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INCORPORATION OF KAURENOIC ACID INTO GIBBERELLINS BY CHLOROPLAST PREPARATIONS OF *BRASSICA OLERACEA*

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Abstract—Sonicated chloroplast preparations from *Brassica oleracea* var. *acephala* showed increased gibberellin-like activity after incubation with (−)-kaur-16-en-19-oic acid. Hormone activity increased with time and was not due to release from a protein-bound form. Studies with 17-^{14}C-labelled substrate indicated that the product was a gibberellin chromatographically similar to gibberellin A_3.

INTRODUCTION

IT HAS been shown that a significant portion of the gibberellin-like activity present in leaf homogenates of barley and kale could be associated with the chloroplast fraction[1] and that increased amounts of activity could be released into the supernatant by ultrasonic disintegration of the plastids.

Recent studies have also demonstrated that the specific activity of the primary photosynthetic enzyme, ribulose-1,5-diphosphate carboxylase (RuDPC), can be increased by raising the endogenous gibberellin level of the leaf.[2]

Thus, it is possible that the hormone may exert a regulatory function in the photosynthetic activity of the plastid and that the rate of carbon fixation may depend, to some extent, upon the rate of gibberellin synthesis or degradation. In examining this proposition, it is first necessary to establish whether the gibberellin present in the chloroplast is synthesized *in situ* or translocated across the membrane from an extra-chloroplastidic source. These studies are concerned with this aspect.

To study biosynthesis successfully, it is necessary to provide the system with a suitably specific precursor. The conversion of mevalonate to (−)-kaurene has been demonstrated with endosperm homogenates of *Echinocystis macrocarpa* and the further steps resulting in (−)-kauren-19-oic acid have also been achieved by using a microsomal preparation from the same source.[3] Incorporation of mevalonate into a fraction chromatographically similar to (−)-kauren-19-ol has been observed in intact roots of *Helianthus annuus*.[4] However, as the mevalonate activating enzymes were likely to be removed by the chloroplast isolating procedures[5] it was thought that a later stage intermediate would be a better choice.

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[1] J. L. STODDART, *Planta, Berlin* **81**, 106 (1968).

[2] K. J. TREHARNE and J. L. STODDART, *Nature, Lond.* **220**, 457 (1968).

[3] C. A. WEST, M. OSTER, D. ROBINSON, F. LEW and P. MURPHY, *Proc. 6th Internat. Conf. on Plant Growth Substances*, in press.

[4] D. SITTON, A. RICHMOND and Y. VAADIA, *Phytochem.* **6**, 1101 (1967).

[5] J. E. GRAEBE, *Phytochem.* **7**, 2003 (1968).

Geissman and co-workers[6] have shown that (−)-kaur-16-en-19-oic acid is used efficiently by *Gibberella fujikuroi* as a precursor of gibberellic acid and it was, therefore, considered that this compound might provide the most suitable substrate for plastid gibberellin synthesis.

RESULTS

Incubation of (−)-kaur-16-en-19-oic acid (KA) with sonicated chloroplast suspensions resulted in an increased level of gibberellin-like activity in the ethyl acetate soluble fraction (Table 1).

TABLE 1. INCREASES IN THE GIBBERELLIN-LIKE ACTIVITY OF CHLOROPLAST PREPARATIONS AFTER INCUBATION WITH (−)-KAUR-16-EN-19-OIC ACID FOR 1 hr AT 30°

Treatment	Experiment number 1	2	3
	[Barley endosperm assay (α-amylase units)]		
Sonicate only (+co-factors)	17·6	13·4	7·8
Sonicate + kaurenoic acid	114·4	88·0	57·2
Boiled sonicate + kaurenoic acid	38·4	29·2	14·3
Kaurenoic acid only (+co-factors)	15·9	3·3	9·0
Gibberellin A_3 (0·05 μg)	138·2	100·7	92·0
Blank	4·9	3·8	6·5
Volume of chloroplast suspension (ml)	5·0	5·0	5·0
Protein content (mg)	30·75	29·50	35·25
Total chlorophyll (mg)	0·96	0·72	1·09

It is evident that KA does not exhibit significant biological activity under the assay conditions employed, which suggests a similarity to previous work indicating that kaurene was only active in the barley endosperm sugar-release system when the incubation was allowed to proceed for more than the normal 24 hr.[7] The initial endogenous activity level of the sonicate was also low enough to preclude interference with, or obscuration of, gibberellin increases resulting from the incorporation of added KA. Comparison of the activity increases with those obtained using gibberellin A_3 suggest that approximately 0·1 per cent of the added KA could be accounted for in terms of increased gibberellin-like activity.

Boiling caused a marked reduction in the incorporating ability of the chloroplast preparations and omission of NADPH tended to reduce the final gibberellin (GA) level, although this effect was not consistent.

Incubations carried out in light (6600 lux, fluorescent) or darkness gave similar results but intact (non-sonicated) suspensions were noticeably inferior, giving 30–55 per cent of the activity found in sonicates from the same source.

The low endogenous activity level present in the sonicates after a 1-hour incubation makes it unlikely that the increases detected in other treatments could be due to the release of GA

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[6] T. A. GEISSMAN, A. J. VERBISCAR, B. O. PHINNEY and G. CRAGG, *Phytochem.* **5**, 933 (1966).

[7] K. C. JONES, *Planta, Berlin* **78**, 366 (1968).

from a bound form during the incubation period. However, this possibility was examined by carrying out a time-course experiment in which changes in the sonicate activity level were followed in the presence and absence of KA (Fig. 1).

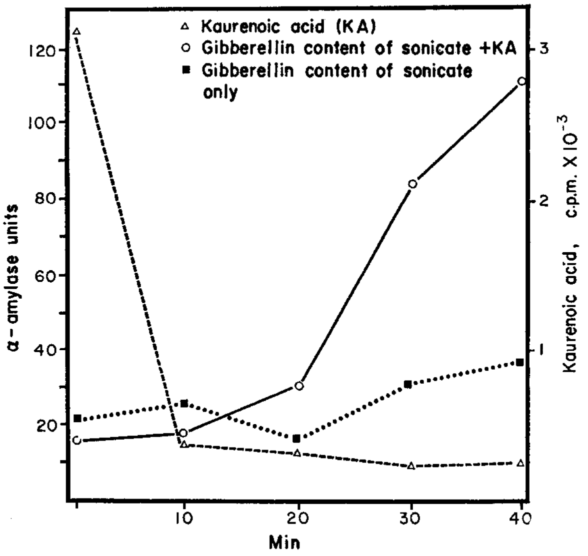

FIG. 1. CHANGES WITH TIME IN THE GIBBERELLIN ACTIVITY AND KAURENOIC ACID CONTENT OF CHLOROPLAST INCUBATES.

Incubated at 30° with shaking at 60 oscillations per min. Gibberellin activity estimated by the barley half-seed α-amylase method and kaurenoic acid by recovery of the 17-^{14}C label.

The disappearance of KA from the ethyl acetate soluble fraction of the sonicate was followed by using (−)-17-^{14}C-kaurenoic acid and a rapid loss of radioactivity was found to occur during the initial 10 min of the incubation. Gibberellin activity showed the first significant increase after 10–20 min and then rose rapidly for the remainder of the experimental period. There was also a slight increase with time in the basal endogenous activity, suggesting that some release of protein-bound hormone might be occurring.

The form of the KA curve is consistent with a rapid binding process and it is interesting to note that gibberellin synthesis is associated with the period during which the reduction in KA level is more gradual.

To assess the specificity of the KA binding effect, 1000 × *g* pellets were incubated with mevalonate, KA and gibberellin A_1. At the end of the incubation period (15 min) the pellets were repeatedly washed with fresh buffer and the supernatant activity determined at each step. After three re-suspensions the residual activity in the pellet was estimated to give a measure of binding for each compound (Table 2).

The concentration of radioactivity in the KA treated pellet was of a different order to that obtained with mevalonate or gibberellin. Nevertheless, it is interesting to note that, even after compensation for the different degrees of labelling, gibberellin was bound marginally more than mevalonate.

Substitution of ^{14}C-KA for the "cold" compound in the sonicate incubations made it possible to follow the qualitative redistribution of the label and to detect incorporation into biologically active compounds. Results from this type of study are described in Fig. 2.

TABLE 2. BINDING OF LABELLED COMPOUNDS BY CHLOROPLASTS DERIVED FROM YOUNG LEAVES OF *Brassica oleracea*

Compound	% of total d.p.m. recovered				Total d.p.m.
	Supernatant				
	1	2	3	Pellet	
DL-Mevalonate-2-^{14}C	90·25	8·56	0·94	0·26	85,320
Kaurenoic acid-17-^{14}C	42·84	8·49	0·62	42·45	12,955
Gibberellin A$_1$-3,4-^{3}H	88·74	8·60	1·66	0·99	18,030

Pellets had mean protein and chlorophyll contents of 29·2 mg and 0·77 mg respectively. Re-suspension medium; 0·4 M sucrose, Tris 0·04 M, EDTA 0·25 mM, MgCl 0·01 M, GSH 2·0 mM, pH 7·8.

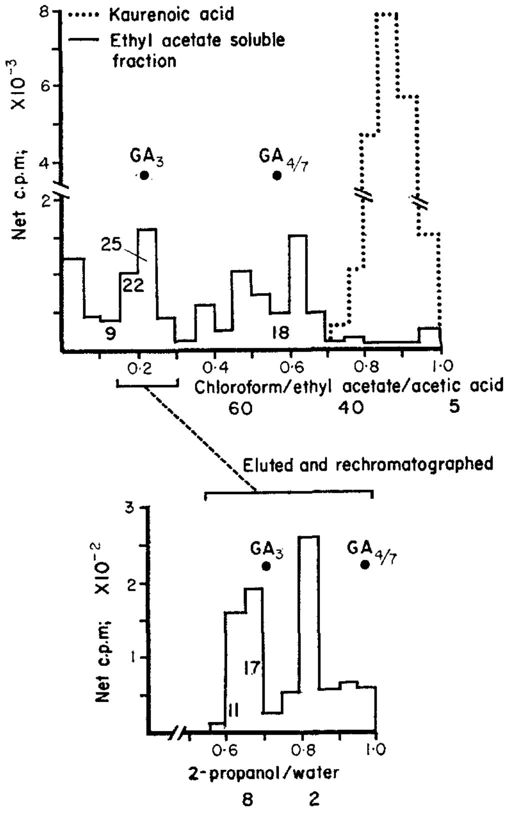

FIG. 2. TLC OF PRODUCTS OF 17-^{14}C-KAURENOIC ACID INCORPORATION BY CHLOROPLAST SONICATES. Adsorbent, silica gel H. Radioactivity assessed by liquid scintillation acounting. Figures located in histogram blocks indicate level of biological activity in the barley endosperm assay (GA$_3$ 0·005 μg = 39 units).

Several new radioactive peaks could be detected in the ethyl acetate soluble acidic fraction after TLC in chloroform/ethyl acetate/acetic acid (60:40:5), but only one clearly corresponded with an area of biological activity. The R_f of this zone coincided with that of authentic GA_3. Adsorbent from the R_f 0·15–0·30 region was removed and eluted with ethanol. After reduction to a suitable volume the eluate was rechromatographed in 80% 1-propanol to yield two major radioactive components, one of which again corresponded to the location of the biological activity on the plate. This peak ran slightly behind GA_3.

It is considered that these data indicate the incorporation of the ^{14}C label from KA into a gibberellin-like compound similar to, but possibly not identical with, GA_3.

DISCUSSION

The sonicate studies show that the level of gibberellin activity increased progressively with time in the presence of added KA and the ^{14}C data confirm that this represented active incorporation. Furthermore, pre-boiling drastically reduced the response to added KA indicating thermolability of some component of the system and suggesting an enzymatic or protein-based mechanism. Reduced incorporation in the absence of NADPH also tends to support this conclusion. Illumination during the incubation period had no demonstrable effect but it is possible that the light regime under which the original material was grown would be of great importance. This aspect is, at present, under examination.

The poor incorporation exhibited by "intact" chloroplast preparations is consistent with permeability restrictions on the intake of KA and it is possible that the residual level of incorporation could be entirely accounted for in terms of contamination by ruptured plastids. This would be in accord with findings indicating that other gibberellin precursors (e.g. mevalonate) can only pass through the plastid membrane with difficulty.[8] On the other hand, the effects of applied GA_3 on RuDPC activity[2] can be interpreted as evidence of permeability of the membrane to the hormone, and this is supported by the finding that plastids derived from leaves infiltrated with labelled gibberellin contain significant amounts of radioactivity, even after rigorous purification on sucrose density gradients.[9] If the apparent permeability to gibberellin is non-directional, then the possibility exists that plastid GA synthesis may also fulfil the requirements of extra-chloroplastidic sites.

The rapid binding of KA in the time-course experiments could indicate that gibberellin synthesis utilizing this precursor is, at least, a two-stage process; with an initial binding to an active site preceding the oxidative rearrangements. Support for this could be drawn from the observation that the increase in the biological activity of the incubates commenced after the completion of the KA binding. However, it is equally probable that only a small fraction of the added precursor was utilized for gibberellin synthesis and that the bulk was incorporated into other compounds. It has been noted that non-fractionated incubates, subjected to TLC, showed a marked association of radioactivity with some of the pigmented zones.

Because of the small amounts of activity involved, it was not practicable to carry out structural studies on the gibberellin formed by incubates in the presence of added KA. The chromatographic behaviour of the principal biologically active peak resembled that of GA_1 or GA_3 but a more detailed description will depend upon further studies using gas chromatography/mass spectrometry.

[8] L. J. Rogers, S. P. J. Shah and T. W. Goodwin, *Biochem. J.* **96**, 7 (1965).

[9] J. L. Stoddart, unpublished data.

EXPERIMENTAL

Materials

Plants of *Brassica oleracea* var. *acephala* cv. Cansons were grown in John Innes No. 1 compost in an unheated glasshouse during the period from May to August 1968. Samples consisted of young, fully expanded leaves selected for uniformity of age and harvested immediately before use.

Samples of (−)-17-^{14}C-kaurenoic acid (1·44 mc/m-mole) and unlabelled (−)-kaur-16-en-19-oic acid were generously donated by Professor T. A. Geissman, University of California at Los Angeles, U.S.A. The Radiochemical Centre, Amersham, Bucks., supplied the mevalonic acid-2-^{14}C (5·03 mc/m-mole) and also carried out the initial selective hydrogenation of GA_3 to provide a crude preparation of gibberellin A_1-3,4-^{3}H. The final stages of purification were carried out by the author, in a similar manner to that described by Kende,[10] to yield a final specific activity of 351 mc/m-mole.

Isolation of the Chloroplast Fraction

Leaf samples (*ca.* 50 g fresh weight) were homogenized for 45 sec in a BTL top-drive homogenizer with 100 ml of ice-cold sucrose buffer (0·4 M sucrose, Tris 0·04 M (Sigma 7–9), EDTA 0·25 mM, GSM 2·0 mM (Sigma), MgCl 0·01 M, pH 7·8) and the brei filtered through two layers of cheesecloth. The filtrate was centrifuged for 2 min at 200 × *g* to remove cell debris and the supernatant was then re centrifuged at 1000 × *g* for 20 min to provide a crude chloroplast pellet. After re-suspending in fresh buffer the pellet was subjected to 200 × *g* for 2 min to remove aggregates. The final chloroplast preparation was then obtained by a further centrifugation at 1000 × *g*.

Ultrasonic Disintegration

Chloroplast pellets, re-suspended in buffer (Tris, EDTA, GSH, MgCl, pH 7·8, no sucrose) were subjected to ultrasonic treatment with an M.S.E. Model 7120 disintegrator for a total of 30 sec at a frequency of 21 kc/s and a peak-to-peak signal amplitude of 6–8 μ. Over-heating was prevented by embedding the tubes in ice.

Conditions of Incubation

Incubations were carried out in 25-ml Erlenmayer flasks on a Gallenkamp water-bath shaker at 30° and a shaking rate of 60 oscillations per min. Each flask contained 5 ml of sonicated chloroplast suspension, 0·8 μmole NADPH (Boehringer), 0·5 μmole $MgCl_2$, 0·14 μmole kaur-16-en-19-oic acid in 10 μl of acetone (= 0·103 μc when added with the 17-^{14}C label). Incorporations were routinely terminated after a period of 1 hr.

Boiled sonicates were prepared by holding the temperature at 100° for 1 min followed by cooling to incubation temperature before use.

Determination of Chlorophyll and Protein Contents

A 1 ml aliquot of the chloroplast sonicate was passed through a 1 cm layer of Hyflo Supercel filter aid supported on a 2·5 cm disc of Metricel GA-3 membrane. The filter aid was then extracted with acetone and the chlorophyll solution was cleared by centrifugation (2000 × *g* for 5 min) before determination of absorptivities. Pigment content was estimated by the Arnon[11] method. Protein content was assessed on similar aliquots by the Kjeldahl procedure.

Estimation of Gibberellin-like Activity

At the end of the incubation period 0·5 ml of 5% TCA was added to each flask and the protein precipitate removed by centrifugation (2000 × *g* for 5 min). After decanting into a 15 ml centrifuge tube, the supernatant was adjusted to pH 2·8 with NaOH and 2 ml of redistilled ethyl acetate was added. The two phases were mixed for 20 sec on a Whirlimix and reseparated by centrifuging for 5 min at 1000 × *g*. A 1 ml aliquot of the upper (organic) phase was then transferred to a small specimen vial and reduced to dryness in a stream of warm air (*ca.* 35°). The residue was taken up in 1 ml of sterile distilled water and used in the barley α-amylase release assay.

Seed of the cultivar "Himalaya", supplied by Professor R. A. Nilan of the Genetical Institute, Copenhagen, was used as the test material and the detailed procedures were as described by Jones and Varner.[12]

Measurement of Radioactivity

Liquid scintillation counting was employed throughout using a Beckman LS-100 instrument. The scintillation fluid consisted of 5 g PPO (diphenyl oxazole) and 100 g naphthalene dissolved in 1 l. of dioxan.

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[10] H. KENDE. *Plant Physiol.* **42,** 1612 (1967).

[11] D. I. ARNON, *Plant Physiol.* **24,** 1 (1949).

[12] R. L. JONES and J. E. VARNER, *Planta, Berlin* **72,** 155 (1967).

Aliquots of ethyl acetate (0·1 ml) were added to 5-ml volumes of scintillation fluid for determination of 17-^{14}C-kaurenoic acid levels. The distribution of activity on thin-layer plates was determined by scraping the adsorbent from the relevant regions directly into the vials. Counting efficiencies were determined using an external standard and values of the order of 60–65% were normally obtained.

Thin-layer Chromatography

All chromatograms were run on 250-μ layers of silica gel H (Merck) using either $CHCl_3$/ethyl acetate/acetic acid, 60:40:5, or 2-propanol/water, 8:2, as the developing solvent.

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