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BIOSYNTHESIS OF ABSCISIC ACID BY A CELL-FREE SYSTEM

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Abstract—Abscisic acid (ABA) is synthesized from labelled mevalonate by a preparation of lysed chloroplasts isolated from ripening avocado fruit, a number of cofactors are required A similar preparation from bean and avocado leaves was also active and chloroplasts from the green outer, and white, inner parts of the fruit were equally effective

INTRODUCTION

AVOCADO fruits are the richest source of abscisic acid (up to 10 mg/kg) and they also convert labelled mevalonate into abscisic acid (ABA) more efficiently than any other tissue known at present [1] Leaves increase their ABA content about 40-fold when they wilt[2] but the percentage of added mevalonate incorporated into ABA, even by wilting leaves, is considerably less[3] in comparison with fruit and at least 10 hr must elapse between feeding and wilting for any incorporation to be detectable [4] Thus leaves are unfavourable organs to use for biosynthetic studies but the apparent existence of a strong barrier to penetration of mevalonate into the site of ABA biosynthesis in leaves parallels the results of Goodwin and colleagues[5–8] who found that the chloroplast membrane is highly impermeable to mevalonate and that good incorporation into carotenoids occurred only when the chloroplasts had been lysed The barrier to incorporation of mevalonate, and the similarity of the structures of ABA and carotenoids, suggested that ABA is formed within chloroplasts

Synthesis of ABA in ripening avocado (*Persea gratissima*) fruits occurs at the same rate whether the mesocarp slices are turgid or wilted,[4] and synthesis is rapid in spite of the high existing ABA content It appears from this that in avocado fruit, at this stage of maturity, biosynthesis is not subject to the regulatory controls that operate in leaves [9] Consequently it was possible that the biosynthesis of ABA from added mevalonate in avocado fruit might be accentuated by damaging the chloroplasts, and that lysed chloroplasts of the fruit were a possible cell-free system for synthesis of ABA from mevalonate

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[3] MILBORROW, B V and NODDLE, R C (1970) *Biochem J* **119,** 727
[4] MILBORROW, B V and ROBINSON D R (1973) *J Exp Botany* **24,** 537
[5] GOODWIN, T W (1958) *Biochem J* **68,** 26p
[6] GOODWIN, T W (1958) *Biochem J* **70,** 612
[7] ROGERS, L J, SHAH, S P J and GOODWIN, T W (1966) *Biochem J* **99,** 381
[8] CHARLTON, J M, TREHARNE, K J and GOODWIN, T W (1967) *Biochem J* **107,** 205
[9] MILBORROW, B V (1972) in *Plant Growth Substances 1970* (CARR, D J, ed), p 281, Springer, Berlin

RESULTS

The results of the first experiment (Table 1) show that labelled mevalonate was incorporated into ABA by the chloroplast preparation and, as expected, intact chloroplasts synthesized less labelled ABA than did broken ones omission of the cofactors drastically reduced incorporation (Tables 2 and 3) The chloroplasts appeared undamaged in electron micrographs and the outer membranes could be seen clearly A few mitochondria were present in the E M preparations but they occurred abundantly (together with some broken and intact chloroplasts) in the original supernatant fractions which synthesized 0 137 of the amount of labelled ABA formed by the chloroplast preparation (Table 3) Ten randomly chosen electron micrographs of a subsample of the preparation used in the experiment in Table 3 gave mean relative areas of 100, 30 and 6 8% for chloroplasts, protoplast fragments within membranes and mitochondria respectively

TABLE 1 INCORPORATION OF LABELLED MEVALONATE INTO ABSCISIC ACID BY INTACT AND LYSED CHLOROPLASTS ISOLATED FROM RIPENING AVOCADO FRUIT TISSUE

Treatment	ABA isolated as methyl ester (μg)	dpm in methyl ABA	dpm in 1 4-*cis*-diol of methyl abscisate	dpm in 1 4-*trans*-diol of methyl abscisate
2 hr Incubation intact chloroplasts	20 8	440	187	175
2 hr Incubation intact chloroplasts acetone and Tween-20 added	22 2	156	60	52
2 hr Incubation chloroplasts lysed in 0 1 M K phosphate	22 1	1000	380	450
17 hr Incubation intact chloroplasts	23 6	546	238	201
17 hr Incubation intact chloroplasts acetone and Tween-20 added	21 7	910	300	280
17 hr Incubation, chloroplasts lysed in 0 1 M K phosphate	24 2	2860	1376	1197

Complete medium, intact and lysed chloroplasts, with and without acetone (0 05 ml 3 5 M H_2O) and Tween-20 (0 05 ml, 20% H_2O) (±)-[2-$^3H_2$]Mevalonolactone (2 0 × 10^7 dpm) was added to each tube and the incubation was carried out under laboratory light (480 lx) (±)-ABA (28 μg) and 2 6-di-*t*-butyl-4-methyl phenol (10 μg) were added at the end of the incubation period in the methanol used to kill the preparation The recovery of the ABA was calculated from the absorptivity of solutions of methyl abscisate (λ_{max} 265 nm = 20 900) the contribution of the endogenous (+)-ABA did not exceed 5% of this value All the samples of methyl abscisate from this experiment were reduced to the isomeric 1 4-diols chromatographed and counted The equal distribution of the radioactivity between the two diols and the absence of any other labelled materials show that the [2-$^3H_2$] mevalonate had been incorporated into abscisic acid Earlier work has shown[21] that approximately half of the tritium from C-2 of mevalonolactone is retained in methyl abscisate

Although halved avocado fruit can incorporate mevalonate into ABA in darkness, and no stimulatory effect of light has been observed the disrupted chloroplasts incorporate less mevalonate into ABA in darkness than when illuminated (Tables 2 and 3) It is possible

that photosynthesis can maintain the supply of ATP or reduced co-enzymes in the lysed chloroplasts An attempt to overcome the effect of phosphatases by adding twice the usual amount of ATP led to reduced incorporation (Table 4) but supra-optimal concentrations of ATP have been reported to inhibit biosynthetic reactions in chloroplasts [8] Addition of polyvinylpyrrolidone and the antioxidant 2,6-di-*t*-butyl-4-methyl phenol (BHT) was without effect (Table 4)

TABLE 2 EFFECT OF COFACTORS ON THE INCORPORATION OF MEVALONATE INTO ABSCISIC ACID

Treatment	dpm in methyl ABA
Complete medium	1260
Complete medium incubated in darkness	55
–ATP	152
–FAD–FMN	152
–NAD–NADH	152
–NADP–NADPH	486

The complete medium and the medium with various co-factors omitted were used (±)-[2-^{14}C]Mevalonolactone (1.7×10^7 dpm) was used each time but in this, and all subsequent experiments, the amount of (±)-ABA added at the end of the 17 hr incubation was lowered to 14 μg, 480 lx

The progressive reduction of the amount of labelled mevalonolactone added, from 67 μg in Table 1 to 17 μg in Table 3, reduced the amount of label incorporated into ABA and as radioautograms of the neutral and acid extracts showed that a large number of other labelled products are formed it is possible that the (+)-[2-^{14}C]mevalonate is used up early on in the incubation Furthermore, the extension of the duration of incubation to 27 hr reduced the amount of label in ABA suggesting that synthesis had slowed or ceased and degradation was occurring Consequently the labelled mevalonolactone was ballasted with 70 μg of unlabelled material (Table 4) This treatment gave the highest incorporation (even higher stimulations have been obtained in other preparations) so the addition of the label in 87 μg (±)-mevalonate was used routinely thereafter

TABLE 3 EFFECT OF TIME AND OTHER FACTORS ON ABSCISIC ACID FORMATION

Incubation time	Treatment	dpm in methyl ABA
A		
9 hr	Complete medium	241
17 hr	Complete medium	906
27 hr	Complete medium	638
17 hr	1×10^6 dpm Mevalonolactone, complete medium	107
17 hr	Complete medium, incubated in darkness	310
17 hr	Supernatant added to complete medium	124
B		
17 hr	Complete medium	260
17 hr	2 × Concentration of ATP added in complete medium	83
17 hr	1×10^6 dpm Mevalonolactone, complete medium	97
17 hr	2,6-Di-*t*-butyl-4-methyl phenol (10 μmol) and polyvinylpyrrolidone (10 mg) added	250
17 hr	Complete medium–NADP	83
17 hr	Complete medium–NADPH	53

(±)-[2-^{14}C]Mevalonolactone (4.4×10^6 dpm) was added to all except where stated 13 000 lx

In 1972 Croteau *et al* [10] increased the yield of labelled peppermint terpenes by supplying sucrose with the mevalonate fed to the tissues and attributed the several fold increases to the relief of an energy deficiency in the sites of synthesis Addition of 10 mM sucrose did not stimulate incorporation, but the sorbitol used contains reducing sugars (0 3%) and so any sugar required could have been supplied from this source An oxygen-free atmosphere (Table 4) gave results similar to the control Centrifugation of a lysed chloroplast preparation at 2750 *g* for 20 min after addition of cofactors and [^{14}C]mevalonate gave a pellet of chloroplast fragments (0 2 ml) and an almost colourless supernatant (0 8 ml) fraction Both subsamples incorporated [^{14}C]mevalonate into ABA to a similar extent showing that the biosynthetic system for ABA can be solubilized

TABLE 4 EFFECT OF VARIOUS FACTORS ON THE BIOSYNTHESIS OF ABSCISIC ACID

Treatment	dpm in methyl ABA
Complete medium	144
O_2-free atmosphere, complete medium	151
Supernatant of lysed chloroplasts	137
Pellet of lysed chloroplasts	105
Sucrose (10 μmol) added	137
Unlabelled (±)-mevalonolactone (0 5 μmol) added	163

(±)-[2-^{14}C]Mevalonolactone (4 4 × 10^6 dpm) plus unlabelled MVA (70 μg) (17 hr) was added in each case One sample of lysed chloroplasts was centrifuged at 2750 *g* for 20 min, this gave a supernatant fraction (0 2 ml) and a pellet (0 8 ml) The oxygen free atmosphere was produced by connecting an inverted U-tube containing pyrogallol and KOH to the sample tube 3500 lx

The predominance of chloroplasts with starch granules and lamellae suggests that the majority came from green parts of the fruit Nevertheless, electron micrographs of colorless mesocarp showed that etioplasts were present, and as such colourless chloroplasts were found to biosynthesize ABA as rapidly as those from the outer green parts, and as slices of the central, white mesocarp have also been found to make ABA [11] it would appear that the etioplasts are equally effective (Table 5)

TABLE 5 EFFECT OF DIFFERENT CHLOROPLASTS AND OTHER FACTORS ON THE BIOSYNTHESIS OF ABSCISIC ACID

Treatment	dpm in methyl ABA
Chloroplasts from green mesocarp complete medium	430
Etioplasts from white mesocarp complete medium	486
Normal chloroplast preparation complete medium	500
(±)-Abscisic acid (0 19 μmol) added	1020
3-Amino-1 2 4-triazole (0 12 μmol) added	460
Chloroplasts from young leaves of an avocado seedling complete medium	734

(±)-[2-^{14}C]Mevalonolactone (4 4 × 10^4 dpm 0 5 μmol) (17 hr) was added to all samples The bean chloroplasts were prepared as shown either from the outer green parts of the mesocarp or the white inner part or a mixture as before Tube 6 contained chloroplasts extracted from green leaves of an avocado seedling (+)-Abscisic acid (0 19 μmol) was added to tube 4 and 3-amino-1 2 4-triazole (0 12 μmol) to tube 5 All chloroplasts were lysed as usual 3500 lx

[10] CROTEAU R BURBOTT A J and LOOMIS W D (1972) *Phytochemistry* **11**, 2932
[11] Unpublished data

Lysed chloroplasts isolated from young avocado leaves were also able to make abscisic acid, as was a complete preparation to which 3-amino-1,2,4-triazole had been added (Table 5) This compound is widely used as a herbicide and causes the development of colourless plants it did not have a noticeable effect on ABA synthesis An inhibitor of carotenoid biosynthesis, diphenylamine,[12] did not inhibit ABA biosynthesis in lysed bean leaf chloroplasts, nor did Sandoz 6706 (4-chloro-5-dimethylamino-2-α,α,α-trifluoro-3-tolyl-3-dihydropyrazinone), also reported[13] to inhibit carotenoid biosynthesis in higher plants (Table 6) Thus ABA appears to be made by reactions other than those of carotenoid biosynthesis

TABLE 6 EFFECT OF INHIBITORS ON THE BIOSYNTHESIS OF ABSISIC ACID

Treatment	dpm in methyl ABA
Complete medium	83
Sandoz 6706 (0 03 μmol) added	135
Diphenylamine (0 06 μmol) added	138

(±)-[2-^{14}C]Mevalonolactone (4 4 × 10^6 dpm, 0 5 μmol) (17 hr) was added to all samples The chloroplasts were prepared from the leaves of bean seedlings and lysed as before, 3500 lx

As less labelled ABA was present in the 27 hr incubation in Table 2 than in the 17 hr one it appears that the newly formed ABA is degraded Consequently unlabelled (±)-ABA (0 19 μmol) was added to reduce the loss by diluting the labelled ABA and thereby to delay its destruction The precedure yielded the highest dpm of the experiment (Table 5) and so ABA cannot inhibit its own synthesis in this tissue as it appears to do in wheat leaves[9]

It is possible that lysis allows the cofactors (rather than mevalonate) to penetrate into the chloroplast and stimulate the biosynthesis of ABA but this is considered unlikely because of the difficulty with which mevalonate penetrates into intact chloroplasts The intact organelles would be expected to have an adequate supply of cofactors to support the normal rapid rate of synthesis because intact leaf chloroplasts can synthesize carotenoids from CO_2 (and acetate to a lesser extent) but not from added mevalonate[7] The amount of mevalonate incorporated into ABA by avocado fruit pieces is extremely variable, even between fruits of one batch at apparently identical stages of ripening Each experiment was carried out on tissue from one fruit and the variations between the control (complete medium) values are attributed to differences between the fruits

DISCUSSION

A cell-free preparation is now available that can biosynthesize abscisic acid from mevalonate and so the constraints of cell and tissue permeability on its biosynthesis can be eliminated In the ripening avocado fruit it appears that the majority of the ABA is biosynthesized within the chloroplasts but Goodwin *et al*[7] have shown that there is a chloroplastic and an extrachloroplastic system able to convert mevalonate into terpenoids, so the occurrence of ABA synthesis outside chloroplasts, and in other tissues, cannot be discounted

[12] GOODWIN, T W and OSMAN, H G (1954) *Biochem J* **56,** 222
[13] BARTELS, P G and MCCULLOUGH, C (1972) *Biochem Biophys Res Commun* **48,** 16

Goodwin *et al*.[5-8] have found that the intact chloroplast membrane is highly impermeable to mevalonate and this barrier may account for the separation of newly synthesized ABA from that absorbed by the cell from the outside.[14] The occurrence of ABA in xylem[15] and phloem[16] saps and nectar[17] shows that it must be able to pass out of the chloroplasts (unless it can also be made outside them). The occurrence of this flux and the mechanism by which wilting causes the dramatic increase in the ABA content of leaves remain for investigation.

EXPERIMENTAL

Intact chloroplasts were prepared according to the methods of Walker[18] from a mixture of the inner white and peripheral, green zones of a peeled avocado fruit or from green leaves of bean (*Phaseolus vulgaris*) and avocado seedlings. A tissue sample (20 g) homogenized in 150 ml of an ice-cold solution of sorbitol (0·35 M), $MgCl_2$ (0·01 M), ascorbic acid (0·01 M) K phosphate buffer pH 7·2 (0·1 M) was filtered through eight layers of cheese cloth. The filtrate (6 × 10 ml) was centrifuged for 1 min (2750 *g* max) and the 6 pellets of intact chloroplasts were combined resuspended in fresh medium (10 ml) and recentrifuged. The packed volume of the mixture of chloroplasts and etioplasts varied between 0·15 and 0·25 ml, 20 g fruit tissue in different experiments. The protein content of a 0·2 ml plastid pellet (Biuret method, bovine plasma albumin as a standard) prepared from 20 g peeled fruit tissue was 0·48 mg. The chlorophyll content of the pellet was 15·2 μg (by Arnon's method[19]) whereas 196 μg were present in an equivalent sample of fruit (20 g). Thus *ca* 8% of the chloroplasts from 20 g fruit were present in an assay tube. The pellet was lysed in 0·5 ml $KHPO_4$ buffer pH 7·2 (0·1 M) for 1 hr and then combined with other preparations and subdivided to give chloroplasts extracted from 20 g fruit in each tube. Aqueous solutions of ATP (0·1 ml, 0·02 M), $MgCl_2$ (0·02 ml, 0·05 M), mercaptoethanol (0·05 ml, 0·2 M) solutions and a mixture of FAD, FMN, NAD, NADH, NADP, NADPH (each 0·02 M in the same 0·1 ml $KHPO_4$ buffer pH 7·2, 0·05 M) were added to each aliquot together with (±)-mevalonolactone ([2-$^3H_2$] 17 μCi/μM or [2-^{14}C] 17 μCi/μM) in 0·05 ml and H_2O (to 1·0 ml total vol). The reaction mixture was incubated for 17 hr in a stoppered glass tube in a waterbath at 28° and illuminated by fluorescent lamps or photoflood tungsten lamps. The reaction was stopped by adding MeOH (1 ml) and isolating the ABA as before.[4] Incorporation of the [^{14}C]mevalonate into ABA was determined according to the criteria described previously.[1,3] Briefly the ABA purified by TLC on silica gel in toluene–EtOAc–HOAc (50:30:4) should be methylated, chromatographed in hexane–EtOAc (3:1), eluted and reduced by $NaBH_4$ in ice-cold aqueous methanol to an equal mixture of the 1,4-*cis*- and 1,4-*trans*-diols of methyl abscisate. The presence of labelled material co-chromatographing with the respective markers, the equal distribution of the radioactivity between the diols together with the absence of any other labelled materials at this stage are considered to be adequate proof of the incorporation of labelled mevalonate into ABA. The UV absorption of the methyl abscisate isolated (Table 1) showed that between 74 and 86% (mean 80%) of the 28 μg added was recovered. The natural (+)-ABA content of a methyl abscisate fraction prepared without the addition of 14 μg (±)-ABA until the preparation was killed showed that 0·5 μg (+)-ABA and 12·0 μg of the racemate were recovered respectively. A sample of the fruit tissue contained 8 μg ABA g fr wt so about 4·6% of the (+)-ABA in the fruit can be calculated to have been present in the chloroplasts. However, this figure may have been distorted by ingress or egress of (+)-ABA during the isolation procedure.

Subsamples of the chloroplast preparation used in Table 3A and pieces of internal white and peripheral green avocado mesocarp were prepared for electron microscopy by fixation with glutaraldehyde (0·5 M) sucrose (0·263 M) Na phosphate buffer (pH 7·2, 0·2 M), stained with osmium tetroxide (0·079 M) in sucrose (0·058 M) embedded in araldite and sectioned at 50 nm.[20] Of the isolated chloroplasts 58% contained thylakoid membranes and starch grains; the remaining 42% were similar to etioplasts in sections of the white parts of the fruit which lacked these features.

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