Phytochemistry, 1969, Vol. 8, pp. 115 to 122. Pergamon Press. Printed in England

THE EFFECTS OF ABSCISIC ACID ON THE BIOSYNTHESIS OF ISOPRENOID COMPOUNDS IN MAIZE

E. I. MERCER and J. E. PUGHE

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth

(*Received* 20 *June* 1968; *in revised form* 27 *July* 1968)

Abstract—(±)-Abscisic acid does not inhibit the incorporation of [2-^{14}C]mevalonic acid into sterols in maize leave tissue. It does, however, depress the synthesis of chlorophylls, carotenoids and chloroplastidic isoprenoid quinones which normally takes place when etiolated maize leaf tissue is illuminated. It is suggested that abscisic acid may depress chloroplast formation.

INTRODUCTION

ABSCISIC acid, previously known as abscisin II[1] or dormin,[2] is a naturally occurring plant hormone for which the structure shown in Fig. 1 has been proposed[3] and confirmed by synthesis.[4] The synthetic hormone is a racemic (±) mixture[4] but the natural compound has

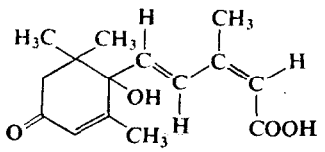

FIG. 1. ABSCISIC ACID.

been shown, by optical rotatory dispersion measurements, to be the dextro-rotatory enantiomorph.[5] The concentration of (+)-abscisic acid in several plant tissues has been determined by Millborrow[6] and shown to fall within the same range of concentrations as reported for other plant hormones. The highest concentration of abscisic acid so far recorded is 4·1 mg/kg fresh weight in the pseudocarp of the fruit of *Rosa arvensis*.[6]

The hormone has several physiological effects. It induces dormancy in the seedlings of several species, e.g. birch[2] and inhibits germination in seeds, e.g. *Arabidopsis thaliana*.[7] It accelerates abscission of cotton fruit and leaves.[1] It encourages tuberization in *Solanum andigena*[8] and inhibits flower initiation in two obligate long day species, *Lolium temulentum* and *Spinacia oleracea*.[8]

[1] F. T. ADDICOTT, H. R. CARNS, J. L. LYON, O. E. SMITH and J. L. MCMEANS, Regulateurs naturels de la Croissance Vegetale, 687, Centre Natl. Recherche Sci., Paris (1964).
[2] C. F. EAGLES and P. F. WAREING, *Nature* **199**, 874 (1963).
[3] K. OHKUMA, F. T. ADDICOTT, O. E. SMITH and W. E. THIESSEN, *Tetrahedron Letters* **29**, 2529 (1965).
[4] J. W. CORNFORTH, B. V. MILLBORROW and G. RYBACK, *Nature* **206**, 715 (1965).
[5] J. W. CORNFORTH, B. V. MILLBORROW and G. RYBACK, *Nature* **210**, 627 (1966).
[6] B. V. MILLBORROW, *Planta (Berlin)* **76**, 93 (1967).
[7] N. SANKHLA and D. SANKHLA, *Z. Pflanzenphysiol.* **58**, 402 (1968).
[8] H. M. M. EL-ANTABLY, P. F. WAREING and J. HILLMAN, *Planta (Berlin)* **73**, 74 (1967).

Little is known of the way in which (+)-abscisic acid brings about these effects. It appears to inhibit at least some of the actions of indole acetic acid,[9] gibberellic acid[9–11] and the kinins.[12] The finding that the gibberellin content of abscisic acid-treated tissues is lower than normal[12] suggests that abscisic acid may be inhibiting the synthesis of the gibberellins. Since the gibberellins are isoprenoid in nature the possibility that (+)-abscisic acid may inhibit the biosynthesis of other isoprenoid compounds arises. This paper reports the effects of synthetic (±)-abscisic acid on the synthesis of the sterols, carotenoids, chlorophylls and isoprenoid quinones in maize leaf tissue.

RESULTS AND DISCUSSION

Effect of Abscisic Acid on the Synthesis of Sterols by Maize Shoots

In order to determine the effect of abscisic acid on sterol synthesis, the incorporation of [2-^{14}C]mevalonic acid into the sterol fraction of abscisic acid treated and control maize shoots was measured over a finite experimental period. This was necessary since the endogenous pool of sterol in maize shoots is large relative to that which could be reasonably expected to be synthesized during a relatively short experimental period. A comparison of the specific activities of the sterol extracted from the abscisic acid treated and control maize would then indicate whether or not abscisic acid had inhibited sterol synthesis.

Etiolated, 8-day-old maize seedlings were excised at the node and divided into two batches of equal wet weight. The seedlings of each batch were gathered together into a bundle held loosely by an elastic band. On bundle (the test) was placed in a suitably sized beaker with the cut ends of the shoots dipping into an aqueous solution containing 10 ppm (±)-abscisic acid ($3{\cdot}8 \times 10^{-5}$ M). The control bundle was placed in a similar beaker with the cut ends dipping into water. The two bundles were allowed to remain in the beakers for 2 hr during which time they were illuminated by two 60-W tungsten bulbs and subjected to a steady draught of air generated by an electric fan sited 24 in. away. After 2 hr the control bundle was transferred to a beaker containing 5 μc *DL*-[2-^{14}C]mevalonic acid dissolved in 15 ml water and the test bundle to a beaker containing 5 μc *DL*-[2-^{14}C]mevalonic acid dissolved in 15 ml of an aqueous solution containing 10 ppm (±)-abscisic acid. The bundles remained dipping into these solutions for 24 hr under the same illumination and air stream as before. When the solutions decreased in volume due to uptake by the shoots, the volume was made up to 15 ml by the addition of water in the case of the control and the 10 ppm abscisic acid solution in the case of the test. After the incubation procedure was complete the bundles were removed from the beakers and washed thoroughly with water to remove residual *DL*-[2-^{14}C]mevalonic acid. The unsaponifiable materials, 3β-hydroxysterols and sterol-free unsaponifiable material were isolated from each bundle and assayed for radioactivity. Table 1 shows the results of two such experiments.

Since solutions of abscisic acid are somewhat unstable in the light[13] two further experiments were carried out. The experimental procedure in these experiments was the same as that just described save that the etiolated maize shoots were kept in complete darkness throughout the incubation period. Table 2 shows the results of these experiments.

[9] F. T. ADDICOTT, O. E. SMITH and J. L. LYON, *Plant Physiol.* **40**, (Suppl.), Abstr., XXVI (1965).
[10] J. W. CORNFORTH, B. V. MILLBORROW, G. RYBACK and P. F. WAREING, *Nature* **205**, 1250 (1965).
[11] T. H. THOMAS, P. F. WAREING and P. M. ROBINSON, *Nature* **205**, 1269 (1965).
[12] J. GOOD, Ph.D. Thesis, University of Wales (1967).

TABLE 1. EFFECT OF ABSCISIC ACID ON THE INCORPORATION OF [2-^{14}C]MEVALONIC ACID INTO THE STEROLS OF MAIZE SHOOTS IN THE LIGHT

Expt. No.	Material	Control			Test			Ratio of specific activity C:T
		Weight (mg)	Counts/min	Specific activity (cpm/mg)	Weight (mg)	Counts/min	Specific activity (cpm/mg)	
1	Maize shoots	38,500*	—	—	38,700*	—	—	—
	Lipid	104·6	—	—	105·7	—	—	—
	Unsaponifiable material	35·1	153,600	4,376	45·3	216,910	4,786	1:1·091
	Sterol-free unsap.	31·2	88,625	2,840	38·5	119,000	3,090	1:1·087
	3β-Hydroxysterol	8·3	67,660	8,150	8·7	89,350	10,260	1:1·260
2	Maize shoots	59,100*	—	—	59,100*	—	—	—
	Lipid	153·8	—	—	148·6	—	—	—
	Unsaponifiable material	59·7	414,615	7,410	63·8	274,080	4,294	1:0·579
	Sterol-free unsap.	44·9	193,750	4,310	47·8	117,900	2,465	1:0·572
	3β-Hydroxysterol	9·7	154,230	15,900	10·1	120,330	11,900	1:0·780

* Wet weight.

Table 2. Effect of abscisic acid on the incorporation of [2-^{14}C]mevalonic acid into the sterols of maize shoots in the absence of light

Expt. No.	Material	Control			Test			Ratio of specific activity C:T
		Weight (mg)	Counts/min	Specific activity (cpm/mg)	Weight (mg)	Counts/min	Specific activity (cpm/mg)	
3	Maize shoots	30,000*	—	—	30,000*	—	—	—
	Lipid	73·3	—	—	73·9	—	—	—
	Unsaponifiable material	21·7	138,600	6,387	21·4	93,500	4,369	1:0·686
	Sterol-free unsap.	14·8	34,020	2,299	16·8	27,020	1,608	1:0·698
	3β-Hydroxysterol	4·0	58,880	14,720	4·0	53,540	13,385	1:0·910
4	Maize shoots	50,000*	—	—	50,000*	—	—	—
	Lipid	123·8	—	—	126·1	—	—	—
	Unsaponifiable material	40·3	317,450	7,880	41·4	327,205	7,900	1:1·002
	Sterol-free unsap.	32·8	164,730	5,020	33·7	168,400	4,998	1:0·995
	3β-Hydroxysterol	6·8	152,800	22,460	6·9	171,700	24,880	1:1·108

* Wet weight

It was found that the rate of uptake of the solution containing the [2-^{14}C]mevalonic acid was not the same in the test and control maize shoots in spite of the incubation conditions being identical. This happened to some extent in all four experiments but was most marked in experiments 2 (Table 1) and 3 (Table 2). Because the specific activity of the extracted sterol is governed by the uptake of radioactive precursor into the tissues, it was difficult to draw any conclusion about the effect of abscisic acid on sterol synthesis by simply comparing the specific activities of test and control sterol. In experiment 2, for example, the specific activity of the control sterol was 15,900 counts/min/mg whilst that of the test sterol was 11,900 counts/min/mg but it was observed that the control maize took up more of the [2-^{14}C]mevalonic acid solution than did the test maize. So, although comparison of the sterol specific activities indicated that abscisic acid was depressing sterol synthesis, the difference between these values could be equally well explained by the difference in uptake of precursor.

In an attempt to overcome this difficulty the ratios of the specific activities of the test and control fractions were compared at each stage during the fractionation process. In experiment 2 the ratio of the specific activities of the unsaponifiable materials from the control and test maize was 1:0·579. If the control:test specific activity ratio of the sterol and sterol-free unsaponifiable fractions were also 1:0·579 it would indicate either that abscisic acid does not have any effect upon the incorporation of mevalonic acid into the components of the unsaponifiable material (in which case the difference between the specific activities of the control and test maize components was due to the different uptake of labelled precursor), or that abscisic acid is blocking isoprenoid biosynthesis in that part of the pathway which is common to all isoprenoid compounds. If the control:test specific activity ratio of the sterol fractions were lower (say 1:0·3) than those of the unsaponifiable and sterol-free unsaponifiable fractions (i.e. 1:0·579) it would indicate that abscisic acid is blocking sterol biosynthesis specifically. If the control:test specific activity ratio of the sterol fractions were higher (say 1:0·7) than those of the unsaponifiable and sterol-free unsaponifiable fractions (i.e. 1:0·579) it would indicate either (*a*) that abscisic acid is stimulating sterol biosynthesis or (*b*) that abscisic acid is having no effect upon sterol biosynthesis but could be depressing the synthesis of some other isoprenoid component of the unsaponifiable material. Since the control:test specific activity ratio of the sterol in experiment 2 is higher (1:0·78) than those of the unsaponifiable and sterol-free unsaponifiable materials (1:0·579 and 1:0·572 respectively) the decision is between (*a*) and (*b*). Alternative (*b*) is preferred because physiological evidence suggests that abscisic acid is an inhibitor rather than a stimulator.

It is significant that in all four experiments the control:test specific activity ratio for the sterol fraction is higher than those of their unsaponifiable material counterparts. This indicates that the lack of effect of abscisic acid on sterol synthesis is not due to its inactivation by light during the incubation period.

In experiments 1 (Table 1) and 4 (Table 2) the specific activity of the test sterol was higher than that of the control in contrast to their lower values in the other two experiments. These discrepancies are almost certainly due to differences in uptake of labelled precursor.

It is concluded, therefore, that abscisic acid does not have any inhibitory effect upon sterol biosynthesis in maize.

Effect of Abscisic Acid on the Synthesis of Chlorophylls, Carotenoids and Isoprenoid Quinones

The chlorophylls, carotenoids and chloroplastidic isoprenoid quinones have in common the fact that they are present in low concentration in etiolated maize shoots and are synthesized

[13] P. F. WAREING, personal communication.

in considerable quantity when the shoots are subsequently illuminated.[14, 15] In this respect they differ from the sterols. It was possible, therefore, to test the effect of abscisic acid on the synthesis of these compounds without recourse to radioactive tracers simply by incubating etiolated maize shoots in the light with and without abscisic acid and subsequently comparing the levels of these compounds in the tissues.

Etiolated, 8-day-old maize seedlings were excised at the node and the leaf sheaths removed. The resulting leaf tissue was divided into two batches of equal wet weight and then cut into small pieces (0·5 cm^2 area). One batch of leaf pieces (the control) was distributed evenly amongst a number of Petri dishes (12 cm dia.) containing 0·01 % (v/v) Nonidet P42 (non-ionic detergent) in water. The other batch (the test) was similarly distributed amongst an equal number of Petri dishes containing 0·01 % (v/v) Nonidet P42 in 10 ppm (±)-abscisic acid solution. The depth of the solution in each Petri dish was 0·75 cm. The leaf pieces were totally immersed in the solutions. The Petri dishes were covered to prevent evaporation, placed on a sheet of aluminium foil and illuminated by four 60-W tungsten lamps and a 1000-W Philips "Cold-light" set at a distance of 24 in. for 30 hr. After the incubation the content of chlorophylls, carotenoids and isoprenoid quinones was determined in the test and control tissue.

In an attempt to observe a greater effect of abscisic acid on the synthesis of these compounds, a second experiment was carried out in which the control and test leaf pieces were incubated with the same solutions as described above for 6 hr in complete darkness before the 30 hr incubation period in the light. It was hoped that the pre-incubation of the test leaf pieces in the dark would enable a considerable amount of abscisic acid to be absorbed before synthesis of the chlorophylls, carotenoids and quinones could begin. Apart from this difference the experimental procedure was the same as that of the previous experiment.

The results of these experiments are shown in Table 3.

In both experiments (5 and 6) abscisic acid depressed the synthesis of chlorophylls, carotenoids, plastoquinone, α-tocopherolquinone and α-tocopherol by 12–60 per cent. The inhibition of chlorophyll synthesis was more marked in experiment 5 (58 per cent) than in experiment 6 (25 per cent) whilst the inhibition of carotenoid synthesis was of the same order (30 per cent) in both experiments. There was a greater inhibition of the isoprenoid quinones in experiment 6 (28–43 per cent) than in experiment 5 (12–18 per cent). Abscisic acid had no effect upon the levels of ubiquinone in either experiment. The reason for this is that ubiquinone is located in the mitochondria rather than the chloroplast and its relatively high level in etiolated maize shoots does not change when the shoots are illuminated.[14]

General Effect of Abscisic Acid

In maize leaf tissue abscisic acid appears to inhibit the synthesis of those isoprenoid compounds which are located in the plastids. It has no inhibitory effect upon the synthesis of either the sterols, the bulk of which are located outside the chloroplast, in the microsomal and mitochondrial fractions,[16] or ubiquinone which is located in the mitochondria.[14] However, it depresses the synthesis of the chlorophylls, carotenoids, plastoquinone, α-tocopherol and α-tocopherol quinone which are located in the chloroplasts.

There is considerable evidence that there are two sites of synthesis of isoprenoid compounds within the plant cell, the chloroplastidic site where the chlorophylls, carotenoids and chloroplastidic isoprenoid quinones are synthesized and the extrachloroplastidic site where

[14] W. T. GRIFFITH, D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* **103**, 589 (1967).
[15] T. W. GOODWIN and E. I. MERCER, in *The Control of Lipid Metabolism* (edited by J. K. GRANT), Biochemical Society Symposium No. 24, p. 37 (1963).
[16] R. J. KEMP and E. I. MERCER, *Biochem. J.*, in press (1968).

TABLE 3. EFFECT OF ABSCISIC ACID ON THE SYNTHESIS OF THE CHLOROPHYLLS, CAROTENOIDS AND TERPENOID QUINONES OF MAIZE SHOOTS

	Experiment 5		Experiment 6	
	Control	Test	Control	Test
Wet weight of tissue (g)	100	100	100	100
Time of incubation (hr)	30	30	6 dark; 30 light	6 dark; 30 light
Temp. of incubation (°)	20	20	20	20
Lighting	Philips 1000-W Coldlight + 4 60-W tungsten lights			
Age of maize (days)	8	8	8	8
(±)-Abscisic acid (ppm)	10	10	10	10
Weight of lipid (mg)	225·5	221·1	237·5	237·5
Weight of chlorophyll a (μg)	2493	988	4040	3094
Weight of chlorophyll b (μg)	1065	481	2044	1435
Weight of chlorophyll a + b (μg)	3555	1469	6084	4529
Weight of carotenoid (μg)	1015	741	460	318
Weight of β-carotene (μg)	66	40	51	44
Weight of xanthophyll (μg)	949	701	409	274
Weight of lutein (μg)	620	458	268	179
Weight of violaxanthin (μg)	171	127	73	49
Weight of neoxanthin (μg)	158	116	68	46
Weight of plastoquinone (μg)	740	651	763	437
Weight of ubiquinone-9 (μg)	201	201	161	161
Weight of α-tocopherol quinone (μg)	163	134	143	103
Weight of α-tocopherol (μg)	100·5	88·5	95·1	64·5

the sterols, cyclic triterpenes and other non-chloroplastidic isoprenoid compounds are synthesized.[15, 17] It is possible that abscisic acid inhibits an enzyme, concerned in isoprenoid biosynthesis, which is specific to the chloroplastidic site.

An equally possible and perhaps more likely explanation of the observed effects of abscisic acid on the synthesis of isoprenoid compounds in maize is that it depresses the development of the etiolated plastid into the mature chloroplast.

EXPERIMENTAL

Biological Material

Maize (*Zea mays* var. South African White Horse Tooth) seeds were purchased from Gunson (Seeds) Ltd., London, E.C.3.

Cultivation of Maize Seedlings

Maize seeds were soaked for 18 hr in water prior to planting 0·5 in. deep in moist vermiculite contained in stainless-steel trays (200 seeds/14 in. × 12 in. × 2 in. tray). The trays were then placed in light-tight growth cabinets maintained at 28°. The humidity of the cabinets was not controlled but there was a constant, slow flow of air through them. The seeds were allowed to germinate in darkness for 8 days. During this period they were watered on alternate days under dim green lighting. At the end of the germination period the seedlings were about 20 cm high, showed no secondary growth and were devoid of any green colour.

Extraction of Lipid

The shoots from experiments 1–4 were cut into small pieces and homogenized in acetone with an Ultra-Turrax homogenizer. The leaf pieces from experiments 5 and 6 were filtered, washed several times with water and then homogenized in acetone. The acetone extracts were then filtered off and the residues re-extracted twice with acetone and once with ether. The bulked extracts from each tissue were diluted with several volumes of water and extracted four times with ether. The ethereal extracts were washed with water, dried (Na_2SO_4) and then evaporated to dryness under N_2. In experiments 5 and 6, known aliquots were removed from the ethereal extracts for chlorophyll assay.

[17] T. W. GOODWIN, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. 2, p. 721, Academic Press, London and New York (1967).

Saponification of Lipid and Isolation of Sterols

In experiments 1–4 the lipid was saponified[18] and the 3β-hydroxysterols isolated from the unsaponifiable material by precipitation as their digitonides[19] followed by regeneration with pyridine.[20] The sterol-free unsaponifiable material was recovered.[21] It should be noted however that the term sterol-free unsaponifiable material is, strictly speaking, a misnomer because the 4,4′-dimethyl-3β-hydroxy- and 4α-methyl-3β-hydroxysterols are incompletely precipitated by digitonin and are present in the sterol-free unsaponifiable material; however the more abundant 4-demethyl-3β-hydroxysterols (e.g. β-sitosterol, stigmasterol, campesterol) are completely precipitated and are not present in the sterol-free unsaponifiable material.

Chromatography on Alumina Columns

In experiments 5 and 6 the lipid was chromatographed on columns of Brockmann Grade III, acid-washed alumina (Woelm, anionotropic) which were developed by stepwise elution with 0·25% E/P*, 1% E/P, 3% E/P, 5% E/P, 8% E/P, 12% E/P and 20% E/P,[22] followed by ethanol to elute the xanthophylls. The fractions were evaporated to dryness under N_2, desiccated to remove last traces of solvent and weighed.

Quantitative Estimation of Components (Experiments 5 and 6)

Chlorophylls a and b were determined spectrophotometrically on a solution of the lipid in 80% (v/v) aqueous acetone.[23] β-Carotene, which was present in the 0·25% E/P fraction of the alumina chromatogram, was determined spectrophotometrically.[24] Plastoquinone, ubiquinone and α-tocopherolquinone, which were present in the 1% E/P, 5% E/P and 20% E/P fractions of the alumina chromatogram respectively, were assayed spectrophotometrically.[22] α-Tocopherol, which was present in the 3% E/P fraction of the alumina chromatogram, was oxidized to α-tocopherolquinone and assayed spectrophotometrically as such.[25] The xanthophylls, eluted from the alumina chromatogram in the ethanol fraction, were dissolved in benzene and a known aliquot chromatographed on silica gel impregnated paper (Whatman SG 81) using methanol:benzene:ethyl acetate mixture in the proportions 5:75:20 for development.[26] This separated the three major xanthophylls found in maize, lutein, violaxanthin and neoxanthin (R_f values 0·4, 0·35, 0·15 respectively). The carotenoid zones were rapidly cut out of the paper chromatogram, cut into small pieces which were packed tightly into small glass chromatography columns. Ethanol (5 ml) was then run through the columns in order to elute the xanthophyll. The individual xanthophylls were then estimated spectrophotometrically in ethanolic solution. The assumption was made that the $E^{1\%}_{1cm}$ at λ_{max} of each xanthophyll was 2500. This is not strictly accurate (see Davies[27]) but is a sufficient close approximation for comparative purposes.

Radioassay

The sterol and unsaponifiable fractions in experiments 1–4 were assayed for radioactivity with a Packard Tricarb Scintillation Spectrometer Series 314E. Suitable aliquots were introduced into the glass counting phials and dissolved in 10 ml scintillator fluid (0·3 g dimethyl POPOP (1,4-*bis*-(4-methyl-5-phenyloxazol-2-yl)-benzene) + 5 g PPO (2,5-diphenyloxazole) per l. toluene). They were usually counted for 10 min. The instrument was set to count ^{14}C with an efficiency of 50 per cent and a background of 25 counts/min.

Acknowledgements—We wish to thank Professor P. F. Wareing for a generous gift of synthetic (±)-abscisic acid, Mrs. A. Warren for skilled technical assistance and Drs. B. H. Davies and D. R. Threlfall for their advice on carotenoid and quinone estimation procedures respectively.

* Abbreviation: E/P, solution of diethyl ether in light petroleum (b.p. 40–60°).

[18] R. J. KEMP, L. J. GOAD and E. I. MERCER, *Phytochem.* **6**, 1609 (1967).
[19] A. WINDAUS and A. WELSCH, *Ber. Dtsch. Chem. Ges.* **42**, 612 (1909).
[20] R. SCHOENHEIMER and H. DAM, *Hoppe-Seyl. Z.* **215**, 59 (1933).
[21] K. J. TREHARNE, E. I. MERCER and T. W. GOODWIN, *Phytochem.* **5**, 581 (1966).
[22] D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* **103**, 573 (1967).
[23] J. T. O. KIRK, *Planta (Berlin)* **78**, 200 (1968).
[24] T. W. GOODWIN, in *Modern Methods of Plant Analysis* (edited by K. PAECH and M. V. TRACEY), Vol. 3, p. 272, Springer-Verlag, Heidelberg (1955).
[25] G. R. WHISTANCE, D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* **105**, 145 (1967).
[26] B. H. DAVIES, personal communication.
[27] B. H. DAVIES, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 489, Academic Press, London and New York (1965).