminated cotyledons fed with exogenous L-phenylalanine. In experiments with ^{14}C-labelled L-phenylalanine kinetin induced a sharp rise in the labelling (resp. in the utilization of exogenous substrate for biosynthesis) of anthocyanins and rutin in the dark and a slight increase in the radioactivity of C-glycosylflavones. Similar labelling changes occurred in the illuminated cotyledons. However, both kinetin and light still more effectively promoted biosynthetic use of the endogenous substrate. As a result the relative portion of flavonoids formed from exogenous L-phenylalanine under these conditions showed a decrease as compared with the ratio of precursor use in the untreated cotyledons. The results show that low accumulation rates of anthocyanins and other flavonoids in the dark are conditioned by the limited access of substrate (L-phenylalanine) molecules to the flavonoid enzymes lending further support to the idea that flavonoid biosynthesis is normally controlled at the substrate rather than at the enzymic level.

Introduction

It is becoming increasingly evident that a change in the structure and properties of cell membranes is the first or an early step in the photocontrol of plant growth, development, and metabolism including biosynthesis of flavonoids [1, 2]. Resulting from treatments with kinetin, *n*-propanol, dimethylsulfoxide and other reagents which are believed to increase membrane permeability, considerable stimulation of anthocyanin formation was obtained in dark-grown material [3 – 10], exogenous shikimic and cinnamic acids promoted intense accumulation of anthocyanins in red cabbage seedlings in the dark while remaining without effect in the untreated material [4], and incorporation of exogenous L-phenylalanine into phenolic units of poplar xylem lignin in the dark became identical to that in the light [11]. By contrast, Ca^{2+} ions, which are known as membrane stabilizers, reduced or completely prevented the effects of kinetin and *n*-propanol as well as the similar effects of the light [6, 11].

These data suggest that light acts through the modification of membrane permeability, improving conditions for intracellular transport and facilitating passage of substrate molecules through membrane barriers to the site of flavonoid biosynthesis. Further they allow to conclude that the main limitation to the formation of flavonoids in plant material unexposed to light is substrate availability rather than the activity of enzymes involved in that biosynthesis (see also [12 – 14]).

In this paper new supporting evidence for the limiting role of substrate availability in the synthesis of flavonoids in dark-grown material is presented. Using preliminary short treatments with kinetin and subsequent incubation of material in a solution of L-phenylalanine we were able to show that in buckwheat tissues with increased membrane permeability both endogenous and exogenous substrates became much more accessible for flavonoid biosynthesis resulting in that the accumulation of these compounds in the dark could practically reach the level characteristic of their production under continuous illumination.

Materials and Methods

Plant material and treatment procedures

The experiments were carried out with isolated buckwheat (*Fagopyrum esculentum* Moench) cotyle-

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dons excised from 80 h old etiolated seedlings grown in water. The excised material was soaked for 5–30 min in a saturated solution of kinetin, was then rinsed with water and thereafter transferred to a dark chamber for a 40 h incubation on filter paper moistened with water or with a 10^{-2} M solution of L-phenylalanine. In control material instead of treatment with kinetin a similar treatment with distilled water was used. Excision of cotyledons and the following soaking procedures were performed under dim green safelight. In some series of experiments a portion of similarly treated cotyledons was incubated under continuous illumination (40 h). For illumination white fluorescent tubes were employed, light intensity – 28000 erg · cm^{-2} · s^{-1}. The temperature was held constant at 25 ± 1 °C both in the dark as well as in the light.

In preliminary experiments the influence of *n*-propanol (1%), dimethylsulfoxide (1.5%) and EDTA (0.5 mM) was also tested, and the effect of kinetin on the accumulation of anthocyanins in intact buckwheat seedlings was studied. In the latter case growth medium (water) of the 56 h old etiolated seedlings was for 5–15 min replaced by or the entire seedlings were for 10 min totally submerged into a solution of kinetin after which the treated seedlings were allowed to continue their growth in the dark in water.

All experiments were run in 3 to 5 replications in space per treatment and per variant of incubation and were also replicated in time on at least 3 occasions. The results were subjected to evaluation by the statistical techniques of Student's significance test.

Flavonoid assay

Anthocyanins were determined photocolorimetrically by measuring the optical density of clear 1% HCl-ethanolic extracts from plant material in a photoelectric colorimeter using a green filter with maximum transmission at 540 nm. Rutin and C-glycosylflavones were determined by two-dimensional ascending paper chromatography combined with a subsequent measurement of the optical density of the ethanolic eluates of individual flavonoid spots spectrophotometrically at 360 nm, 350 nm, and 335 nm for rutin, luteolinic C-glycosylflavones orientin and isoorientin, and their apigeninic analogues vitexin and isovitexin, respectively. For chromatography Filtrak FN-11 paper and solvent systems (1) isoamyl alcohol/petrol ether/acetic acid/water (3:1:3:3, v/v/v/v, upper phase) and (2) 10% acetic acid were used. Flavonoid spots were detected under UV light using exposure of chromatograms to ammonia vapours [15]. The content of flavonoids was expressed in nmols per seedling using for calculations the following extinction coefficients: anthocyanins 2.7×10^4 [16], rutin – 1.40×10^4, orientin and isoorientin – 1.59×10^4, vitexin and isovitexin – 1.94×10^4 [15].

Isotope experiments

For labelling of the initial substrate a preparation of [1-^{14}C]-D,L-phenylalanine was added to incubation solutions containing this amino acid. The specific activity of L-phenylalanine in the resulting labelled solutions was 0.25 Ci/mol.

Radioactivity of individual flavonoids was assayed after two-dimensional paper chromatography of plant extracts, either directly on flavonoid spots cut off from the developed chromatograms or in their ethanolic eluates. In the case of anthocyanins the elution was carried out with ethanol acidified to 1% with HCl. For chromatographic separation of rutin and C-glycosylflavones the solvent systems mentioned above were used, chromatograms for anthocyanins were developed using solvent systems (1) *n*-butanol/acetic acid/water (4:1:5, v/v/v, upper phase) and (2) 30% acetic acid. Radioactivity of chromatogram spots was measured in a special device for direct paper radiometry furnished with a quadratic (5 × 5 cm) SBT-10 Geiger counter. Each spot was measured from both sides. For radioactivity assay of eluates a Beckman LS-100C liquid-scintillation counter was used.

Phenylalanine ammonia lyase (PAL) assay

The activity of PAL was determined by the amount of cinnamic acid formed in an assay mixture during its incubation for 4 h at 35 °C. The assay mixture consisted of 0.1 M borate buffer (2.7 ml; pH 8.8), 0.1 M solution of L-phenylalanine (0.1 ml), and a sample (0.2 ml) of enzyme extract prepared using 0.1 M borate buffer for homogenization of plant material [17].

Results

Preliminary experiments

n-Propanol (1%), dimethylsulfoxide (1.5%), and EDTA (0.5 mM) which were effective in promoting dark synthesis of anthocyanins in red cabbage [4, 6], mung bean [5], and mustard seedlings [10], and also in *Spirodela oligorrhiza* fronds [8], showed no measurable effect on anthocyanin accumulation in buckwheat seedlings. However, treatments with kinetin were highly stimulatory and brought about an increase in the accumulation of anthocyanins many times greater relatively than it was observed in plant material investigated by earlier authors. When the roots of intact seedlings were for 5 or 15 min kept in contact with a solution of kinetin an about 3- to 5-fold stimulation of anthocyanin formation was observed in the cotyledons during subsequent 40 h incubation of seedlings in the dark. A total submersion of intact seedlings into the treatment solution (for 10 min) gave rise to even a 7-fold increase in the accumulation of cotyledonary anthocyanins (Table I). In excised cotyledons a stimulation of about the same order of magnitude was obtained after kinetin treatment.

Marked pigmentation occurred in kinetin-treated cotyledons in the dark also in those cases when the material in the etiolated state showed no visible anthocyanins. By contrast, neither excised hypocotyls nor the hypocotyls of intact buckwheat seedlings, normally incapable of synthesizing anthocyanins when unexposed to the light, showed any positive response to treatment with kinetin or with the other compounds tested. A similar unresponsiveness to treatment with membrane-active compounds was reported also in the hypocotyls of etiolated mung bean seedlings [5].

Table I. Effect of kinetin treatment on the accumulation of anthocyanins in the cotyledons of intact buckwheat seedlings grown in the dark.

Treatment conditions	Anthocyanins, nmol/seedling	
	Control	Kinetin
Only roots treated with a solution of kinetin		
5 min	0.60	1.85
15 min	0.62	3.05
Seedlings totally submerged into kinetin solution		
10 min	0.58	4.12

Experiments with excised cotyledons

For the ease of manipulation all further experiments were carried out with excised cotyledons. The results obtained were in agreement with preliminary observations and indicated convincingly that limited biosynthesis of anthocyanins as well as of other buckwheat flavonoids in the dark was conditioned by insufficient availability of substrate materials while the enzymic apparatus involved was capable of operating with full efficiency without any light action. An improvement of conditions for transmembrane transport resulting from a short treatment with kinetin made possible a rise in the formation of anthocyanins in etiolated cotyledons equal to about 50–60 percent of the level of anthocyanin accumulation characteristic of excised buckwheat cotyledons under continuous illumination (Fig. 1). Since the activity of PAL did not show a comparable change in the treated material (data not presented; see also [4]) the rise observed could only be due to an increased access of endogenous substrates to the site of their biosynthetic use obviously hindered in the dark. L-phenylalanine supplied externally enhanced to some extent anthocyanin biosynthesis in the dark but had not the capacity to substitute light or kinetin treatment completely although at least a 30-fold increase in the pool of free L-phenylalanine could be expected

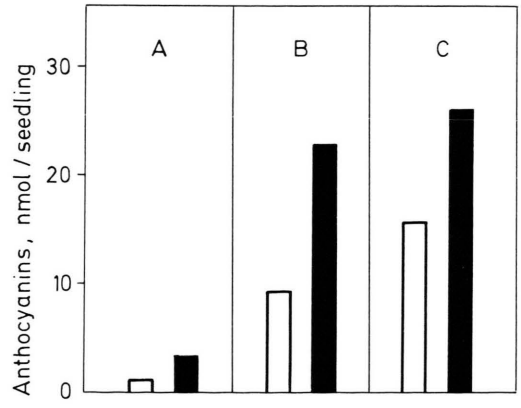

Fig. 1. Effect of kinetin treatment on the accumulation of anthocyanins in excised buckwheat cotyledons incubated for 40 h in the dark in water (white bars) or in a 10^{-2} M solution of L-phenylalanine (black bars). Duration of the treatment – 15 min (treatments for 5 or 30 min gave similar results). A – untreated material, incubation in the dark; B – material treated with kinetin, incubation in the dark; C – untreated material, incubation under continuous illumination.

to occur in cotyledons under these conditions [18]. However, in cotyledons with their membrane structures destabilized by kinetin the promoting effect of exogenous L-phenylalanine was about 6–7 times greater than in the untreated material resulting in that the accumulation of anthocyanins in the dark attained practically the same high level as it was observed in the illuminated material fed with L-phenylalanine (Fig. 1).

Rutin and especially C-glycosylflavones are synthesized in buckwheat cotyledons in quite a large amounts without any exposure to the light. In spite of that kinetin, similar to its effect on anthocyanin accumulation, promoted also formation of these flavonoids in the dark although the stimulation achieved was not more than 50 percent over the control level in the case of rutin and did not exceed a 30 percent level on the average in the case of C-glycosylflavones (Fig. 2). Exogenous L-phenylalanine slightly increased kinetin effect on rutin but was not able to produce an additional accumulation rise of C-glycosylflavones.

It must be noted that the relatively small stimulatory effect of kinetin on the accumulation of rutin and C-glycosylflavones in the dark as compared with its effect at the level of anthocyanins

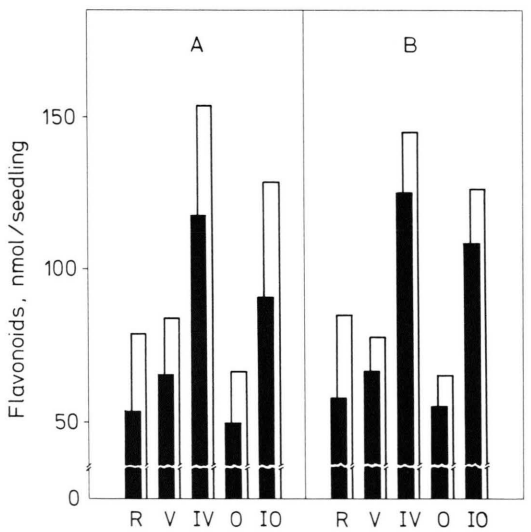

Fig. 2. Effect of kinetin treatment on the accumulation of rutin and C-glycosylflavones in excised buckwheat cotyledons incubated for 40 h in the dark in water (A) or in a 10^{-2} M solution of L-phenylalanine (B). Duration of the treatment – 15 min. Untreated material – black bars, kinetin-treated material – white bars. Rutin (R), vitexin (V), isovitexin (IV), orientin (O), isoorientin (IO).

Table II. Content and radioactivity of flavonoids in excised buckwheat cotyledons treated with kinetin[a] and incubated for 40 h in a 10^{-2} M solution of labelled L-phenylalanine.

Flavonoid	Incubation in the dark		Incubation under continuous illumination	
	Control	Kinetin	Control	Kinetin
Anthocyanins[b]	3.67 3100	23.3 7890	25.9 9683	82.1 17205
Rutin	80.5 11940	124 19178	148 28523	209 43088
Vitexin	98.9 9983	111 10403	107 11048	122 11865
Isovitexin	190 17730	210 16598	210 18525	231 20895
Orientin	70.8 11918	88.1 12825	88.1 14580	89.9 14430
Isoorientin	148 20678	167 21218	185 25485	181 24585

[a] Duration of the treatment – 15 min.
[b] Within the data on every separate flavonoid the content in nmols per seedling is presented in the upper row, the radioactivity in cpm per seedling in the lower one. Specific activity of labelled L-phenylalanine introduced to seedlings – 388 cpm/nmol.

corresponded actually to a very large change in terms of absolute units. The total absolute increase in the content of these flavonoids in the treated material incubated in water was equal to about 135 nmol per seedling (Fig. 2) while the respective increase in the content of anthocyanins did not exceed 8 nmol (Fig. 1). Thus, that increase, in fact, accounted for about 95 percent of the total overall increase in the absolute content of flavonoids generated in etiolated cotyledons by kinetin treatment.

Isotope experiments

Posttreatment incubation of material in a solution of ^{14}C-labelled L-phenylalanine made possible to follow changes in the availability of substrates for flavonoid biosynthesis in a more direct manner and, also, to differentiate between changes in the utilization of exogenous and endogenous substrates.

Substantial rise in the radioactivity of anthocyanins and rutin paralleling high increase in their absolute content in kinetin-treated cotyledons in the dark clearly showed that just the increased access of substrate materials to the site of biosynthesis of

these flavonoids was the main intracellular condition that was responsible for the stimulation observed in their accumulation after kinetin treatment (Table II). General accumulation levels of anthocyanins and rutin remained under these conditions only little lower than it was characteristic of cotyledons under continuous illumination. However, enzymatic potentialities for producing these flavonoids in cotyledons were still far from being exhausted. When the material treated with kinetin was exposed to the light, a sharp additional increase in the incorporation of exogenous L-phenylalanine into anthocyanins and rutin was observed and a similar or even a more distinct increase in the absolute content of both flavonoids occurred. Thus, kinetin proved to be able to increase substrate availability not only in the dark but also in the illuminated material creating, under sufficient supply of substrates, conditions for a better realization of the actual capacity of tissues for flavonoid synthesis.

Within the group of C-glycosylflavones a marked incorporation of label from exogenous L-phenylalanine occurred in the dark-incubated cotyledons without any treatment (Table II). When the cotyledons were subjected to the action of kinetin an up to 25 percent rise was found in the content of C-glycosylflavones but that, as a rule, was not accompanied by an increase in their radioactivity. Under conditions of continuous illumination only a slight but mostly insignificant tendency for an additional increase of the total amount as well as of the radioactivity of all four C-glycosylflavones could be observed in cotyledons irrespective of whether the material was or was not previously treated with kinetin.

An interesting aspect of these experiments was that changes in the radioactivity of flavonoids reflecting biosynthesis from exogenous L-phenylalanine did not fall quantitatively into line with the general changes in the accumulation of the respective flavonoids that under these experimental conditions, of course, comprised synthesis from

Table III. Comparison of kinetin and light effects on the production of flavonoids in excised buckwheat cotyledons during their incubation for 40 h in a 10^{-2} M solution of labelled L-phenylalanine.

Flavonoid	Origin of substrate	Incubation in the dark, untreated material		Incubation in the dark, kinetin-treated material[a]		Incubation in the light untreated material	
		Absolute production, nmol/seedling	% of total production	Absolute production, nmol/seedling	% of total production	Absolute production, nmol/seedling	% of total production
Anthocyanins	ex[b]	2.75[c]	~ 100	20.6	85.8	23.0	90.9
	end	~ 0		3.4		2.3	
Rutin	ex	30.7	70.1	49.4	56.9	73.4	66.1
	end	13.1		37.4		37.6	
Vitexin	ex	25.7	63.6	26.8	50.8	28.4	59.0
	end	14.7		26.0		19.7	
Isovitexin	ex	45.6	61.9	42.7	46.0	47.7	51.0
	end	28.1		50.2		45.8	
Orientin	ex	30.7	90.3	33.0	64.3	37.5	73.1
	end	3.3		18.3		13.8	
Isoorientin	ex	53.2	71.9	54.6	58.5	65.6	58.9
	end	21.3		38.8		45.8	

[a] Duration of the treatment – 15 min.
[b] Ex – exogenous substrate, end – endogenous substrate.
[c] Calculated by dividing the total radioactivity (cpm/seedling) found in a separate flavonoid by the specific activity of labelled L-phenylalanine (in this experiment 388 cpm/nmol) introduced to cotyledons. The production from endogenous substrate was calculated by substracting the value so found from the total amount of the corresponding flavonoid produced during incubation.

both endogenous and exogenous substrate materials. It prompted us to set up special experiments for a closer study of substrate origin of flavonoids synthesized in cotyledons after treatment with kinetin and under different conditions of their subsequent incubation in a solution of labelled L-phenylalanine. For that purpose we determined the content of flavonoids in cotyledons before and at the end of the incubation period and fixed the absolute amount of flavonoids produced during incubation. Based on the special activity of L-phenylalanine used for exogenous feeding and the total amount of label found in separate flavonoids we then calculated what portion of each of them was formed from the substrate supplied exogenously. The results of a representative series of these experiments ar presented in Table III.

One can clearly see, first of all, that exogenous L-phenylalanine with a remarkably high efficiency served as the substrate for the synthesis of buckwheat flavonoids contributing to, in most cases, more than 50 percent of the total production of separate derivatives during incubation. The most important point, however, was that the relative portion of labelled flavonoids in the total amount of respective compounds produced during incubation showed a marked decrease in the kinetin-treated material and did so even in those cases (see data on anthocyanins and rutin) when the absolute amount of the labelled fraction actually increased (Table III). When we calculated similar distribution ratios for untreated cotyledons incubated under continuous illumination we discovered that kinetin, in fact, imitated light action: the relative amount of labelled flavonoids showed a tendency of decreasing also in illuminated material (Table III). In a further experiment with seedlings which exhibited pronounced responsiveness to kinetin treatment also in the light, the same tendency manifested itself even more distinctly (Table IV).

This phenomenon can only be explained by a considerable improvement of using endogenous substrates for flavonoid biosynthesis both in kinetin-treated as well as in light-incubated material notwithstanding the cells of cotyledons were abundantly supplied with exogenous L-phenyalalanine and its biosynthetic use, too, showed an increase under these conditions. It suggests that limitations on the availability of endogenous substrate materials inherent in etiolated cotyledons are effectively removed by both treatments, so that even with large excess of substrate molecules supplied exogenously the process of forming flavonoids can switch over to a much greater use of endogenous substrate.

Table IV. Effect of light on the production of flavonoids in kinetin-treated[a] buckwheat cotyledons during their incubation for 40 h in a 10^{-2} M solution of labelled L-phenylalanine.

Flavonoid	Origin of substrate	Incubation in the dark		Incubation in the light	
		Absolute production, nmol/seedling	% of total production	Absolute production, nmol/seedling	% of total production
Anthocyanins	ex[b]	20.6[c]	85.8	53.6	59.8
	end	3.4		36.1	
Rutin	ex	60.1	93.2	121.5	66.9
	end	4.4		60.1	
Vitexin	ex	24.2	61.6	29.6	41.1
	end	15.1		42.5	
Isovitexin	ex	40.9	60.0	53.2	38.7
	end	27.3		84.2	
Orientin	ex	24.7	75.1	39.1	63.5
	end	8.2		22.5	
Isoorientin	ex	45.4	73.8	67.9	54.6
	end	16.1		56.4	

[a] Duration of the treatment – 15 min.
[b] Ex – exogenous substrate, end – endogenous substrate.
[c] Calculated as in Table III. Specific activity of labelled L-phenylalanine used for feeding in this experiment – 347 cpm/nmol.

Discussion

As imposed by obligatory participation of protein catalysts in almost every biochemical reaction taking place in a living cell and by easy affectability of their enzymatic activities by various environmental influences modern research generally tends to emphasize the role of enzymes in controlling the rate of metabolic processes. However, the occurrence of even marked changes in the activity of a particular enzyme does not necessarily imply that the changes are rate-determining for the related sequence of reactions. In a recent survey of the senior author it has been shown that the activity of phenylalanine ammonialyase (PAL), the key enzyme of phenolic biosynthesis, is in most tissues many times higher than it is necessary to satisfy requirements for building flavonoids (resp. phenylpropanoic polyphenols) and that considerable increase in the formation of these compounds can occur even at lowered PAL activities. Based on these data and several other arguments a conclusion has been drawn that flavonoid biosynthesis is controlled, most likely, at the substrate rather than at the enzymic level [14].

The results of the present investigations lend further support to the idea and show unequivocally that substrate changes probably underlie even such widely discussed phenomenon as light-dependent stimulation of synthesis of anthocyanins and other flavonoids. Considerable increase in the biosynthesis of flavonoids in etiolated plant material after kinetin treatment, marked enhancement of that kinetin effect at the level of anthocyanin accumulation by post-treatment administration of exogenous L-phenylalanine, and, finally, much increased incorporation of label from radioactive exogenous L-phenylalanine into anthocyanins and rutin in kinetin-treated cotyledons as compared with the extent of their labelling in untreated material leave no doubt that not the activity of enzymes but just the supply of initial substrate is the main intracellular factor limiting flavonoid biosynthesis in the dark.

Since kinetin exhibited marked stimulatory effect also without administration of exogenous L-phenylalanine the data obtained here stress, in general, that substrate limitations in the etiolated material must be primarily due to its limited access to the flavonoid enzymes while the total endogenous supply of L-phenylalanine available for flavonoid biosynthesis in dark-grown cotyledons seems to be large enough, at least in certain seedling populations, to ensure formation of simpler flavonoids (C-glycosylflavones) nearly at rates typical of seedlings at normal light conditions. However, in the case of rutin and especially of anthocyanins maximal accumulation in the dark could be achieved only in the presence of exogenous L-phenylalanine. Accumulation of both of them regularly showed an additional increase when the treated material, instead of being incubated in the dark, was continuously illuminated. A similar tendency occasionally occurred also in the accumulation of C-glycosylflavones. It is possible therefore that stimulatory action of light, in a great part adequately imitatable by kinetin treatment, actually involves two effects: removal of an internal restraint (probably related to membrane permeability) which is placed on the transfer of L-phenylalanine molecules to the site of flavonoid biosynthesis, on the one hand, and stimulation of L-phenylalanine production in the cells, on the other. Experimental evidence was recently obtained that shikimic acid pathway, the unique route for synthesizing aromatic amino acids in plants, can indeed be stimulated by light action [19, 20].

It cannot be said at present what is the relative role of both of these effects. There seems to be no doubt, however, that even in continuously illuminated cotyledons the total available supply of endogenous L-phenylalanine apparently remains below the level that may be saturating for the whole complex of enzymes involved in flavonoid biosynthesis although for several independent branches of this pathway that supply may prove to be nearly sufficient to allow accumulation rates very close to their maximums. Hence it follows that in buckwheat cotyledons total enzymatic capacity of synthesizing flavonoids is normally much higher than are maximal endogenous resources of their natural substrate, and can scarcely find a full realization within the range of conditions which do not cause extreme pathological shifts in the physiological state of plants. A similar situation is presumably characteristic also of most other plant tissues.

Unsaturating supply with the natural endogenous substrate provides a reasonable explanation why accumulation rates of flavonoids and particularly of anthocyanins can be so readily modified by a great variety of influencing factors most of which un-

doubtedly exerts its action on that process through a change in the intracellular level of available substrate materials. It allows also to realize why separate members of a flavonoid complex usually show differential accumulation pattern with a marked quantitative prevalence of simpler flavonoids such as flavones (including C-glycosylflavones) over flavonols, and the latter, in turn, over anthocyanins [21, 22]. An obvious reason is that the simpler forms compete much more effectively for their common precursor than do the related flavonoids with a more complex biosynthetic pathway resulting in that substrate materials from a common source inevitably follow uneven distribution in favour of simpler pathways. Under abundant substrate supply these pathways may approach saturation and accumulation differences between different flavonoids become less distinct. This tendency could be clearly seen in the present experiments. In illuminated cotyledons fed with exogenous L-phenylalanine and treated with kinetin to remove barriers interfering with intracellular transport of substrate molecules, production of anthocyanins practically equalled production of other flavonoids by its order of magnitude (Table IV, cf. Table III).

A brief consideration must be given to the differential biosynthetic use of exogenous and endogenous L-phenylalanine. Molecules of this amino acid supplied exogenously easily reach flavonoid enzymes but it is obvious that they do not equilibrate with the endogenous pools of L-phenylalanine before the precursor molecules are captured by the enzymes (see also [23, 24]). Hence, the path of exogenous L-phenylalanine to the site of flavonoid biosynthesis must be different from that which is normally operating in plant cells for L-phenylalanine of endogenous origin. Favouring action of light (imitatable by kinetin) seems to be specifically directed towards the mechanisms of channelling of the endogenous precursor although both light and kinetin facilitate also access of exogenous L-phenylalanine to the flavonoid enzymes. Whether the channelling mechanisms are identical both for L-phenylalanine derived directly from shikimic acid pathway and for its molecules released from proteins during their catabolic degradation remains to be clarified.

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[1] W. R. Briggs and H. V. Rice, Ann. Rev. Plant Physiol. **23,** 293–334 (1972).
[2] A. W. Galston and R. L. Satter, Recent Advances in Phytochemistry **5,** 51–80 (1972).
[3] R. C. Pecket and T. A. H. Bassim, Phytochemistry **13,** 815–821 (1974).
[4] R. C. Pecket and T. A. Bassim, Phytochemistry **13,** 1395–1399 (1974).
[5] F. Dumortier and J. Vendrig, Z. Pflanzenphysiol. **87,** 313–324 (1978).
[6] T. A. H. Bassim and R. C. Pecket, Phytochemistry **14,** 731–733 (1975).
[7] O. Servettaz, D. Castelli, and C. P. Longo, Plant Sci. Letters **4,** 361–368 (1975).
[8] D. C. Elliott, Aust. J. Plant Physiol. **4,** 39–49 (1977).
[9] N. Nakamura, H. Nakamae, and S. Maekawa, Z. Pflanzenphysiol. **98,** 263–270 (1980).
[10] G. C. Whitelam and C. B. Johnson, Phytochemistry **20,** 9–11 (1981).
[11] C. Grand, R. Ranjeva, A. M. Boudet, and G. Alibert, Planta **146,** 281–286 (1979).
[12] D. B. Harper, D. J. Austin, and H. Smith, Phytochemistry **9,** 497–505 (1970).
[13] G. Engelsma, Inhibition of phenylalanine ammonia-lyase by cinnamic acid derivatives. In: FEBS 12th Meeting Dresden 1978, vol. 55: Regulation of Secondary Product and Plant Hormone Metabolism (M. Luckner and K. Schreiber, eds.), p. 163–172, Pergamon Press, Oxford and New York 1979.
[14] U. Margna, Phytochemistry **16,** 419–426 (1977).
[15] U. Margna and E. Margna, ENSV TA Toimet. Biol. **18,** 40–50 (1969).
[16] H. Scherf and M. H. Zenk, Z. Pflanzenphysiol. **57,** 401–418 (1967).
[17] M. Zucker, Plant Physiol. **40,** 779–784 (1965).
[18] U. Margna and T. Vainjärv, Biochem. Physiol. Pflanzen **176,** 44–53 (1981).
[19] N. Amrhein and H. Holländer, Naturwissenschaften **68,** 43 (1981).
[20] A. Tohver and U. Õnnepalu, ENSV TA Toimet. Biol. **31,** 213–218 (1982).
[21] J. B. Harborne, Chemicogenetical studies of flavonoid pigments. In: The Chemistry of Flavonoid Compounds (T. A. Geissman, ed.), p. 593–617, Pergamon Press, Oxford, London, New York, Paris 1962.
[22] U. Margna, ENSV TA Toimet. Biol. **26,** 302–316 (1977).
[23] U. Margna and E. Margna, Biochem. Physiol. Pflanzen **173,** 2–10 (1978).
[24] U. Margna and E. Margna, Biochem. Physiol. Pflanzen **173,** 347–354 (1978).