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Gibberellins: Synthesis, Compartmentation and Physiological Process [and Discussion]

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Gibberellins: synthesis, compartmentation and physiological process

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This paper has two objectives: (1) to examine the nature of regulation of gibberellin (GA) biosynthesis and metabolism with respect to GA biological activity, and (2) to identify cellular compartments which play a rôle in biosynthesis, metabolism and release of GAs. The importance of *ent*-kaurene synthetase and its regulation for the production of GAs is emphasized. Similarly, factors regulating the enzymes mediating oxidation steps to GA_{12}-aldehyde and hydroxylation steps to GAs are identified. Specificity of hydroxylation for high and low biological activity as well as conjugation reactions resulting in deactivation of GAs are related to the problem of regulation of GA concentration in cells.

Compartmentation of GA synthesis and sequestration and release of GAs from organelles are discussed. The need for careful separation of organelles and membrane systems to assess properly their rôles in GA synthesis and compartmentation is stressed. The rôle of plastids in biosynthesis and release of GAs is analysed. Red light is known to enhance GA levels in intact tissues and plastid preparations; whether the increase is due to red light promoted conversion from 'bound' to 'free' GAs or to altered permeability characteristics of the chloroplast membranes is not known.

Metabolism of [^{3}H]GA_1 in protoplasts and vacuoles is described in a progress report. Protoplasts are capable of metabolizing GA_1 to GA_8, GA_8-glucoside and other metabolites, whereas isolated vacuoles appear to metabolize GA_1 only to GA_8.

I. Introduction

A review of the status of knowledge of the gibberellins (GAs) reveals a disconcerting dichotomy: much is known about the synthesis of GAs in the fungus *Gibberella fujikuroi* and in a few vascular plants, whereas the fundamental mechanism of action of these compounds is still obscure.

With regard to biosynthesis and metabolism† we now have a fairly cohesive picture of how gibberellins are formed but we have only a hazy image of the regulation of the steps which result in a maze of 52 currently identified GAs, including what Hedden *et al.* (1978) have called 'primary gibberellins'. These occur at or near the terminus of a pathway and possess high biological activity which is generally lost as a result of hydroxylation, conjugation, or both.

Underscoring the limited knowledge of the regulation of these pathways, and ultimately our perception of the physiological action of GAs, is the meagre understanding of the subcellular sites of biosynthesis, metabolism and compartmentation of these hormones and their derivatives. Such understanding is significant to the questions of (1) which pathway and, therefore, which GAs will be dominant in a species; (2) how cell and tissue concentration of GAs is determined; (3) whether pool size of endogenous GAs and their turnover rate influence subsequent biosynthesis and metabolism, and ultimately, physiological action of the hormone;

† No adequate terminology has as yet been developed to distinguish between synthesis of GAs and metabolism to inactive forms. Unless otherwise indicated, biosynthesis will mean the formation of all compounds up to and including the first GA precursor, GA_{12}-aldehyde. Metabolism will refer to all subsequent steps through the pathway, including conjugation. Conjugates will be identified by their specific structures, e.g. GA-glucosyl ethers, GA-glucosyl esters.

and (4), of major interest to plant biologists, whether applied gibberellins enter the same pathways as do the endogenous ones. It is the objective of this paper to consider selected aspects of regulation of biosynthesis and metabolism as they relate to compartmentation and release of GAs in cells.

Detailed reviews on biosynthesis and metabolism of GAs have been published by Cross (1968), Lang (1970), MacMillan (1971, 1974, 1977*a*, *b*), MacMillan & Pryce (1973), Barendse (1975), Bearder & Sponsel (1977), Hedden *et al.* (1978), and Graebe & Ropers (1978).

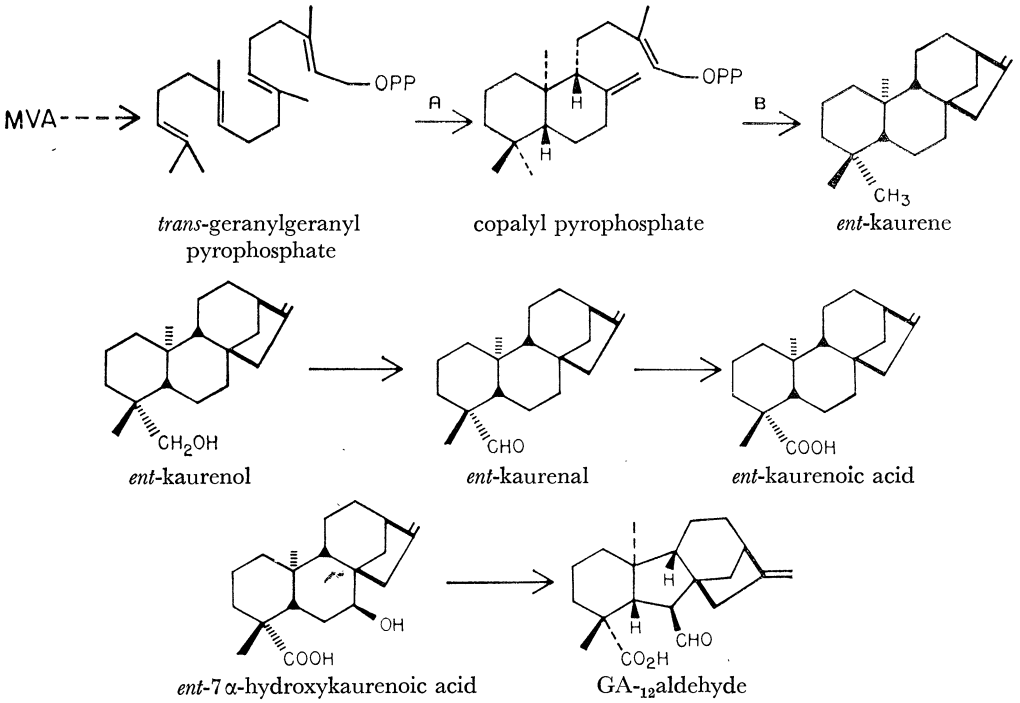

FIGURE 1. The biosynthetic pathway of gibberellin A_{12}-aldehyde.

II. THE BIOSYNTHETIC PATHWAY TO GA_{12}-ALDEHYDE

The GAs are diterpenoids which share with steroids, carotenoids and higher isoprenoids mevalonate as a common precursor. The main features of the biosynthetic pathway to the formation of the first gibberellin, GA_{12}-aldehyde, are depicted in figure 1. The first committed step in the biosynthesis of GAs is the formation of *ent*-kaurene from GGPP†. This reaction requires two cyclization steps, A and B, mediated by the complex enzyme *ent*-kaurene synthetase (West 1973). *ent*-Kaurene synthetase has been partly purified from *Gibberella fujikuroi* and *Marah* (*Echinocystis*) *macrocarpus*, and similarities and differences compared (Fall & West 1971; Frost & West 1977). In both studies it was difficult to separate activity A (conversion of GGPP to CPP) from activity B (conversion of CPP to *ent*-kaurene). A and B activities of *ent*-kaurene synthetase from both sources have different pH optima (7.3 and 6.9, respectively), quantitatively different responses to concentration of divalent ions, especially Mg^{2+}, Mn^{2+} and Ni^{2+}, and different responses to sulphydryl inhibitors (Frost & West 1977).

† The following abbreviations are used in this paper: IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; MVA, mevalonate; GGPP, geranylgeranyl pyrophosphate; CPP, copalyl pyrophosphate; Phosphon D, tributyl-2,4-dichlorobenzylphosphonium chloride; SKF-525A, *N*,*N*-dimethylaminoethyl-2,2-diphenyl pentanoate.

Examples of the sensitivities of steps in the biosynthetic pathway to divalent cations are shown in table 1. In *M. macrocarpus*, divalent ions, Mg^{2+} and Mn^{2+} in particular, were essential for A and B activities (Frost & West 1977). Mg^{2+} at 1 mM maximized activity A and Mn^{2+} at small concentration (0.1 mM) promoted an optimal response (table 1). A tenfold greater concentration of Mg^{2+} was necessary to optimize activity B; Mn^{2+} and Co^{2+} were less effective than Mg^{2+}. It is noteworthy that these results were obtained with systems *in vitro*. Whether ion flux participates in regulation of biosynthesis *in vivo* is not known, but would seem to be worth investigating.

TABLE 1. THE EFFECTS OF DIVALENT IONS ON BIOSYNTHESIS AND METABOLISM OF GIBBERELLINS

ion	concentration/mM	reaction	effect
Mn^{2+}	—	condensation of IPP with FPP	+
	—	condensation of IPP with GPP	+
	—	condensation of IPP with DMAPP	+
	0.1	GGPP → *ent*-kaurene	+
	>0.1	GGPP → *ent*-kaurene	−
	0.1	CPP (1.1 μM as substrate) → *ent*-kaurene	+
	>0.1	CPP (1.1 μM as substrate) → *ent*-kaurene	−
	—	GA_{12} aldehyde → GAs	−
Mg^{2+}	—	GPP → CPP → *ent*-kaurene	+
	1	GGPP (1 μM as substrate) → *ent*-kaurene	++
	10	CPP (1.1 μM as substrate) → *ent*-kaurene	++
Fe^{2+}	—	GA_{12} oxidation → GAs	+
Fe^{2+}	—	GA_1 hydroxylase → GA_8	+

+, Enhances; ++, maximal rate; −, inhibits.

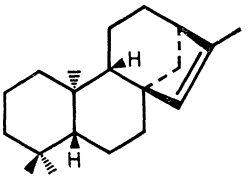

FIGURE 2. *iso*-Kaurene (shown here) is produced in high ratio to *ent*-kaurene in the gibberellin-deficient single gene recessive dwarf maize mutant *d-5*. *iso*-Kaurene accumulates and is not further metabolized to GAs (from Hedden & Phinney 1976).

Another significant insight into regulation of *ent*-kaurene synthetase activity has been obtained from studies of growth retardants which inhibit GA biosynthesis in plants in systems *in vitro* (Lang 1970). Inhibition of stem growth by a retardant is usually reversible by applied GA. Certain retardants were shown by Frost & West (1977) to affect differentially A and B activities of *ent*-kaurene synthetase. Using the *Marah macrocarpus* endosperm cell free system (Graebe *et al.* 1972), Frost & West found, for example, that Phosphon D inhibited primarily activity A, whereas SKF-525A and several other inhibitors blocked both A and B activities. Whether 'endogenous' retardants occur naturally and play a rôle in regulation of the biosynthetic pathway is not known. West (private communication) has stated that an intensive search for such compounds has not been fruitful.

How critical the regulation of *ent*-kaurene synthesis is in the gibberellin pathway is seen in the novel research of Hedden & Phinney (1976) and Hedden *et al.* (1978). Using a cell-free system from coleoptiles of a gibberellin-deficient single gene recessive corn (*Zea mays*) mutant,

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d-5, they found preferential accumulation of *iso*-kaurene (figure 2), an isomer of *ent*-kaurene that is apparently biologically inactive. *iso*-Kaurene is not further metabolized to gibberellins by *G. fujikuroi*. However, the limited synthesis of *ent*-kaurene detected in the *d-5* cell-free system indicates that the mutant is leaky and that the amount produced is available for base level growth of the dwarf plant.

Further oxidation of *ent*-kaurene leads to synthesis of *ent*-7α-hydroxykaurenoic acid (figure 1), the steps catalysed by mixed function oxidases associated with the microsomal fraction (Dennis & West 1967; Graebe *et al.* 1965; Murphy & West 1969; West 1973). The reaction requires a reduced pyridine nucleotide (NADPH) and O_2. The participation of cytochrome P450 is indicated by inhibition of the reaction by CO and its maximum reversibility at an irradiation of 450 nm. Heat-stable soluble and heat-labile non-dialysable cofactors in the supernatant from immature *M. macrocarpus* seeds were reported to enhance the oxidase activity by Hasson & West (1976). They also found that biosynthetic capacity changed with stage of seed development. Thus, biosynthetic steps may be regulated by as yet unidentified factors and biosynthesis of GA precursors may be correlated with stage of development.

III. Metabolism of gibberellins

1. *Metabolic pathways*

The purpose of this section is to identify the major steps leading to formation of biologically active GAs and the reactions leading to loss of biological activity. These are important as they relate to the problem of compartmentation (§ IV). The pathway leading to synthesis of GA_{12}-aldehyde appears to be identical in several plant species and in *G. fujikuroi*. The formation of GA_{12}-aldehyde results from ring B contraction of *ent*-7α-hydroxykaurenoic acid (figure 1). This compound is also at a branch point for two other pathways, but these do not yield GAs (Hedden *et al.* 1978). Thus identification of the factors favouring the prevailing pathway should give important insight into regulation of GA metabolism.

Hedden *et al.* (1978) and Graebe & Ropers (1978) have summarized the known steps in the metabolic pathways in several species, particularly *G. fujikuroi*, *C. maxima*, *P. sativum* and *P. vulgaris*. The information has been obtained by studies of cell-free systems with [^{14}C]MVA and labelled *ent*-kaurenoids. They have been established by gas chromatography–mass spectroscopy analysis and stepwise refeeding of the metabolites. The differences in pathways after GA_{12}-aldehyde stem primarily from capacity of each species to hydroxylate at the C-3 or C-13 positions, or both. For example, C-3 and C-13 hydroxylations of GA_{12}-aldehyde occur early in *Phaseolus vulgaris*, only 3-hydroxylation is found in *C. maxima* (Graebe *et al.* 1974) and, primarily, the C-13 position is hydroxylated in pea (Ropers *et al.* 1978).

In *C. maxima* the conversion of [^{14}C]MVA to GA_{12}-aldehyde and GA_{12} is mediated by the low-speed supernatant of an endosperm homogenate. Inclusion of Mn^{2+} in the medium to favour *ent*-kaurene synthetase activity, however, inhibited GA metabolism beyond GA_{12}-aldehyde by competing with Fe^{2+}/Fe^{3+} which is required for the reaction (Graebe & Hedden 1974; Patterson & Rappaport 1974; Patterson *et al.* 1975). When Mn^{2+} was eliminated from the incubation, C-20 and C-19 GAs were produced (Graebe *et al.* 1974; Hedden *et al.* 1978). Again, divalent ions appear to play a regulatory rôle in GA synthesis.

Metabolism beyond GA_{12}-aldehyde may involve many steps; however, relatively few of the GAs produced may be classified as primary GAs, those having high biological activity and

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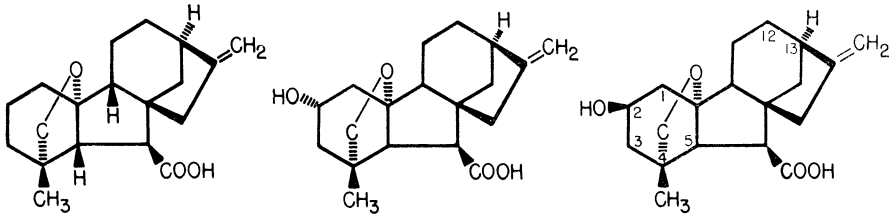
which are subsequently metabolized to inactive GAs. It appears that relatively few of the identified GAs are significant in physiological processes.

2. *Hydroxylation reactions*

The extent and position of hydroxylation markedly influence biological activity and capacity for subsequent hydroxylation and conjugation. Although there is ample evidence that many of the hydroxylating enzymes are non-specific in that they will accept a number of substrates, it is now certain that 2β-hydroxylation is under tight stereochemical constraint and the reaction requires NADPH and O_2. It is also favoured by Fe^{2+}. These conclusions are drawn from feeding studies with GA_1 and its analogues, analysis of the capacity of a cell-free enzyme system to hydroxylate GA_1 but none of its biologically inactive analogues, and from studies of the effect of 2α- and 2β-hydroxylation of GA_9 on its biological activity.



	GA_9	GA_{40} (2α)	GA_{51} (2β)
lettuce hypocotyl	+ + + +	+	0
pea stem	+	0	0
cucumber hypocotyl	+ + +	+ +	0
dwarf rice	+ +	+	0

FIGURE 3. The effects of 2α- and 2β-hydroxylation of GA_9 on biological activity detected on four bioassays. 2α-hydroxylation of GA_9 (GA_{40}) results in reduced biological activity while 2β-hydroxylation nullifies activity completely (from Sponsel *et al.* 1977).

In two studies *in vivo*, Stolp *et al.* (1973, 1977) measured uptake and metabolism of [^{3}H]GA_1 and its biologically inactive 3-hydroxy epimer (pseudo-GA_1) by barley half seeds and aleurone layers. Although a greater percentage of pseudo-GA_1 than of GA_1 was taken up, virtually none of the epimer was metabolized, whereas GA_1 was metabolized, primarily to ampho-GA_1, a highly polar, amphoteric conjugate postulated to be a peptide (Nadeau & Rappaport 1974*a*). The investigation was extended by Stolp *et al.* (1977) with a series of analogues of [^{3}H]GA_1 which, like the 3-OH epimer, had no biological activity in the barley aleurone system. Whereas GA_1, GA_1-methyl ester, dihydroGA_1 and '16-keto-GA_1' (the acid-hydrolysed Wagner–Meerwein rearrangement product of GA_1), were metabolized by barley aleurone layers, none of the metabolites were found to be GA_8 derivatives.

Evidence that stereochemistry of the GA_1 structure plays a major rôle in 2β-hydroxylation was obtained by Patterson & Rappaport (1974) and Patterson *et al.* (1975). They partly purified an enzyme from cotyledons of *P. vulgaris* which specifically hydroxylates the 2β position of GA_1. Using GA_1, pseudo-GA_1 and 16-keto-GA_1 as substrates, they found that only the bioactive GA_1 was metabolized to GA_8. Thus it was apparent from these studies that alteration of the stereochemistry of the C-3 hydroxyl, methyl esterification of the C-7 carboxyl, rearrangement of the C/D rings, or dehydration of the C-16 methylene group resulted in inhibition of 2β-hydroxylation.

Sponsel *et al.* (1977) made a detailed study of the effect of 2-hydroxylations of GA_9 on

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biological activity. The data in figure 3 compare the effects of GA_9, 2α-hydroxyl GA_9 (GA_{40}) and 2β-hydroxyl GA_9 (GA_{51}) in four bioassay systems. Activity of GA_9 ranged from low to high and 2α-hydroxylation reduced activity overall. However, as with 2β-hydroxylation of GA_1, 2β-hydroxylation of GA_9 totally nullified its biological potency.

Analogies with the effects of 2β-hydroxylation of GA_1 and GA_9 on biological activity are seen in conversion of GA_{20} to GA_{29} in pea (Railton *et al.* 1974; Frydman & MacMillan 1975), and *Bryophyllum daigremontianum* (Durley *et al.* 1975) and of GA_4 to GA_{34} in pollen of *Pinus attenuata* (Kamienska *et al.* 1976).

3. *Conjugation*

Gibberellin conjugates are natural products found in many tissues, most copiously in maturing seeds, e.g. *Phaseolus multiflorus* (Sembdner *et al.* 1968), *Pharbitis nil* (Yokota *et al.* 1971), *Phaseolus vulgaris* (Hiraga *et al.* 1974), and *Cystisus scoparius* (Yamane *et al.* 1975, 1977). Yamane *et al.* (1977) were unable to detect either glucosides or glucosylating enzymes in immature seeds of *P. vulgaris*. Conjugates have also been isolated after radioactive precursors were fed to various tissues and organs (Nadeau & Rappaport 1972, 1974; Nadeau *et al.* 1972; Davies & Rappaport 1974; Rappaport *et al.* 1974; Stoddart & Jones 1977). All natural conjugates so far identified in plant extracts are either C-7 glucosyl esters or glucosyl ethers. The glucosyl ethers such as GA_8-*O*-(2)-β-D-glucosylpyranoside (GA_8-glucoside) show virtually no activity in biological assays (Sembdner *et al.* 1968, 1976; Yokota *et al.* 1971; Yamane *et al.* 1973) although glucosyl esters often have activity similar to that of the underivatized parent GA (Yokota 1971; Sembdner *et al.* 1976). Activity of GA glucosyl esters was attributed by Yokota *et al.* (1971) to deesterification by microorganisms in the growing medium since sterile plants showed virtually no response on a number of assays.

Glucosyl ethers have been considered to be storage forms (Sembdner *et al.* 1968, 1976); however, evidence of recycling of such conjugates is not overwhelming (Musgrave & Kende 1970; Davies & Rappaport 1974; Rappaport *et al.* 1974). It is likely that these products are sequestered as part of a 'detoxification process', thereby contributing to the overall maintenance of the level of GA_1 in the system (Rappaport *et al.* 1974). How important conjugation is for regulation of physiological processes will be better understood when their ultimate fate in the cell is clarified.

IV. Compartmentation

From the foregoing sections it is evident that a plethora of GAs are assembled along pathways which vary with species, that their biosynthesis and metabolism are mediated by soluble and microsomal enzymes, and that the reactions are modified by cofactors, cations and availability of precursors. To obtain a better understanding of the factors determining a particular pathway and of regulation of concentration in cells it is necessary to understand the subcellular sites of biosynthesis, metabolism and compartmentation of the precursors and products as well as the conditions governing uptake and efflux from the cell.

In this section we discuss current knowledge of compartmentation of GAs and present previously unpublished data indicating a rôle for the vacuole as a site of active metabolism. The focus is on organelles and their contribution to the oxidation and hydroxylation processes in the GA pathway as well as significance for action. This is a relatively new field, and interpretation of results is tenuous because of the problems of obtaining pure organelles, assaying activity of extracted hormones and characterizing the products of metabolism.

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1. *Endoplasmic reticulum*

The stepwise oxidation reactions from *ent*-kaurene to *ent*-7α-hydroxykaurenoic acid (figure 1) are mediated in *G. fujikuroi* and a number of plant species by mixed function oxidases. Mixed function oxidases are generally associated with endoplasmic reticulum (e.r.) and this appears to be true for the *ent*-kaurenoid oxidative system as well (West 1973). It should be stressed here, however, that membrane fragments other than those from e.r. may occur in usual microsomal preparations. Madyastha *et al.* (1977) showed that the tonoplast membrane preparation accounts for 95 % of the C-10 hydroxylation of the monoterpene alcohols nerol and geraniol in *Catharanthus* cells. There is no evidence for e.r.-associated enzymes responsible for hydroxylation past GA_{12}-aldehyde; such enzymes remain in the high speed supernatant although they possess many of the characteristics of mixed function oxidases (Patterson & Rappaport 1974; Patterson *et al.* 1975).

2. *Mitochondria*

The contribution of mitochondria as possible compartments in gibberellin metabolism and sequestration of derivatives has barely been explored. Green *et al.* (1975) showed mitochondria from *Ricinus communis* contain the necessary enzyme complement to synthesize GGPP. However, GGPP is a precursor of other products synthesized in mitochondria and its occurrence is not sufficient evidence from which to draw conclusions about participation of mitochondria in GA biosynthesis.

Simcox *et al.* (1975), who explored mitochondria as a possible compartment for the production of *ent*-kaurene, concluded that little or no *ent*-kaurene was produced by mitochondria. Whether mitochondria play a rôle in sequestration of metabolites is not known.

3. *Plastids*

Chloroplasts are important in terpene biosynthesis (Goodwin & Mercer 1963) and a growing literature implicates plastids in gibberellin metabolism. Hedden *et al.* (1978) have detailed the evidence for this conclusion. Etioplasts are capable of utilizing cytoplasmically synthesized MVA, but illumination results in loss of permeability, and chloroplasts are incapable of taking up MVA (Wellburn & Hampp 1976; Cockburn & Wellburn 1975). Synthesis of GA by illuminated plastids would require them to become autotrophic with respect to MVA synthesis.

Simcox *et al.* (1975) found that proplastid fractions from etiolated pea (*Pisum sativum*) shoot tips, castor bean (*R. communis*) endosperm and *M. macrocarpus* endosperm all catalysed *ent*-kaurene synthesis from CPP. Isolated proplastids from *M. macrocarpus* endosperm synthesized *ent*-kaurene from either GGPP or CPP. However, plastids from etiolated pea shoot tips or castor bean endosperm were essentially incompetent to convert GGPP to *ent*-kaurene. Recently, Moore & Coolbaugh (1976) were able to show conversion of GGPP to *ent*-kaurene in extracts of sonicated chloroplasts from *P. sativum*.

Direct proof that MVA is converted to *ent*-kaurene in chloroplasts is difficult to obtain because of technical difficulties. When isolated in aqueous medium, plastids lose the enzyme MVA kinase which is necessary for activation of early steps of terpenoid synthesis. Isolated in non-aqueous medium, however, the chloroplasts retain MVA kinase activity (Buggy *et al.* 1974), making it likely they can synthesize *ent*-kaurene from MVA.

Stoddart (1968, 1969) presented the first evidence that chloroplasts of barley and kale, *Brassica oleraceae*, contain significant amounts of GA-like substances. Sonicated chloroplasts

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converted 17-[^{14}C] *ent*-kaurenoic acid into GA-like substances. Similarly, Murphy & Briggs (1975) found that barley chloroplasts incorporate *ent*-kaurenol into *ent*-kaurenal. *ent*-Kaurenoic acid was converted into *ent*-hydroxykaurenoic acid. Thus it is likely that the chloroplast is capable of supporting the entire GA biosynthetic pathway.

That isolated chloroplasts are competent to metabolize GAs is seen in the studies of Railton & Reid (1974) and Railton (1976, 1977). [^{3}H]GA_{20} was metabolized by sonicated chloroplasts from 'Alaska' pea shoots into [^{3}H]GA_{29}, and [^{3}H]GA_{9} was converted into [^{3}H]GA_{10}, possibly an artefact, and 16,17-dihydro 16,17-dihydroxy [^{3}H]GA_{9}. From their experiments and our own observations (Adams & Rappaport, unpublished), it appears that the amounts of labelled GAs taken up by chloroplasts are very small.

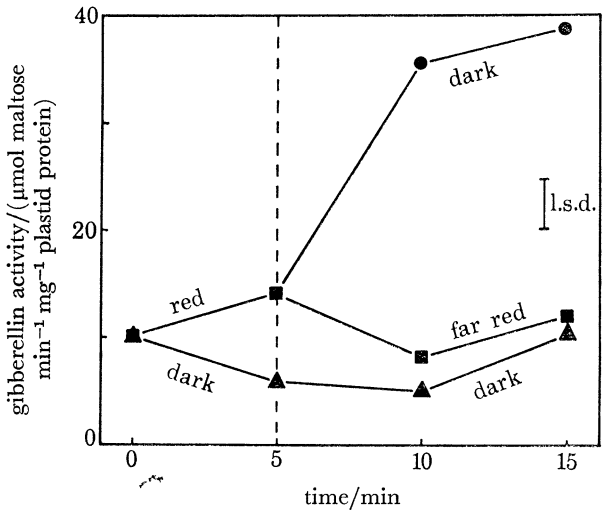

FIGURE 4. Red and far-red light effects on gibberellin activity extractable from intact barley etioplasts. Activity was measured by a modification of the barley aleurone bioassay of Jones & Varner (1967) (from Evans & Smith 1976).

The above discussion points to plastids as organelles involved in the earliest stages of GA biosynthesis. There is also some evidence that release of GAs from plastids may be governed by the chloroplast envelope. This was first suggested by the fascinating observation by Reid *et al.* (1968) that brief exposures of barley leaves to red light (660 nm) for as little as 15 min resulted in a rapid increase in endogenous GA level, followed by an equally rapid decline in level. In a similar vein, Stoddart & Lang (1968) found that daylength altered concentration of endogenous GAs in *Trifolium pratense*. The effects of red light on concentration of GAs in leaves indicated the participation of phytochrome. Studies by Beevers *et al.* (1970) with wheat leaves, Kohler (1971) with dwarf and tall peas and Reid *et al.* (1972) with homogenates of etiolated barley leaves, indicated that red light enhanced production of GAs. Far-red light partly reduced GA production in etiolated barley leaf homogenates (Reid *et al.* 1972).

These results prompted research into the effect of red light on release of gibberellins by etioplast membranes (Cooke *et al.* 1975; Cooke & Saunders 1975; Cooke & Kendrick 1976; Evans & Smith 1976*a*; and Browning & Saunders 1976). Cooke *et al.* (1975) measured an increase in concentration of acidic ethyl acetate soluble substances in response to 20 min red irradiation of intact and sonicated etioplasts. The effect was enhanced by 15 min of darkness after red light exposure, but a far-red light treatment reversed the effect of red light. Cooke *et al.* (1975)

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extended these observations by showing that the GA activity resulting from exposure to red light was localized mainly in the etioplast fraction and that similarly treated etiolated wheat leaves behaved in an identical manner. They also found that etioplast preparations exhibited photoreversible changes in absorbance at 660 nm and 730 nm characteristic of the response of phytochrome.

TABLE 2. GIBBERELLIN-LIKE ACTIVITY (micromoles maltose per minute per milligram protein) IN INTACT ETIOPLAST PREPARATIONS SONICATED BEFORE EXTRACTION (FROM EVANS & SMITH 1976*a*)

	dark	5 min red light	5 min red light + 5 min dark		
etioplasts ...	T	T	T	P	S
intact	4.0	10.8	22.0	3.0	24.1
sonicated	17.5	17.9	24.9	—	—

T, total suspension; P, pellet; S, supernatant.

Probing further the nature of GA release from barley etioplasts, Evans & Smith (1976*a*) found that far-red light reversed the red light induced increase in acidic ethyl acetate soluble GA-like substances detected by the lettuce hypocotyl assay (figure 4). In addition, they found that both light and dark-treated sonicated etioplast suspensions yielded considerably more than intact GA activity etioplasts (table 2). Cooke *et al.* (1975), Cooke & Kendrick (1976) and Evans & Smith (1976*a*) showed that phytochrome was associated only with the etioplast fraction, making it probable that phytochrome is contained within or is bound to etioplasts. Moreover, in an analysis of the subcellular localization of phytochrome, Evans & Smith (1976*b*) showed that etioplast envelopes accounted for essentially all of the phytochrome extracted from crude etioplast homogenates.

The results of these investigations were in general agreement that release of GAs from etioplasts after red illumination was due to the action of phytochrome altering permeability of the envelope to GA.

Cooke & Kendrick (1976) added a new twist to the story with the observation that red light altered the metabolism of GAs in isolated etioplasts. In results reminiscent of those of Loveys & Wareing (1971) and Cooke *et al.* (1975), they showed a decrease in polar 'bound' GAs and an increase in ethyl acetate-soluble 'free' GAs after red irradiation of leaves. Cooke & Kendrick (1976) concluded that red light acts by promoting conversion of 'bound' to 'free' GAs. Unfortunately, all the GA measurements in the above investigations were made with biological assays and the differences detected were usually very small. Larger yields of hormone and physical methods of identification are required to validate the results.

If GAs are associated with lipophilic sites in the membrane, it might be possible to release the GAs by altering membrane structure. To test this possibility, Browning & Saunders (1976) compared GA activity obtained from pelleted wheat chloroplasts extracted with (*a*) buffered 2 % Triton X-100, a nonionic detergent known to disperse membranes, (*b*) with 80 % methanol or (*c*) with methanol after sonication. Treatment with the detergent alone resulted in a startling 1000-fold increase in the amount of GA released as compared with the other methods. Enough was obtained from 10 g of leaves to identify GA_4 and GA_9 by gas chromatography–mass spectroscopy. At the concentration they reported in the detergent extract, there is approximately one molecule of GA_9 present per 10 molecules of chlorophyll. Regrettably, these results

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have not been reproduced in the literature. It is yet to be determined that the method is generally applicable for extraction of GAs. Moreover, the questions of whether GAs are produced in plastids, where they are localized, and how much is actually present remain to be answered.

It is obvious that chloroplasts should receive the attention they have had with respect to GA formation and release. However, the participation of other organelles in GA biosynthesis, metabolism and compartmentation should not be overlooked. Each of the major organelles is a biosynthetic unit, so it will come as no surprise if other organelles than plastids are found to participate in synthesis, sequestration or release of GAs.

4. *Vacuoles*

The participation of vacuoles in hormonal regulation has been inferred from physiological studies (Goldsmith 1977), but direct studies on isolated vacuoles have been possible only recently as a result of the breakthrough by Wagner & Siegelman (1975) who isolated intact vacuoles on a large scale from petals and leaves. Vacuoles have been viewed variously as (*a*) 'sewers' in which cytoplasmically synthesized products are sequestered, (*b*) as lysosomes and (*c*) as organelles capable of sustaining metabolic activity.

Sequestration in the vacuoles of a product synthesized in the cytoplasm is illustrated by the work of Saunders & Conn (1978). They showed that the vacuoles of sorghum, *Sorghum bicolor*, accumulate up to 90 % of the cyanogenic glucoside dhurrin, which comprises 25–30 % of the dry mass of the cell. Similarly, Buser & Matile (1976) detected a large percentage of malic acid in the vacuole of *Bryophyllum* leaves, but all the enzyme activity associated with synthesis of malic acid was found in the cytoplasm. Thus, malic acid was synthesized in the cytoplasm but accumulated in the vacuole. They also found that K^+ and Ca^{2+} ions accumulated in the vacuoles as well as about 50 % of the amino acids measured in the protoplasts.

Evidence that the vacuole has a regulatory rôle is found in the work of Lin *et al.* (1977) who detected high ATPase activity in tonoplast preparations from *Hippeastrum* and *Tulipa* petals. There was a difference in sensitivity of the tonoplast and protoplast ATPases to oligomycin. Only the vacuolar ATPase was insensitive, an observation that may be useful in characterizing tonoplast membranes. Lin *et al.* (1977) concluded that ion balance may be regulated by vacuoles. Participation of the tonoplast in ion regulation may, in turn, be significant for regulation of GA synthesis in view of the cation requirements for biosynthesis and metabolism (table 1).

Excellent evidence that vacuoles play an active metabolic rôle was provided by Madyastha *et al.* (1977) who showed that vacuoles of *Catharanthus* contain a monooxygenase with an associated haem protein, cytochrome P-450, which hydroxylates C-10 methyl groups of geraniol and nerol. This hydroxylation is an important step in the biosynthesis of indole alkaloids.

As a step toward improving our understanding of the rôle of organelles in GA biosynthesis and metabolism, we are investigating uptake and metabolism of [^{3}H]GA_1 by isolated protoplasts and vacuoles. Current objectives are to determine whether isolated vacuoles have the capacity to metabolize GAs and whether they play a rôle in the compartmentation of GAs or GA derivatives. We incubated barley (*Hordeum vulgare*) protoplasts and vacuoles with tritiated GA_1 according to the methods of Nadeau & Rappaport (1974*a*). Probable occurrence of GA_1 has been reported in *Hordeum vulgare* (Faull *et al.* 1974) and we have studied the metabolism of [^{3}H]GA_1 in barley aleurone layers and half seeds (Nadeau & Rappaport 1972, 1974*a*; Stolp *et al.* 1973, 1977).

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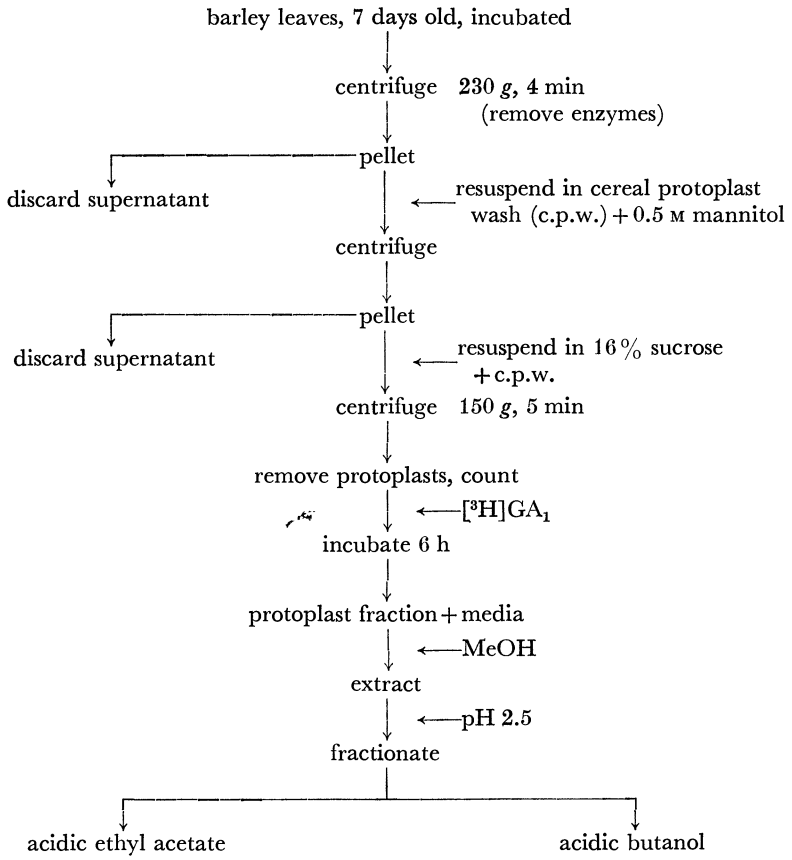
(*a*) *Protoplasts*

Protoplasts were enzymically isolated from barley leaves 7 days old and incubated in [^{3}H]GA_1 (a count of 1.23×10^7 min^{-1}) for 6 h according to the procedures shown in table 3. After incubation for 6 h in [^{3}H]GA_1, protoplasts and the incubation medium were extracted with 80 % methanol which was evaporated to the water phase. This phase, adjusted to pH 2.5,

TABLE 3. PROTOCOL FOR THE ISOLATION OF PROTOPLASTS FROM BARLEY LEAVES AND FOR INCUBATION WITH [^{3}H]GA_1

(for details see § VI)



was partitioned first with ethyl acetate, and then with butanol. The extracts were concentrated and streaked on ChromAR strips (5 cm × 20 cm) which were developed with isopropanol–3N NH_4OH (8:1 by volume) and then with benzene–acetic acid (4:1 by volume). The chromatograms were cut into ten equal segments and radioactivity of each measured by liquid scintillation spectrometry.

The ethyl acetate fraction had a large percentage (48 %) of unmetabolized [^{3}H]GA_1. There was a broad region of radioactivity with a peak at R_f 0.7 which corresponded with that of a GA_8 standard. This radioactivity amounted to 29.5 % of the total radioactivity in the incubation medium. The butanol fraction had a major zone of radioactivity at R_f 0.5–0.6, the R_f of GA_8-glucoside, and no indication of the presence of GA_8 (figure 5). GA_8-glucoside accounted for 8.9 % of the total radioactivity. Figure 6 presents gas chromatography–radiocounting

(g.c.r.c.) data for the provisional identification of GA_8 and GA_1 at R_f 0.7–0.8. Methods were reported by Nadeau & Rappaport (1972). These results indicate that isolated protoplasts are competent to metabolize GA_1 to GA_8 and GA_8-glucoside and to other as yet unidentified products.

In another experiment, protoplasts were incubated in [^{3}H]GA_1 (count of 6.3×10^6 min^{-1}) for

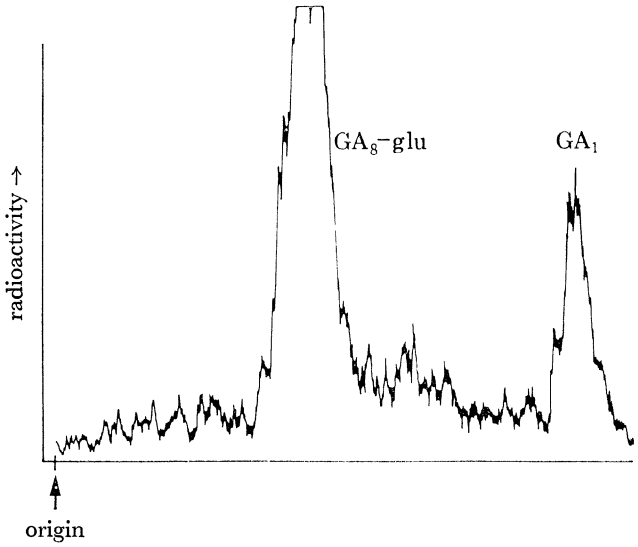


FIGURE 5. Radioscan of the acidic butanol fraction obtained from a methanol extract of barley protoplasts that had been incubated with [^{3}H]GA_1. Only unmetabolized [^{3}H]GA_1 and [^{3}H]GA_8-glucoside were detected.

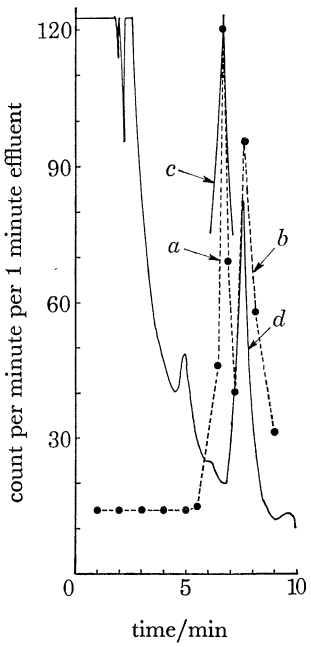


FIGURE 6. G.c.r.c. of the methyl esters–trimethylsilylated ethers of radioactive GA_1 (*a*) and GA_8 (*b*) obtained from R_f 0.7–0.8 of a thin layer chromatogram. Derivatives of standard GA_1 (*c*) and GA_8 (*d*) were co-injected for comparison. The samples were obtained from the acidic ethyl acetate fraction of the aqueous phase of an extract of barley protoplasts which had been incubated with [^{3}H]GA_1. The column was 2% QF-1 and the temperature was 215 °C.

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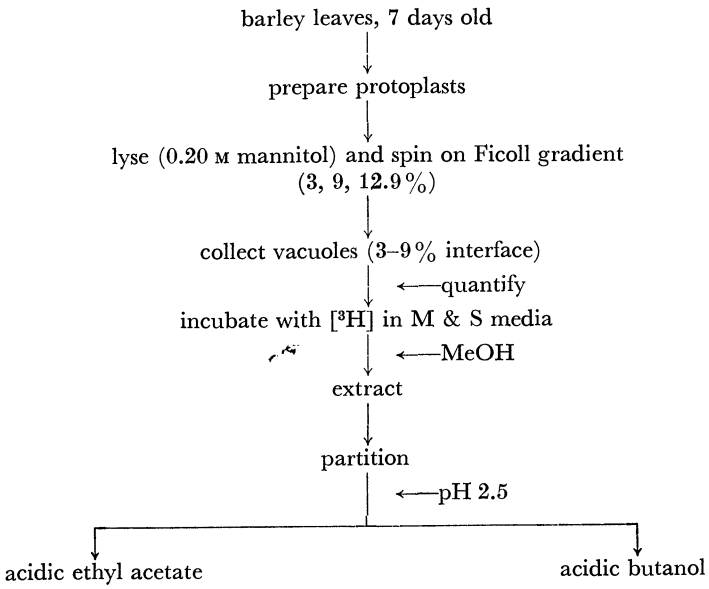
12 h and the medium was partitioned and chromatographed on t.l.c.–ChromAR as described above. The chromatograms were cut into ten segments and the eluted radioactive compounds were counted by scintillation spectrometry. Of the GA_1, 64 % remained unmetabolized. GA_8 accounted for 15.3 % and GA_8-glucoside for 2 % of the total radioactivity fed as GA_1. GA_8-glucoside was provisionally identified by g.c.r.c. Interestingly, significant radioactivity was found at R_f 0.4–0.6 in the ethyl acetate fraction which did not correspond with GA_8 either on t.l.c. or on g.c.r.c. Similarly, radioactivity was detected at R_f 0.1–0.2 and 0.6–0.7 in the butanol fraction which did not correspond with a GA_8-glucoside standard. Identification of these compounds is currently being pursued. The results indicate that GA_8 is the major metabolite, and that a small but significant amount of GA_8-glucoside is formed in protoplasts. However, other products amounting to 19 % of the total radioactivity remain to be accounted for.

TABLE 4. PROTOCOL FOR ISOLATION OF VACUOLES AND INCUBATION IN [^{3}H]GA_1



(*b*) *Vacuoles*

Vacuoles were prepared and incubated according to procedures of table 4 and as described in § VI. Figure 7 is a photomicrograph of a typical preparation of vacuoles. The vacuoles with the incubation medium were extracted with methanol and the aqueous phase partitioned into acidic ethyl acetate and butanol fractions as described above. Identification of metabolites was again by g.c.r.c.

The results from three experimental incubations of vacuoles with [^{3}H]GA_1 are given in table 5. At a pH of 5.7, after 4 h of incubation, 8.9 % of the total radioactivity supplied was metabolized to GA_8. When tabulated as radioactivity per vacuole, we found a count of 0.57 min^{-1} GA_8 per vacuole. In an incubation for 5 h at pH 4.6, this was only 0.17 min^{-1} GA_8 per vacuole, whereas at pH 5.7 there was nearly twelve times as much per vacuole.

Surprisingly, the capacity for further metabolism of GA_8 by isolated vacuoles was almost nil. No more than a trace of the total radioactivity supplied was recovered as GA_8-glucoside

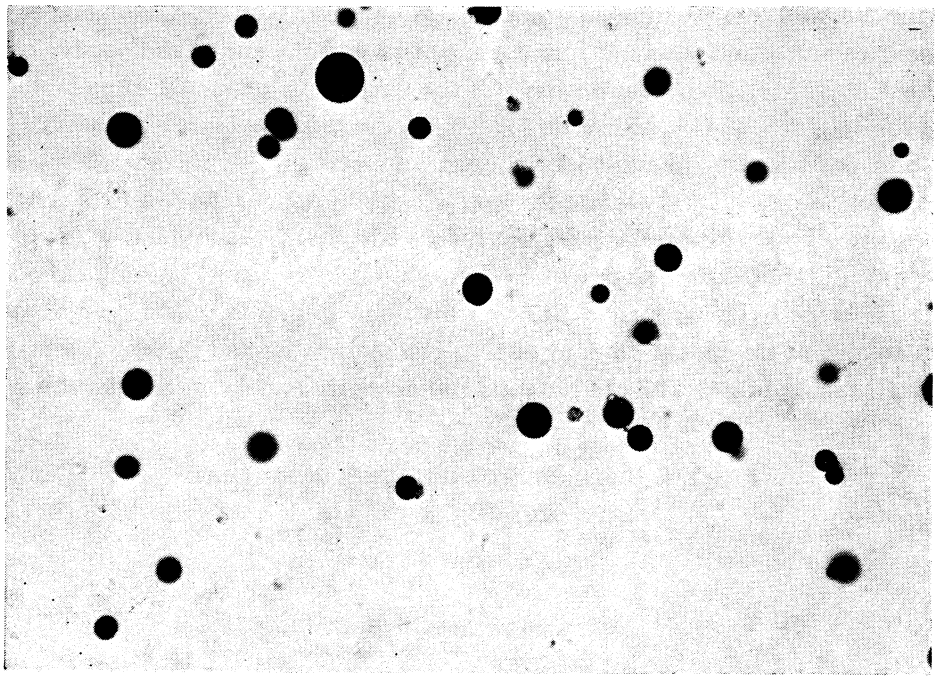


FIGURE 7. Barley vacuoles, stained with neutral red, isolated according to the procedures shown in table 4 and in §VI.

TABLE 5. METABOLISM OF [^{3}H]GA_1 BY VACUOLES FIRST ISOLATED FROM PROTOPLASTS OF 7 DAYS OLD BARLEY LEAVES AS DETECTED BY SCINTILLATION COUNTING OF ELUATES FROM THIN LAYER CHROMATOGRAMS

experiment no.	pH	incubation time h	10^{-5} × no. of vacuoles	10^{-6} × radioactivity in medium (count/min)	% radioactivity as GA_8	% radioactivity as GA_8-gly	radioactivity per vacuole as GA_8 (count/min)
1	5.7	4	7.2	4.6	8.9	trace	0.57
2A	5.7	5	2.9	2.5	22.9	trace	1.97
2B	4.6	5	12.2	1.76	12.1	trace	0.17

(table 5) and no other peaks of radioactivity were detected. This is in contrast to the results with protoplasts in which GA_8-glucoside and other metabolic products were found.

V. DISCUSSION

These results indicate that isolated vacuoles are capable of metabolizing GA_1 to GA_8 and virtually no other products. In contrast, protoplasts metabolize GA_1 to GA_8-glucoside and, apparently, two other products. It is premature to speculate on the implications of these results although it is interesting to note the report of Hartmann *et al.* (1977) that alkylation of cycloartenol, a triterpene, is mediated by a microsomal enzyme, whereas glucosylation is mediated by a transferase associated with plasmalemma. Their results suggest a spatial separation, a kind of compartmentation, between alkylating and glucosylating enzymes. It remains to be seen whether such compartmentation applies to GA_1 metabolism.

Isolation of clean vacuoles is not easy to accomplish and contamination by adherent cytoplasmic material is a particular danger in metabolic studies. The vacuolar preparations from

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barley leaves used in these experiments were checked microscopically for contamination and the vast majority of the vacuoles were found to be free of adherent materials. Not yet accomplished in these studies are specific assays for detecting contamination by cytoplasmic constituents.

VI. Experimental

1. *Preparation of protoplasts and vacuoles*

Protoplasts were prepared by a modification of the procedure of Saunders & Conn (1978) for *Sorghum bicolor* since it permits separation of vacuoles cleanly from chloroplasts and cellular debris. The details of this method are as follows and in table 3.

The apical 15 cm of the first leaf of barley are abraded with carborundum, rinsed with distilled water and plasmolysed with 0.5 M mannitol in phosphate–citrate buffer, pH 5.5. The leaves are incubated with shaking for 4 h at 37 °C in KH_2PO_4–citrate buffer containing macerase (Calbiochem) (5 g/l) and cellulase (Onozuka R-10) (15 g/l).

The digest is centrifuged at 230 *g*, the supernatant discarded and the pellet resuspended in 0.5 M mannitol and 'cereal protoplast wash' (c.p.w.) (M. Banks, private communication), a mixed salt solution. The wash is repeated and the protoplast pellet resuspended in sucrose (160 g/l) and c.p.w., and centrifuged. The clean protoplasts accumulate at the top of the sucrose solution and are removed with a Pasteur pipette.

These protoplasts are then ready for incubation experiments. They are maintained during incubation in Murashige–Skoog medium (1962) minus hormone and containing 0.5 M mannitol, sucrose (20 g/l) and ampicillin (100 μg/ml) to retard bacterial contamination.

Intact vacuoles were prepared by pelleting the protoplasts at low speed and lysing them by diluting the medium to 0.20 M mannitol with Tris–HCl (pH 8.0, containing 10 mg/ml bovine serum albumin) (Saunders & Conn 1978). Purification of the vacuoles was accomplished by centrifuging a protoplast lysate for two hours at 26000 r.p.m. on a discontinuous Ficoll-400 gradient (3 %, 9 %, 12.5 % by mass) containing 0.5 M mannitol. Vacuoles accumulate at the 3–9 % interface, unlysed protoplasts at the 9–12 % interface, and chloroplasts and cellular debris are pelleted. Fractions (1.5 ml) were collected from the gradient by upward displacement with 60 % (by mass) sucrose. Vacuoles were collected in fractions 8 and 9. Protoplasts and vacuoles were counted using an A. O. Spencer bright line haemocytometer with a well 0.1 mm deep. Counting was facilitated by staining the vacuole preparation with neutral red.

2. *Identification of metabolites*

Metabolic products of [^{3}H]GA_1 were provisionally identified by comparing them with authentic standards in t.l.c. and gas chromatography–radiocounting (Nadeau & Rappaport 1972). The chromatograph is equipped with a flame ionization detector and an effluent splitter. The 3.5 × 180 cm stainless steel columns were packed with either 3 % SE-30 or QF-1 on Gas-Chrom Q. Column temperatures are shown in figure 5. Radioactivity was collected in the effluent from the chromatograph. The retention times of the methyl ester trimethylsilyl ether-derivatized standards and radioactive compounds were compared for coincidence.

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Discussion

A. R. Wellburn (*University of Lancaster*). Is there any evidence, in view of the results of Cooke *et al.* (1975), Cook & Kendrick (1976) and Evans & Smith (1976*a*) that red light promotes conversion of 'bound' to 'free' GAs rather than biosynthesis of GAs in plastids?

L. Rappaport. Evidence that red light actually promotes biosynthesis is very limited. It is confined to the observation that GA increases in sonicated etioplast preparations left a few minutes in darkness, or in red-light irradiated intact etioplasts left in darkness. This is almost no proof at all. Red light promoted conversion of 'bound' (polar) GAs to 'free' (ethyl acetate soluble) GAs has been noted; however, the differences, measured by bioassay, are too small to speculate about. In my opinion, in line with Dr MacMillan's comment, a very detailed analysis is needed of the occurrence and identification of GAs from red light irradiated and sonicated etioplasts, coupled with precursor feeding experiments. Good, clean plastids are essential to pursue these investigations.

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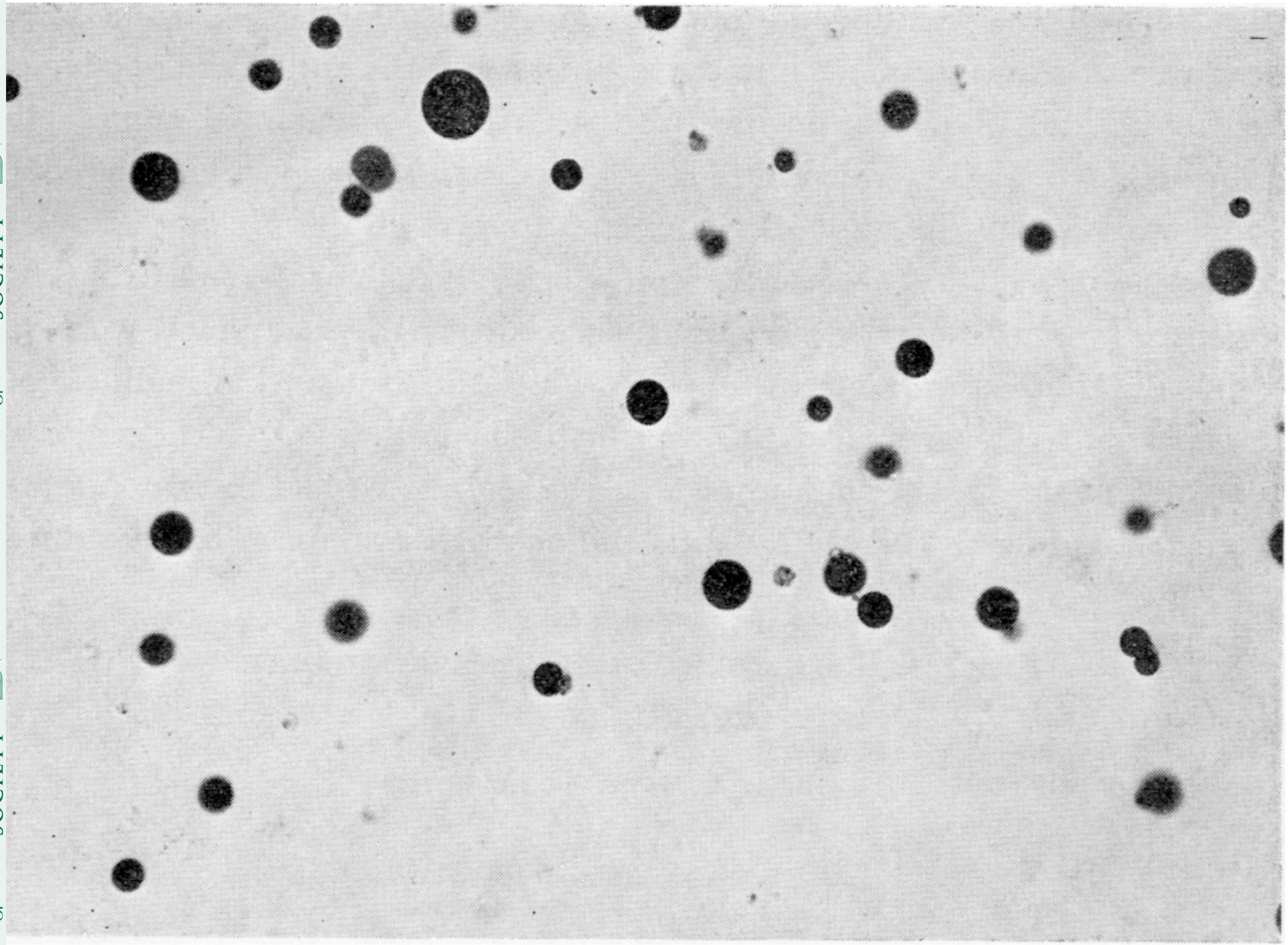

FIGURE 7. Barley vacuoles, stained with neutral red, isolated according to the procedures shown in table 4 and in §VI.

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