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Possible Site of Flavonoid Synthesis in the Photosynthetic Apparatus

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Chloroplasts were prepared in aqueous suspension from buckwheat (*Fagopyrum esculentum* Moench.) seedlings, and the incorporation of [1-^{14}C]acetic acid into quercetin (3,5,7,3′,4′-pentahydroxyflavone) by the isolated chloroplast preparations was investigated.

Although there are several publications concerning the biosynthesis of flavonoid compounds by various plant tissues (Bogorad, 1958; Grisebach, 1965, 1968; Grisebach & Barz, 1969), no information is available about the mechanism of flavonoid synthesis within the chloroplast. In a continuing study of flavonoid ring A biosynthesis with buckwheat seedlings (Saito, 1974), we have now obtained evidence for the incorporation of radioactively labelled acetic acid into quercetin (3,5,7,3′,4′-pentahydroxyflavone) by isolated chloroplast particles.

Experimental

Buckwheat (*Fagopyrum esculentum* Moench.) seeds were obtained locally. Ficoll and Dextran-40 were purchased from Pharmacia, Uppsala, Sweden. [1-^{14}C]Acetic acid (45.4mCi/mmol) was obtained from Dai-ichi Chemical Co. Ltd., Tokyo, Japan. ATP, CoA, NADPH and UDP-D-glucose were from C. F. Boehringer und Soehne G.m.b.h., Mannheim, Germany.

Seeds were germinated in trays with moist Vermiculite under light of 4000lx at 27°C. After about 70h of germination, the cotyledons were collected and washed several times with 70% v/v ethanol and finally with distilled water. Sterilized cotyledons (approx. 500g fresh wt.) were homogenized for 15s in a chilled blender (Ultra-Turrax Typ TP 18/2, Janke & Kunkel K.G.) with 500–600ml of cold Honda medium [Ficoll, 2.5% (w/v); Dextran-40, 5.0% (w/v); sucrose, 250mM; Tris, 25mM, pH7.8; $MgCl_2$, 1mM; 2-mercaptoethanol, 4mM] (Smillie, 1972). The homogenate was squeezed through three layers of nylon cloth and the filtrate was centrifuged at 800*g* for 10min (r_{av}. 7.3cm). The light-green supernatant solution was centrifuged at 2000*g* for 10min and the resulting pellet was washed again with 1vol. of the same medium. The washed pellet was then layered on a discontinuous gradient formed from 5ml each of 2.5M-, 2.0M-, 1.5M- and 1.0M-sucrose (Sager & Ishida, 1963) in Honda medium. The gradient was centrifuged in the Spinco Model L centrifuge for 70min in the SW 25.1 rotor at 20000*g*.

The various bands obtained were examined under a light microscope. The third layer from the top, containing the intact chloroplasts, was collected and diluted with Honda medium without sucrose and again centrifuged at 2000*g* for 10min. This washing procedure was repeated three times. The entire preparation was carried out in a cold-room at 4°C.

Intact chloroplasts were exposed to [1-^{14}C]acetic acid in the reaction mixture described in the legend to Table 1. The reaction was stopped by addition of methanol–HCl [approx. 10ml of 2M-HCl in 70% (v/v) methanol] and the mixture was heated on a water bath at 80°C for 10min. The precipitate collected by centrifugation was washed three times with hot 70% (v/v) methanol and the concentrated extract transferred to a separating funnel. About 100μg of carrier rutin (quercetin 3-rutinoside) was added and the solution extracted exhaustively with ethyl acetate (6–8 times). After concentration to a small volume the extract was chromatographed on Whatman No. 1 paper first with butan-1-ol–acetic acid–water (4:1:5, by vol.) and then with acetic acid–water (1:9, v/v) respectively. The rutin spot on the paper was cut out, eluted with 80% (v/v) methanol and the eluate was concentrated by distillation *in vacuo*. An equal volume of 2M-HCl was added and the acidified solution was heated on a water bath at 100°C for 20min. Carrier quercetin was mixed with the hydrolysate, which was then exhaustively extracted with ether (10–14times). The concentrated extract was then chromatographed first with acetic acid–water (2:3, v/v) and then with ethyl acetate saturated with water. The spot corresponding to quercetin was eluted with methanol. Radioactivity in the quercetin was measured in a Beckman liquid-scintillation spectrometer Model LS-250 with Bray's solution (Bray, 1960). Chlorophyll was determined by the method of Strain *et al.* (1971).

Table 1. *Distribution of radioactivity in quercetin isolated from reaction mixture*

The reaction mixture contained in a final volume of 2ml: 0.24μmol of [1-^{14}C]acetic acid, 7μmol of ATP, 0.3μmol of CoA, 0.9μmol of NADPH, 0.5μmol of UDP-D-glucose, 1.5μmol of Mg^{2+}, 1.0μmol of *p*-coumaric acid, 1.0μmol of $NaHCO_3$, 2.5μmol of 2-mercaptoethanol, 50μmol of Tris–HCl buffer, pH7.8, and chloroplast suspension containing approx. 0.9μg of chlorophyll. The mixture was incubated at 30°C for 3h.

Experiment no.	10^4×Total radioactivity (d.p.m.)	Chlorophyll (μg)	10^4×Specific radioactivity (d.p.m./μg of chlorophyll)
1	1.5	0.91	1.7
2	0.7	0.88	0.8
3	3.5	0.92	3.8

Results and discussion

On the basis of the data presented in Table 1, isolated chloroplasts appear to incorporate radioactive acetic acid into quercetin. Exogenously applied acetic acid is probably activated to its CoA form in the chloroplast and then incorporated mainly into ring A of quercetin according to the acetate hypothesis (Birch & Donovan, 1953). The occurrence of flavonoid compounds in chloroplasts has been demonstrated in many plants (Beck *et al.*, 1966; Zaprometov & Bukhlaeva, 1967; Monties, 1969; Weissenböck *et al.*, 1971; Weissenböck, 1973). By exposing leaves to $^{14}CO_2$ and light for short periods, Zaprometov & Kolonkova (1967) showed that the specific radioactivity in the phenolic compounds in chloroplasts was higher than that in phenolic compounds in the soluble supernatant and they suggested that chloroplasts can participate in the primary synthesis of phenolic substances including flavonoids. Oettmeier & Heupel (1972) separated flavonols and other aromatic acids from ether-treated freeze-dried spinach chloroplasts. Moreover, Löffelhardt *et al.* (1973) reported that the enzymes concerned with the incorporation of phenylalanine into the reaction sequences leading to the synthesis of C_6–C_3 and C_{15} compounds may be associated with intact chloroplasts.

All these findings strongly support the evidence in Table 1 that flavonoids are synthesized within chloroplasts and that flavonoids could play a physiological role in higher plants. Additional studies, however, would be required to clarify these interesting problems further.

Beck, A. B., Kowala, C. & Lamberton, J. A. (1966) *Aust. J. Chem.* **19**, 1755–1757
Birch, A. J. & Donovan, F. W. (1953) *Aust. J. Chem.* **6**, 360–368
Bogorad, L. (1958) *Annu. Rev. Plant Physiol.* **9**, 417–448
Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
Grisebach, H. (1965) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.), pp. 279–308, Academic Press, New York and London
Grisebach, H. (1968) in *Recent Advances in Phytochemistry* (Mabry, T. J., Alston, R. E. & Runeckles, V. C., eds.), vol. 1, pp. 379–406, Appleton Century Crofts, New York
Grisebach, H. & Barz, W. (1969) *Naturwissenschaften* **56**, 538–544
Löffelhardt, W., Ludwig, B. & Kindl, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 1006–1012
Monties, B. (1969) *Bull. Soc. Fr. Physiol. Veg.* **15**, 29–45
Oettmeier, W. & Heupel, A. (1972) *Z. Naturforsch.* **27B**, 177–183
Sager, R. & Ishida, M. R. (1963) *Proc. Nat. Acad. Sci. U.S.* **50**, 725–730
Saito, K. (1974) *Biochim. Biophys. Acta* **343**, 392–398
Smillie, R. M. (1972) *Methods Enzymol.* **24B**, 381–394
Strain, H. H., Cope, B. T. & Svec, W. A. (1971) *Methods Enzymol.* **23A**, 452–476
Weissenböck, G. (1973) *Ber. Deut. Bot. Ges.* **86**, 351–364
Weissenböck, G., Tevini, M. & Reznik, H. (1971) *Z. Pflanzenphysiol.* **64**, 274–277
Zaprometov, M. N. & Bukhlaeva, V. Ya. (1967) *Sov. Plant Physiol.* **14**, 167–177
Zaprometov, M. N. & Kolonkova, S. V. (1967) *Dokl. Akad. Nauk SSSR* **176**, 470–473