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THE SIGNIFICANCE OF CARBOHYDRATE METABOLISM IN FLAVONOID SYNTHESIS IN STRAWBERRY LEAF DISKS

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Abstract—The production of phenylalanine ammonia-lyase and flavonoids in illuminated strawberry leaf disks was dependent on a supply of carbon dioxide, while in darkness carbon dioxide had no effect on the small rates of production. An external supply of sucrose could remove the carbon dioxide dependency of flavonoid and phenylalanine ammonia-lyase production and a supply of carbon dioxide could remove the stimulation of phenylalanine ammonia-lyase and flavonoid production brought about by external sucrose. The uptake and metabolism of external sucrose by leaf disks was stimulated by light and the light-stimulated uptake was inhibited by DCMU. The synthesis of flavonoids and phenylalanine ammonia-lyase were inhibited by DCMU probably through its inhibition of CO_2 fixation or sucrose uptake. Photosynthesis and carbohydrate metabolism were found to be of extreme importance in controlling the synthesis of flavonoids in leaf disks.

INTRODUCTION

THE ROLE of sugars in the synthesis of anthocyanins was suggested by the studies of Overton[1] on a number of plant species. He supplied external sugar and observed an increase in the formation of anthocyanin. Subsequent experiments in which sugar was supplied externally usually resulted in an increase in the formation of anthocyanin,[2–4] although measurement of internal sugar levels generally did not correlate with anthocyanin production.[4,5] The conclusion of Eberhardt,[6] that the essential requirement for anthocyanin synthesis was not the accumulation of sugar but rather the increased turnover of substrate, would appear to account for many of the differences observed between species.

In isolated green plant parts not supplied with an external source of substrate such as sugars, photosynthesis would logically play a significant role in the availability of internal substrate for synthetic activity. Part of the photochemical mechanism of photosynthesis has also been implicated in the light reactions associated with anthocyanin synthesis in apple skin disks.[7]

Control of the amount or rate of flavonoid synthesis probably depends on those factors regulating the supply of initial substrate (e.g. sugar), as well as factors influencing the levels of intermediate precursors (e.g. phenylalanine), those factors controlling the metabolism of intermediate phenolic compounds[8,9] and whatever control mechanisms are functioning on

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[2] L. J. STADLER, *Am. J. Botany* **29**, 17s (1942).
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[4] B. P. EDDY and L. W. MAPSON, *Biochem. J.* **49**, 694 (1951).
[5] A. FREY-WYSSLING and F. BLANK, *Ber. Schweiz. Botan. Ges.* **53A**, 550 (1943).
[6] F. EBERHARDT, *Planta* **43**, 253 (1954).
[7] R. J. DOWNS, H. W. SIEGELMAN, W. L. BUTLER and S. B. HENDRICKS, *Nature* **205**, 909 (1965).
[8] G. ENGELSMA, *Planta* **77**, 49 (1967).
[9] I. RISSLAND and H. MOHR, *Planta* **77**, 239 (1967).

the production of the flavonoid end-product.[10,11] Many problems undoubtedly arise from the assignment of a control mechanism to a specific end-product without knowing where the control exists. In strawberry leaf disks the synthesis of two flavonoids with similar hydroxylation patterns are known to have different light responses[12] so the light response for one of them is not likely to be general for all flavonoids. This work was designed to investigate the control which carbohydrate metabolism exerts directly or indirectly over flavonoid synthesis and to find ways to predetermine this control.

RESULTS AND DISCUSSION

The effect of varying concentrations of carbon dioxide on the synthesis of flavonoids and phenylalanine ammonia-lyase (PAL) is shown in Table 1. When disks were floated on water, little synthetic activity took place without CO_2, and the amounts of flavonoids, carbohydrates and PAL synthesized increased with increasing pCO_2. When leaf disks were supplied with

TABLE 1. THE EFFECT OF CARBON DIOXIDE ON PHENYLALANINE AMMONIA-LYASE (PAL) ACTIVITY, STRAWBERRY LEUCOANTHOCYANIN (SLA) SYNTHESIS, ANTHOCYYNIN SYNTHESIS, AND SYNTHESIS OF NON-EXTRACTABLE, HYDROLYZABLE CARBOHYDRATE

Treatment	48 hr increase in PAL activity μm/hr/100 mg	SLA synthesis μm/48 hr/ 100 cm²	Anthocyanin synthesis μm/48 hr/ 100 cm²	Non-extractable hydrolyzable carbohydrate. mg/48 hr/100 cm²
H_2O, "0" % CO_2	0·02	3·71	0·36	18
H_2O, 0·0032 % CO_2	0·55	7·28	0·73	50
H_2O, 0·012 % CO_2	1·57	15·27	2·06	53
H_2O, 0·049 % CO_2	1·33	15·41	2·45	59
Sucrose, "0" % CO_2	2·74	33·91	3·12	—
Sucrose, 0·0032 % CO_2	2·21	36·19	3·65	—
Sucrose, 0·012 % CO_2	2·29	34·53	3·88	—
Sucrose, 0·049 % CO_2	2·63	30·53	3·33	—

Strawberry leaf disks supplied with 2500 ft-c light for 48 hr with or without 0·15 M sucrose.

an external source of carbohydrates, the beneficial effects of CO_2 were no longer evident. The supply of CO_2 was necessary for the photosynthetic production of carbohydrate in the leaf disk and when an alternate supply was provided photosynthetic fixation was no longer essential. The effect of pCO_2 on the synthetic activity of leaf disks in darkness is shown in Table 2. No stimulation in synthetic ability resulted from the presence of CO_2 without the participation of light. The functioning of photosynthesis in the production of carbohydrates is of prime significance in the synthesis of flavonoids and PAL. Carbohydrates in the green leaf would be necessary as starting material and energy source for all synthetic activity.

Photosynthesis is, therefore, the initial light reaction necessary for the production of flavonoids in this tissue.

The large stimulation in flavonoid synthesis and PAL activity previously reported in this tissue by sucrose[12,13] was obtained by supplying sucrose solutions to leaf disks in petri

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[11] D. HESS, *Planta* **61**, 73 (1964).
[12] L. L. CREASY and T. SWAIN, *Phytochem.* **5**, 501 (1966).
[13] L. L. CREASY, *Phytochem.* **7**, 441 (1968).

TABLE 2. THE EFFECT OF pCO_2 ON THE SYNTHESIS OF FLAVONOIDS AND THE LOSS OF CARBOHYDRATE IN STRAWBERRY LEAF DISKS DURING 72 hr DARKNESS FOLLOWING 24 hr LIGHT

% CO_2	SLA* synthesis μm/100 cm^2/ 72 hr	Anthocyanin synthesis μm/100 cm^2/ 72 hr	Total carbohydrate loss mg/100 cm^2/ 72 hr
"0"	4·80	0·24	33·9
0·006	3·48	0·21	54·7
0·020	2·28	0·18	58·9
0·030	2·11	0·18	69·5
0·10	3·59	0·26	22·7
0·15	2·85	0·17	17·1

* SLA = strawberry leucoanthocyanin.[12]

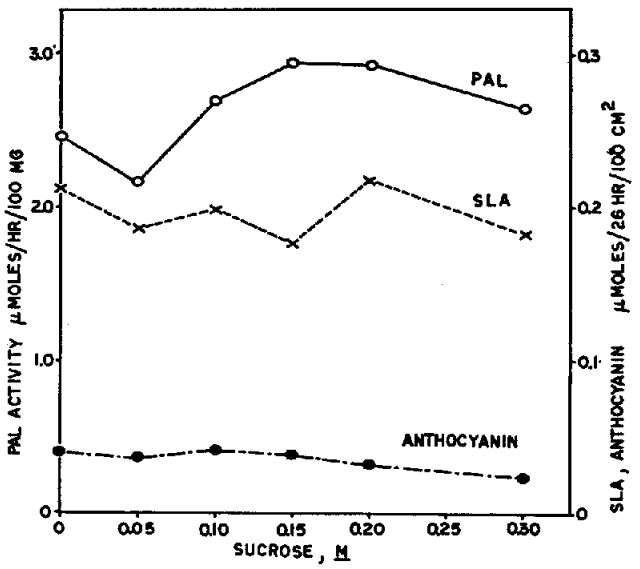

FIG. 1. THE EFFECT OF SUCROSE CONCENTRATION ON THE INCREASE IN THE ACTIVITY OF PHENYLALANINE AMMONIA-LYASE (PAL), THE SYNTHESIS OF STRAWBERRY LEUCOANTHOCYANIN (SLA) AND THE SYNTHESIS OF ANTHOCYANIN. STRAWBERRY LEAF DISKS SUPPLIED WITH 0·03% CO_2 AND 2500 ft-c FLUORESCENT LIGHT FOR 26 hr.

dishes without internal carbon dioxide buffers. Recent results (Fig. 1) with a range of sucrose concentrations but with internal carbon dioxide concentration buffered at 0·03 per cent showed that sucrose was not a stimulator of flavonoid synthesis or PAL activity when the leaf disks had access to carbon dioxide. The photosynthetic ability of the disks was great enough so that the external sucrose was not stimulatory. In petri dishes without internal CO_2 buffer the carbon dioxide was quickly used up and the disks responded to sucrose because photosynthesis was limited by a shortage of carbon dioxide. In experiments of longer than a day, sucrose was stimulatory to flavonoid synthesis even in the presence of carbon dioxide (see Table 1). This was probably due to a breakdown of the photosynthetic ability of the leaf disks with time.

When leaf disks were sealed in dishes without CO_2 and without a source of external carbohydrate, exposure to light had some effect on their synthetic activity even in the absence of net photosynthesis (Table 3). This probably represents a utilization of reserve materials for limited synthetic activity in light. If external sucrose was supplied, then light greatly stimulated synthetic activity. The uptake and metabolism of the external sucrose as measured by conversion to non-extractable but hydrolyzable carbohydrate was also stimulated by light so that it is not possible to say whether the light stimulation of synthetic activity was due to direct light reactions in flavonoid synthesis or to the increase in uptake and metabolism of external sucrose brought about by light. The uptake of glucose by *Nitella* from solution has also been shown to be light stimulated.[14] The action of light on the metabolism of external sucrose in the absence of added carbon dioxide could mean that the light reactions associated with it were not coupled to CO_2 fixation or that sufficient internal carbon dioxide produced by respiration was available in the leaf disks to cycle the photosynthetic mechanism even though net synthesis could not take place.

TABLE 3. THE EFFECT OF LIGHT ON PAL ACTIVITY, FLAVONOID SYNTHESIS AND CARBOHYDRATE IN STRAWBERRY LEAF DISKS WITH "0"% CO_2 AND WITH OR WITHOUT 0·15 M SUCROSE

Treatment	PAL* activity μm/hr/100 mg AcP	SLA synthesis μm/100 cm^2 /48 hr	Anthocyanin synthesis μm/100 cm^2 /48 hr	Hydrolyzed carbohydrate mg/100 cm^2 /48 hr
H_2O, darkness	0·51	0·11	0·0	−21·4
H_2O, 500 ft-c	0·55	0·11	0·043	−21·6
H_2O, 1000 ft-c	0·56	1·02	0·102	−27·7
Sucrose, darkness	2·79	7·99	0·112	− 7·3
Sucrose, 500 ft-c	4·35	11·93	0·278	+38·9
Suctose, 1000 ft-c	6·23	20·83	0·556	+63·2

* PAL = phenylalanine ammonia-lyase, SLA = strawberry leucoanthocyanin.

The action of 3(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU) as an inhibitor of photoreaction II of photosynthesis is well established. The action of DCMU on the synthetic ability of leaf disks at 0 per cent CO_2 with external sucrose is shown in Table 4. DCMU inhibited the synthetic ability of the leaf disks, but also inhibited the uptake of metabolism of external sucrose. DCMU inhibited light-stimulated glucose uptake in *Nitella*.[14] The inhibitory effect of DCMU could be due to a direct effect on the light reactions associated with synthetic activities but also could be due to a direct effect on the metabolism of external sucrose necessary for its utilization in these synthetic activities. If the latter were the case, DCMU would be acting indirectly on flavonoid and enzyme (PAL) synthesis and this would imply some function for photosynthetic light reactions in the metabolism of external sucrose which was demonstrated as being light stimulated (Table 3).[14]

The action of DCMU on the synthetic ability of leaf disks supplied with 0·03 per cent CO_2 is given in Table 5. In the absence of external sucrose, DCMU was a very effective inhibitor of synthetic activity including and probably due to its inhibition of carbohydrate synthesis. In the presence of external sucrose, DCMU was less effective as an inhibitor of

[14] F. A. SMITH, *J. Expt. Botany* **18**, 348 (1967).

synthetic activity, having no effect on the PAL activity and an effect on flavonoids or the metabolism of external sucrose only at the highest concentration.

TABLE 4. THE EFFECT OF DCMU ON FLAVONOID, PAL AND CARBOHYDRATE IN STRAWBERRY LEAF DISKS WITH "0" % CO_2, 0·15 M SUCROSE AND 2500 ft-c LIGHT FOR 40 hr

DCMU* concentration	PAL activity		SLA synthesis		Anthocyanin synthesis		Hydrolyzed carbohydrate	
	μm/hr 100 mg AcP	% inhib.	μm/ 100 cm²	% inhib.	μm/ 100 cm²	% inhib.	mg/ 100 cm²	% inhib.
0	4·15	—	26·3	—	1·76	—	72·2	—
10^{-6} M	3·53	15	25·0	5	1·90	0	63·2	12
10^{-5} M	3·25	22	26·8	0	1·64	7	48·0	34
10^{-4} M	2·14	48	18·4	30	0·93	47	42·9	41
5×10^{-4} M	1·07	74	9·6	64	0·69	61	41·7	47

* DCMU = 3(3,4 dichlorophenyl)-1,1-dimethylurea, PAL = phenylalanine ammonia-lyase, SLA = strawberry leucoanthocyanin.

The effect of DCMU on the metabolism of leaf disks, pretreated with 0·03 per cent CO_2 in the light to permit buildup of internal carbohydrate, is given in Fig. 2. After 24 hr pretreatment, the disks were transferred to 0 per cent CO_2 on water with different concentrations of DCMU. If the only effect of DCMU were on the production of carbohydrate from

TABLE 5. THE EFFECT OF DCMU ON PAL ACTIVITY, FLAVONOID SYNTHESIS AND CARBOHYDRATE IN STRAWBERRY LEAF DISKS WITH 0·03 % CO_2, 2500 ft-c LIGHT AND WITH OR WITHOUT 0·15 M SUCROSE

Treatment*	PAL activity μm/hr/100 mg A.P.	SLA synthesis μm/100 cm² /48 hr	Anthocyanin synthesis μm/100 cm² /48 hr	Hydrolyzed carbohydrate mg/100 cm² /48 hr
H_2O	1·87	17·41	0·72	+82·0
$H_2O + 10^{-5}$ M DCMU	0·82	2·28	0·03	−11·7
$H_2O + 10^{-4}$ M DCMU	0·75	0·91	0	−19·2
Sucrose	3·82	29·50	0·75	+110·0
Sucrose + 10^{-5} M DCMU	4·61	30·42	0·68	+88·3
Sucrose + 10^{-4} M DCMU	4·68	15·23	0·52	−18·0

* DCMU = 3(3,4 dichlorophenyl)-1,1-dimethylurea, PAL = phenylalanine ammonia-lyase, SLA = strawberry leucoanthocyanin.

CO_2 or the uptake or metabolism of external sucrose, then DCMU would have no effect on the metabolism of flavonoids under these conditions. The figure shows no effect of DCMU in darkness. Light stimulated the synthesis of flavonoids but had no effect on the activity of PAL. The exposure to light stimulated the loss of carbohydrate and this light-stimulated carbohydrate loss was inhibited by DCMU. DCMU also caused some inhibition of flavonoid synthesis in the light. The inhibition by DCMU of light-stimulated carbohydrate loss and light-stimulated flavonoid synthesis suggests that the effect of DCMU on flavonoid synthesis could be intimately associated with the metabolism of carbohydrate. Alternatively, the light-stimulated flavonoid synthesis resulted in greater utilization of carbohydrate due to

predetermined higher levels of flavonoid synthesis. The lack of inhibition of the level of PAL activity suggests that PAL synthesis is controlled by the absolute level of carbohydrate rather than the rate of its metabolism when it is present in adequate quantity.

In this experiment (Fig. 2) about 20 per cent of the mass of carbohydrate lost was found in strawberry leucoanthocyanin (SLA) in darkness. In light about 40 per cent of the carbohydrate loss could be accounted for in the synthesis of SLA. DCMU did not affect these proportions of conversion significantly. Since this is a significant conversion of an energy-rich material to a presumed non-recoverable form, it raises the question of the role of these

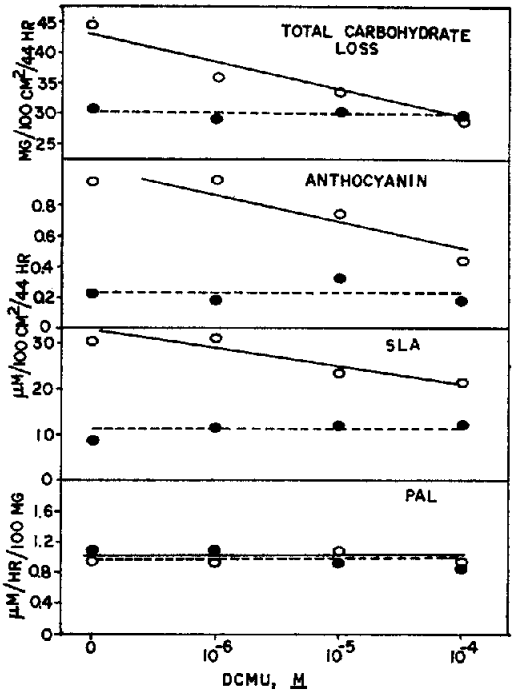

FIG. 2. THE EFFECT OF DCMU ON PHENYLALANINE AMMONIA-LYASE (PAL) ACTIVITY, STRAWBERRY LEUCOANTHOCYANIN (SLA) SYNTHESIS, ANTHOCYANIN SYNTHESIS AND CARBOHYDRATE LOSS IN STRAWBERRY LEAF DISKS WITH "0" % CO_2, WITHOUT SUCROSE AND WITH (OPEN CIRCLES) OR WITHOUT LIGHT (FILLED CIRCLES) (2500 ft-c) FOLLOWING 24 hr EXPOSURE TO LIGHT IN THE PRESENCE OF 0·03 % CO_2.

secondary products in plant metabolism. Could useful work be done during their synthesis or could they serve as storage materials for later recovery? We are presently re-investigating these possibilities with strawberry leaf material.

In a non-storage organ such as the green leaf disk the continued production of carbohydrate is of prime importance for all synthetic activities. In the absence of favorable conditions for internal production of carbohydrate, leaf disks can metabolize external sucrose and utilize it for their synthetic activity. The metabolism of external sucrose is partially dependent on the exposure of the leaf disk to light and the light reactions associated are sensitive to DCMU. The metabolism of internal carbohydrate also appears to be influenced by light, and this could account for part of the effect of light on the synthesis of flavonoids. The light reactions associated with the production and metabolism of carbohydrate are

considered to be the initial essential light reactions necessary for flavonoid synthesis in autotropic organs. Additional roles of light in flavonoid synthesis only function in some tissues after the light requirements for carbohydrate synthesis have been satisfied.

EXPERIMENTAL

Plant Material

Strawberry plants (*Fragaria vesca* var. alpine) were grown in the greenhouse at Ithaca, New York. Leaf disks (1 cm in diameter) 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.[13] Activity is given in micromoles of cinnamic acid produced at 40°/hr/100 mg of acetone powder. The average yield of acetone powder from leaf disks is 2·76 mg/cm².

Flavonoid Measurement

Strawberry leucoanthocyanin (SLA)[15] was measured as previously described.[12] Anthocyanin was measured by determining the visible absorption spectrum of the acidified (1 per cent HCl) methanol extract of leaf disks. The contribution of chloroplast pigments was removed by utilizing the following equation: $A^{anthocyanin} = (A^{535}-A^{620}) - 0{\cdot}1\,(A^{650}-A^{620})$. A molar extinction coefficient of 30,000 for anthocyanin was utilized in the estimation.[12]

Carbohydrate Measurement

The methanol extractable carbohydrates were measured with the Anthrone reagent.[16] An aliquot of the methanol extract was taken to dryness under vacuum and then taken up in 1 ml of methylene chloride and 1 ml of water. After centrifugation, a sample of the water layer was used for Anthrone reaction using glucose as standard. The methanol extracted leaf disks were heated at 90° for 1½ hr with *n*-butanol/HCl reagent (95+5, v/v).[12] An aliquot of the butanol/HCl was added to water layered on Anthrone reagent and the quantity of carbohydrate measured. The standard curve was determined with glucose using the same quantity of butanol/HCl in each tube. Samples of potato starch subjected to methanol extraction did not release measurable carbohydrate. Treatment of the methanol extracted potato starch with butanol/HCl at 90° for 1½ hr resulted in complete solubilization of the starch and full reaction with the Anthrone reagent. Starch would not, however, be the only carbohydrate measured by this treatment.

Carbon Dioxide Buffers

Carbonate–bicarbonate buffers to maintain the partial pressure of carbon dioxide were prepared according to the formula,

$$pCO_2 = \left(\frac{1}{\alpha CO_2}\right)(22{\cdot}4)\left(\frac{K_2}{K_1}\right)\left(\frac{[HCO_3^-]^2}{[CO_3^=]}\right).$$

The solutions were placed in the center well of Conway dishes with the disks floating on appropriate solutions in the outer ring. The maintaining of 0 per cent CO_2 in some treatments was accomplished by placing 0·1 M NaOH in the center wells.

[15] L. L. Creasy and T. Swain, *Nature* **208**, 151 (1965).
[16] D. L. Morris, *Science* **107**, 254 (1948).