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THE BIOSYNTHESIS OF ALL MAJOR PEA GIBBERELLINS IN A CELL-FREE SYSTEM FROM *PISUM SATIVUM*

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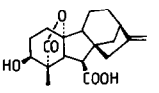
Key Word Index—*Pisum sativum*; Leguminosae; pea; biosynthesis; cell-free system; GC/MS; gibberellins.

Abstract—The soluble fraction of a cell-free system from immature pea embryos converts gibberellin A_{12}(GA_{12}) sequentially to GA_{15}, GA_{24}, GA_9 and GA_{51}, which are all not hydroxylated at C-13. GA_{12} can also be 13-hydroxylated by the microsomal fraction to GA_{53}, which is converted further by the soluble fraction sequentially to GA_{44}, GA_{19}, GA_{20} and GA_{29}, the same series as before but 13-hydroxylated. The microsomal 13-hydroxylation requires oxygen and NADPH, the conversions by the soluble fraction require oxygen, Fe^{2+} and α-ketoglutarate and are stimulated by ascorbate. GA_{15} and GA_{44} serve as substrates in the above sequences after alkaline hydrolysis of the lactone functions only, but such hydrolysis is not required for the conversion of GA_9 to GA_{51} and GA_{20} to GA_{29}. The products of the *in vitro* system are identical with the major GAs found endogenously in immature pea seeds and the sequences obtained are considered representative of the natural biosynthetic pathway of GAs in many plant species.

INTRODUCTION

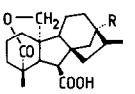
Cell-free systems that can catalyse steps in gibberellin (GA) biosynthesis have been reported from a limited number of higher plants (see reviews [1–3]). Most extensively studied are the systems from endosperm of *Marah macrocarpus* [4] and *Cucurbita maxima* [5], both belonging to Cucurbitaceae. The *Marah* system has been used to study the early steps of the pathway including the formation of 7β-hydroxy-ent-kaurenoic acid [6–9]. The *Cucurbita* system converts MVA past this stage via GA_{12}-aldehyde (**1**) to several C_{20}-GAs and one C_{19}-GA, GA_4 (**2**) [10–13]. The immediate precursor of GA_4 is GA_{36} (**3**) [14], which defines the stage of oxidation at which C-20 is lost when C_{20}-GAs are converted to C_{19}-GAs. This conversion is particularly worthy of attention as it leads to the physiologically most active GAs. Unfortunately, the formation of GA_4 is very low in the *Cucurbita* system, GA_{36} becoming converted mainly to the C_{20}-GAs GA_{13} (**4**) and GA_{43} (**5**).

Immature pea seeds were chosen for the study of GA biosynthesis because they are easily obtained and because they synthesize large amounts of GAs at defined stages. In addition, the main GAs of peas are 13-hydroxylated, a hydroxylation pattern that does not occur in Cucurbitaceae [15]. Cell-free systems from immature pea seeds have previously been shown to convert MVA to ent-kaurene and ent-kaurenol [16–18], and to convert ent-kaurene to GA_{12}-aldehyde, GA_{12} (**6**), GA_{53} (**7**) and GA_{44} (**8**) [19, 20]. GA_{15} was also obtained in one case [unpublished results], but C_{19}-GAs were not obtained in the previous studies. Endogenous GAs and the *in vivo* metabolism of GA_9 (**10**), GA_{20} (**11**) and GA_{29} (**12**) in immature pea seeds have been extensively and precisely studied by Sponsel (née Frydman) and MacMillan [21–26] as well as by Durley *et al.* [27].

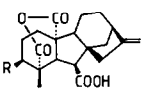


2 GA_4

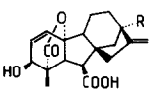
	R_1	R_2	R_3	R_4	
3	CHO	H	OH	H	GA_{36}
4	COOH	H	OH	H	GA_{13}
5	COOH	OH	OH	H	GA_{43}
17	COOH	H	H	H	GA_{25}
20	COOH	H	H	OH	GA_{17}



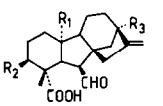
8 R = OH GA_{44}
9 R = H GA_{15}



18 R = H GA_{25} anhydride
22 R = OH GA_{13} anhydride



23 R = H GA_7
24 R = OH GA_3



	R_1	R_2	R_3	
21	Me	H	OH	GA_{53}-aldehyde
25	Me	OH	H	GA_{14}-aldehyde
26	COOH	OH	H	GA_{13}-aldehyde

We now report cell-free conversion of GA_{12} to all major GAs found in immature pea seeds, including the C_{19}-GAs GA_9, GA_{20}, GA_{29} and GA_{51} (**13**). The system is useful for preparing isotopically labeled 13-hydroxylated and non-hydroxylated GAs and for studying the conversion of C_{20}-GAs to C_{19}-GAs.

RESULTS

Preliminary experiments to improve activity

A cell-free system prepared from immature pea seeds as

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described in [19] had strong 13-hydroxylating activity. Thus the low-speed supernatant (S-2) of a homogenate converted [^{14}C]GA_{12}-aldehyde (**1**) to [^{14}C]GA_{44} (**8**) in the presence of NADPH, ATP and PEP. Since these results confirm the ones reported in refs. [19, 20], details are not given here.

The distribution of enzyme activity in the seeds was investigated as a possible means of improving activity and obtaining further conversion. Cell-free extracts were prepared separately from seed coats and embryos (mainly cotyledons) and incubated with [^{14}C]GA_{12} in the presence of NADPH, ATP, PEP and Fe^{2+}. The seed coat preparation converted [^{14}C]GA_{12} (**6**) to a single main product, which was identified by TLC as GA_{53} (**7**) whereas the embryo system yielded several products chromatographing like GA_{53}, GA_{44} (**8**), GA_{19} (**14**) and GA_{20} (**11**). Since the cofactor mixture was not optimal and since results reported later in this paper amply confirm the identifications, details are omitted at this point. Further improvement of activity was achieved by concentration of the high- or low-speed supernatant fractions with dry Sephadex G-25 [28]. Such concentrated preparations of pea embryos were used for all experiments reported in the following, unless otherwise noted. Gel filtration was not used routinely, since it decreased the activity of the preparations even in the presence of all cofactors later known to be required.

Conversion of GA_{12} to 13-deoxy-GAs by soluble enzymes

GA_{12} was the preferred precursor in incubations with high-speed supernatant (S-200), since GA_{12}-aldehyde (a favourite substrate in the *C. maxima* system) was converted mainly to a conjugate by this fraction. The identification of this conjugate as GA_{12}-aldehyde glucosyl ester by high resolution mass spectrometry and Fourier transformed ^{1}H NMR will be reported in a separate paper.

[^{14}C]GA_{12} (**6**) (8.9×10^5 dpm; 42 Ci/mol) was converted by S-200 in the presence of NADPH and Fe^{2+} to two products (Table 1). The main product, less polar than GA_{12} on TLC, was identified by GC/MS as [^{14}C]GA_{15} (**9**). As in all following identifications by GC/MS, the mass spectra contained clear ^{14}C-isotope peaks unequivocally demonstrating that the products originated from the ^{14}C-labelled substrates. The minor product, less polar than GA_{12} on TLC, had the same R_f as GA_{24} but there was not enough material for identification by GS/MS.

In further incubations ascorbate was substituted for NADPH. When [^{14}C]GA_{12} (1.7×10^6 dpm; 118 Ci/mol) was incubated with S-200, ascorbate and Fe^{2+} it was converted to two major compounds, which were identified by GC/MS as [^{14}C] GA_9 (**10**) and [^{14}C]GA_{51} (**13**) (Table 1). Two minor components with R_f-values like GA_{15} (**9**) and GA_{24} (**15**) were also found. Since GA_9 and GA_{51} are the main endogenous 13-deoxy C_{19}-GAs of pea seeds [22, 24], these results establish that a major part of the pathway is active in the cell-free system.

To confirm each step in the sequence (Scheme 1), prospective intermediates were incubated with S-200, ascorbate and Fe^{2+}. The results are quantitated in Table 1. [^{14}C]GA_{15} (**9**) incubated in this manner remained unchanged (not shown). This is not surprising, since it has been proven by the use of [19-^{18}O]GA_{12} that GA_{15} in its normal lactone form cannot be an intermediate in GA biosynthesis [29]. Since it had previously been suggested that the corresponding hydroxy carboxylic acid (**16**) could be the true intermediate [30], [^{14}C]GA_{15} was hydrolysed with 0.5 M potassium hydroxide and incubated as above. Two main products were obtained in the incubation and identified by GC/MS as [^{14}C]GA_9 and [^{14}C]GA_{24}, respectively (Table 1). Thus the open-lactone form of GA_{15} is probably the true but unstable intermediate in the pathway, lactonizing to GA_{15} on extraction. A third minor component migrating like GA_{51} on TLC was detected but not identified by GC/MS in this experiment. The next prospective intermediate, [^{14}C]GA_{24}, was completely converted to [^{14}C]GA_9 as identified by GC/MS and a minor component, migrating like GA_{51} on TLC (Table 1). Since Sponsel and MacMillan [24] have shown that 2β-hydroxylation preferably occurs at later stages of maturation, an S-200 preparation from somewhat older peas (29 days vs 18 days after anthesis) was used to demonstrate the last step. This preparation converted [^{14}C]GA_9 completely to [^{14}C]GA_{51} as identified by GC/MS (Table 1).

Table 1. The conversion of non-hydroxylated [^{14}C]GAs by the soluble fraction (S-200) of *P. sativum* cotyledons

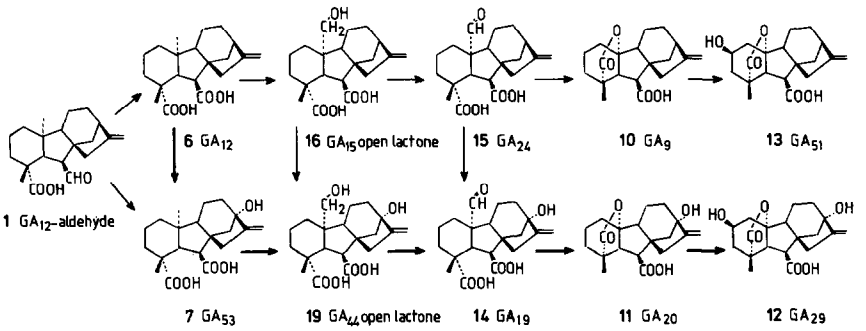
Substrate	Total act. (dpm × 10^{-3})	Sp. act. (Ci/mol)	Products and recovered substrate (dpm × 10^{-3})				
			GA_{12}	GA_{15}	GA_{24}	GA_9	GA_{51}
GA_{12}†	890	43	506	272*	13	0	0
GA_{12}‡	1700	118	8	22	22	672*	449*
GA_{15}-open lactone‡	3400	118	0	598*	151*	1130*	123
GA_{24}‡	150	41	0	0	0	83*	18
GA_9‡	910	100	0	0	0	0	652*

*Identified by GC/MS.

†Incubated with S-200 (1.5 ml), $FeSO_4$ (0.5 mM) and NADPH (1 mM).

‡Incubated with S-200 (4.0, 5.0, 1.5 and 1.0 ml, respectively), $FeSO_4$ (0.5 mM) and ascorbate (5 mM).

Incubations were at 30° for 4 hr. Only qualitative comparisons are possible from substrate to substrate, as the incubations were done with different cell-free preparations and at different substrate concentrations (see Experimental).



Scheme 1. Biosynthetic relationship of GAs obtained in the pea cell-free system.

Theoretically, GA_9 could either be formed directly from GA_{24} or via the tricarboxylic acid GA_{25} (**17**). To distinguish between these possibilities, GA_{24}, GA_{25} and GA_{25} anhydride (**18**) were incubated with S-200 and cofactors as before. Since the enzyme extracts do not contain endogenous GA_9 and GA_{25}, non-radioactive substrates could be used in this experiment. A small amount of ^{14}C-marker was added before extraction of the products to allow quantitation by isotope dilution and mass fragmentography. As shown in Table 2, GA_{24} was converted to GA_9 and GA_{51}, whereas GA_{25} and GA_{25} anhydride were not. Thus each step in the conversion of GA_{12} to GA_{51} by S-200 has been defined (Scheme 1).

Microsomal 13-hydroxylation

The experiments described in the previous section were done with the high speed supernatant (S-200) as a source of enzyme activity. When $[^{14}C]GA_{12}$ (1.5×10^7 dpm; 98 Ci/mol) was incubated with a low speed supernatant (S-2) together with NADPH, ATP and PEP it gave one product only, which was identified by GC/MS as the 13-hydroxylation product $[^{14}C]GA_{53}$ (**7**) (1.3×10^7 dpm; 98 Ci/mol). Also the washed microsomal pellet converted $[^{14}C]GA_{12}$ and $[^{14}C]GA_{12}$-aldehyde to $[^{14}C]GA_{53}$ with NADPH as only cofactor requirement (Table 3). Thus the 13-hydroxylating activity is associated with the microsomal pellet. ATP and PEP had no significant effect on the conversion when the microsomal pellet was used (Table 3) although they increased 13-hydroxylation when S-2 was used [20]. This stimulation is probably due to NADPH-generating or -preserving enzymes in the crude homogenate. For the preparation of 13-hydroxylated intermediates, the use of S-2 in combination with NADPH, ATP and PEP was preferred, since it gave a (*ca* 10-fold) better yield than the washed pellet with NADPH. GA_{12} was always converted better than GA_{12}-aldehyde.

The requirement for oxygen in 13-hydroxylation was studied by the use of $^{18}O_2$. GA_{12} (60 nmol) was labelled slightly with $[^{14}C]GA_{12}$ (1.1×10^5 dpm, resulting in 0.8 Ci/mol) and incubated with S-2, NADPH, ATP and PEP under $^{18}O_2$ ($^{18}O:^{16}O = 4:1$). After extraction and purification by TLC, the methyl TMS derivative of the product GA_{53} gave a strong $[M+2]^+$ ion (m/z 450:m/z 448 = 79.7:20.3) in the mass spectrum, demonstrating that the oxygen of the 13-hydroxyl came from $^{18}O_2$. The ^{14}C-label that had been added to aid the purification was below the level detectable in the mass spectrum and did not interfere with the determination of ^{18}O. Thus 13-hydroxylation requires NADPH and oxygen.

To determine at which points in the pathway 13-hydroxylation can occur, $[^{14}C]GA_{15}$ (**9**), $[^{14}C]GA_{15}$-open lactone (**16**), $[^{14}C]GA_{24}$ (**15**), $[^{14}C]GA_9$ (**10**) and $[^{14}C]GA_9$-open lactone were incubated with S-2, NADPH, ATP and PEP. As shown in Table 4, the lactones remained unchanged, whereas GA_{15}-open lac-

Table 2. Conversion of the C-20 aldehyde GA_{24}, but not the C-20 carboxylic acid GA_{25}, and its anhydride to C_{19}-GAs by the soluble fraction (S-200)

Substrate		Products plus internal standards				
		GA_9			GA_{51}	
GA	nmol	Sp. act. (Ci/mol)	Rel. mass units	pmol	Sp. act. (Ci/mol)	pmol
None	—	100	838	150	118	130
GA_{24}	29	n.m.*	124000	22000	13	1180
GA_{25}	27	100	900	150	118	130
GA_{25} anhydride	29	100	803	150	118	130

*Not measurable because of dilution by unlabeled GA_9 formed as a product.

S-200 (1 ml) was incubated at 30° for 5 hr with substrates as specified, $FeSO_4$ (0.5 mM) and ascorbate (5 mM). After incubation, $[^{14}C]GA_9$ (150 pmol, 100 Ci/mol) and $[^{14}C]GA_{51}$ (130 pmol, 118 Ci/mol) were added as int. standards for determination of the products by isotope dilution, or in the case of GA_9, by the peak area of the mass fragmentogram (see Experimental).

Table 3. Cofactor requirements for 13-hydroxylation by the microsomal fraction

Substrate/cofactors	Recovered substrate (GA_{12} or GA_{12}-aldehyde) (dpm × 10^{-2})	Product (GA_{53}) (dpm × 10^{-2})
GA_{12}		
NADPH	153	349
NADPH, ATP, PEP	91	427
ATP, PEP	337	52
None	360	17
GA_{12}-aldehyde		
NADPH	419	59
NADPH, ATP, PEP	492	53
ATP, PEP	513	5
None	483	5

GA_{12} (556 × 10^2 dpm, 42 Ci/mol) and GA_{12}-aldehyde (562 × 10^2 dpm, 118 Ci/mol) were incubated at 30° for 4 hr with microsomal suspension (0.2 ml) containing KPi (0.05 M, pH 7.0), $MgCl_2$ (2.5 mM), NADPH (1 mM), ATP (10 mM) and PEP (20 mM) in variations as indicated. Separation of products by TLC in solvent system (1c).

Table 4. 13-Hydroxylation of different intermediates in the non-hydroxylated series by the low speed supernatant (S-2)

Substrate	Total act. (dpm × 10^{-3})	Sp. act. (Ci/mol)	Recovered substrate (dpm × 10^{-3})	Products (dpm × 10^{-3})			
				GA_{53}	GA_{44}	GA_{19}	GA_{20}
GA_{12}	1480	98	0	1240	0	0	0
GA_{15}	337	44	303	0	0	0	0
GA_{15}-open lactone	899	44	649	0	188	0	0
GA_{24}	31	12	3	0	0	14	0
GA_9	340	100	292	0	0	0	0
GA_9-open lactone	284	100	142	0	0	0	92

Incubations with S-2 (10.0, 0.6, 2.0, 0.1, 0.3 and 0.3 ml, respectively), NADPH (1 mM), ATP (10 mM) and PEP (20 mM) at 30° for 4 hr. See Experimental concerning different sizes of incubations and the separation by TLC. All products were identified by GC/MS.

tone, GA_{24} and GA_9-open lactone were 13-hydroxylated. A diluted S-2 preparation was used for this experiment to limit the conversion mainly to one step, thus facilitating the identification by GC/MS. As in Table 3, GA_{12} was converted to the greatest extent, which was a general experience throughout this work.

Conversion of GA_{53} to other 13-hydroxylated GAs by soluble enzymes

When [^{14}C]GA_{53} (**7**) was incubated with concentrated S-200, Fe^{2+} and ascorbate, it was converted to [^{14}C]GA_{44} (**8**), [^{14}C]GA_{19} (**14**), [^{14}C]GA_{20} (**11**) and [^{14}C]GA_{29} (**12**) as shown in Table 5. All products were identified by GC/MS. Initial difficulties in detecting the isotope peaks in [^{14}C]GA_{20} and [^{14}C]GA_{29} because of dilution with endogenous material were overcome by the use of more highly labeled substrate [31]. [^{14}C]GA_{12} to be used as a substrate was usually prepared from [2-^{14}C]MVA with a sp. act. of 11 Ci/mol by using the *C. maxima* cell-free system. The mass spectrum of methyl-[^{14}C]GA_{12} thus prepared gives an isotope peak at *m/z* 330 $[M+2]^+$ corresponding to the frequency of molecules with one ^{14}C atom each. In the presence of large amounts of endogenous material, the contributions by natural ^{13}C and silicon isotopes to the corresponding isotope peaks in the products obscure the contribution by ^{14}C. Therefore, [2-^{14}C]MVA of sp. act. 53 Ci/mol was used in such cases, which resulted in [^{14}C]GA_{12} with strong isotope peaks at *m/z* 334 and 336 $[M+6]^+$, $[M+8]^+$, corresponding to three and four ^{14}C atoms/molecule, respectively. These peaks were not affected by endogenous material, thus permitting accurate determination of the sp. act. of the products from their mass spectra [32]. In the experiment shown in Table 5, [^{14}C]GA_{29} obtained from [^{14}C]GA_{12} had a sp. act. of 16 Ci/mol, corresponding to a six-fold dilution by endogenous GA_{29}. The ^{14}C content of [^{14}C]GA_{20} was also reduced (*ca* two-fold), whereas [^{14}C]GA_{44} and [^{14}C]GA_{19} had the same sp. act. as the substrate [^{14}C]GA_{12}.

To confirm each metabolic step, the 13-hydroxylated intermediates were incubated with S-200 using Fe^{2+} and ascorbate as cofactors (Table 5). Like [^{14}C]GA_{15} (**9**), the lactonic [^{14}C]GA_{44} (**8**) was not converted but had to be hydrolysed with 0.5 M potassium hydroxide before incubation. The product of hydrolysis (**19**) was then converted by the enzymes to [^{14}C]GA_{19} (**14**) and [^{14}C]GA_{20} (**11**) in good yields. In the next step in the sequence, [^{14}C]GA_{19} was predominantly converted to [^{14}C]GA_{20}. For further

Table 5. The conversion of 13-hydroxylated [^{14}C]GAs by the soluble fraction (S-200)

Substrate	Total act. (dpm × 10^{-3})	Sp. act. (Ci/mol)	Products and recovered substrate (dpm × 10^{-3})				
			GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_{29}
GA_{53}	11200	98	258	1190*	3380*	3120*	1290*
GA_{44}-open lactone	680	98	0	204*	165*	122*	42
GA_{19}	230	98	0	0	0	151*	28
GA_{20}	560	45	0	0	0	49	427*

*Identified by GC/MS.

Incubations with S-200 (20.0, 2.0, 1.0 and 1.0 ml, respectively), $FeSO_4$ (0.5 mM) and ascorbate (5 mM) at 30° for 4 hr. See Experimental concerning different sizes of incubations and the separation by TLC.

2-hydroxylation to GA_{29} (**12**), the same preparation from 29-day-old peas as before was used. This preparation converted [^{14}C]GA_{20} to [^{14}C]GA_{29} in over 75% yield. All major products were identified by GC/MS. The resulting pathway of 13-hydroxylated GAs is a part of Scheme 1.

Cofactor and oxygen requirements of the soluble enzymes

The protein fraction of S-200 was separated from the small molecule fraction by CC using Sephadex G-25. [^{14}C]GA_{12}, [^{14}C]GA_{15}-open lactone, [^{14}C]GA_{19} and [^{14}C]GA_9 were used as substrates in order to define the cofactor requirements for each type of reaction observed with S-200. Table 6 shows that each step requires α-ketoglutarate and Fe^{2+} and is further stimulated by ascorbate. Table 7 shows that the conversions require oxygen.

DISCUSSION

Two complete sequences of GAs were obtained from GA_{12} in the cell-free system, one with and one without 13-hydroxylation (Scheme 1). Both involve oxidation and subsequent loss of C-20 to form C_{19}-GAs, which are finally hydroxylated in the 2β-positions. This corresponds closely to the situation *in vivo* where the C_{19}-GAs first formed are considered to be the physiologically active hormones, whereas the 2β-hydroxylated GAs are believed to be inactivated end products.

Sponsel (née Frydman) and MacMillan in their extensive study identified GA_9, GA_{17} (**20**), GA_{20}, GA_{29}, GA_{44} and GA_{51} as the native GAs of immature pea seeds [21, 22, 24]. Ingram and Browning, using a different cultivar of peas, identified GA_{19} in addition to GA_{20} and GA_{29} [33]. All these GAs except GA_{17} (**20**) were also obtained in the cell-free system. Even the formation of this GA, which is present at very low levels *in vivo* [22], is not excluded, since several products were present in too small amounts for identification. GA_{12}, GA_{15} and GA_{24}, on the other hand, were obtained in the cell-free system but not *in vivo* where they obviously do not accumulate. This is not surprising, since the 13-deoxy GAs are weakly represented *in vivo*, GA_9 being found at levels *ca* 1% those of GA_{20} and GA_{29} [22]. Also the cell-free system yields mainly 13-hydroxylated products, provided the low speed supernatant with its microsomes is used as a source of enzyme activity. The terminal steps, the conversion of GA_{20} to GA_{29} and of GA_9 to GA_{51}, have also been shown by feeding labeled GA_{20} and GA_9 to peas maturing in their pods [23, 24, 27] and to etiolated pea shoots [34, 35]. These feeding studies yielded three more meta-

Table 6. Cofactor requirements with gel filtered soluble enzymes

Cofactors	^{14}C-labelled products (dpm × 10^{-2}) after conversion of			
	GA_{12} to GA_{15}	GA_{15}-open lactone to GA_{24}	GA_{19} to GA_{20}	GA_9 to GA_{51}
Fe^{2+}, α-KGA*, ascorbate	173†	175†	120	144
Fe^{2+}, α-KGA*, NADPH	55	101†	70	35
α-KGA*, ascorbate	43	68	40	9
Fe^{2+}, α-KGA*	51	78	26	6
Fe^{2+}, ascorbate	6	6	6	8
None	2	4	5	4

*α-Ketoglutarate.

†Includes minor amounts of product from one further step of oxidation.

[^{14}C]GA_{12} (44 400 dpm, 118 Ci/mol), [^{14}C]GA_{15}-open lactone (56 200 dpm, 118 Ci/mol), [^{14}C]GA_{19} (56 800 dpm, 98 Ci/mol) and [^{14}C]GA_9 (90 900 dpm, 100 Ci/mol) were incubated at 30° for 4 hr with the large molecule fraction (0.5 ml, 25 mg protein) of Sephadex G-25 filtered enzyme preparation, Fe^{2+} (0.5 mM), α-ketoglutarate (5 mM), ascorbate (5 mM) and NADPH (1 mM) in combinations as indicated.

Table 7. The requirement for air in the conversions catalysed by the soluble fraction (S-200)

Substrate*	Total act. (dpm × 10^{-2})	Gas phase	Products and recovered substrate (dpm × 10^{-2})							
			GA_{12}	GA_{15}	GA_{24}	GA_9	GA_{51}	GA_{19}	GA_{20}	GA_{29}
GA_{12}	1110	N_2	644	60	3	88	2	0	0	0
		Air	8	44	28	308	527	0	0	0
GA_{15}-open lactone	1400	N_2	0	1030	26	13	12	0	0	0
		Air	0	27	27	272	656	0	0	0
GA_9	1110	N_2	0	0	0	824	4	0	0	0
		Air	0	0	0	237	523	0	0	0
GA_{19}	1230	N_2	0	0	0	0	0	1110	9	7
		Air	0	0	0	0	0	58	792	180

*Sp. act as in Table 6.

The incubations with S-200, $FeSO_4$, α-ketoglutarate and ascorbate are described in the Experimental.

bolites, which were not obtained in the cell-free system. The most prominent of these, a 2-keto compound called GA_{29}-catabolite [25–27], was formed from GA_{29} only in much more mature seeds than were used in our work. Perhaps it can be obtained cell-free by using homogenates from older seeds. The second metabolite, H_2-GA_{31} [23, 24, 34], may have been an artefact of feeding unphysiologically high amounts of the substrate GA_9 [24]. The third metabolite, GA_{10}, obtained from GA_9 in one case only [34], may have been an artefact of the isolation method. We conclude that the pathway, as obtained in the cell-free system, is a good representation of GA biosynthesis in immature peas *in vivo*.

The same pathway probably operates in seedlings. Evidence for 2β-hydroxylation was mentioned above. Furthermore, Durley *et al.* fed [17-^{3}H]GA_{14} to etiolated pea seedlings and identified radioactive GA_{18}, GA_{38}, GA_{23}, GA_1, GA_8 and GA_{28} as products [36]. Substrate and products are not endogenous to pea seedlings and the identifications (by GC/RC on three columns) were not definitive but, since the series differs from the 13-hydroxylated GAs in Scheme 1 only by the 3β-hydroxyl group introduced by the unnatural substrate, the results are in agreement with ours. Only GA_{28}, which corresponds to GA_{17}, has no counterpart in Scheme 1.

The point in the pathway at which 13-hydroxylation occurs has been much discussed. Since GA_9 (**10**) applied *in vivo* to pea shoots becomes converted to GA_{20} (**11**) [34] and since endogenous GA_9 comes to a peak earlier in development than GA_{20} [22], it was first believed that GA_9 was the natural precursor of GA_{20}. However, Sponsel and MacMillan [23, 24] revised this view when they found that the conversion of GA_9 to GA_{20} is very low (0–10%) and takes place at young stages of pea development only, at which time there is little GA_{20} formed. On the basis of this and other evidence, they concluded that the predominant 13-hydroxylation must occur earlier in the pathway. Our results support this view, since both GA_{12}-aldehyde (**1**) and GA_{12} (**6**) are readily 13-hydroxylated (Table 3), whereas GA_9 is converted to GA_{20} after treatment with alkali only (Table 4). The fact that some conversion takes place when GA_9 is fed to intact peas [23, 24] and etiolated seedlings [34], suggests the presence of a hydrolase opening the lactone ring, at least when the substrate is added artificially. Although it now seems certain that 13-hydroxylation occurs early in the pathway, the exact point of this event is still undecided. In our work GA_{12} was the 'best' substrate for 13-hydroxylation (Tables 3 and 4), but this is not definitive since we worked with end-point incubations and did not determine the decisive kinetic parameters. Another possibility would be 13-hydroxylation of GA_{12}-aldehyde to form GA_{53}-aldehyde (**21**), which would then be converted by soluble enzymes to GA_{53} and the series of 13-hydroxylated GAs shown in Scheme 1. Such a sequence would correspond to the formation of 3β-hydroxylated GAs in the fungus *Gibberella fujikuroi* [37], but there is no result in our work to support this.

The conversion of C_{20}-GAs to C_{19}-GAs is an important step in GA biosynthesis since only C_{19}-GAs have high physiological activity. Our results conclusively show that GA_{24} (**15**) is a precursor of GA_9 (**10**), whereas GA_{25} (**17**) and GA_{25}-anhydride (**18**) are not (Table 2). Thus, the conversion from C_{20}-GAs to C_{19}-GAs takes place with C-20 at the stage of an aldehyde, at least for the non-hydroxylated GAs. The corresponding conversion of 3β-hydroxy-GAs has recently been shown with the *C. maxima* cell-free system, in which GA_{36} (**3**) is converted to GA_4 (**2**), whereas GA_{13} (**4**) is not [14]. Thus the conversion to C_{19}-GAs probably universally occurs via the C-20 aldehydes. This has been suggested before (see ref. [36]) although experimental proof has been missing. On the basis of careful analyses of quantitative changes in endogenous GAs in *Spinacia oleracea* grown under long- and short-day conditions, Metzger and Zeevaart [38] also concluded that GA_{19} (**14**) most likely is the direct precursor of GA_{20} (**11**) and, furthermore, that the conversion of GA_{19} to GA_{20} is the step under photoperiodic control. This has also been implied in *Agrostemma githago* [39] and in the G and G2 lines of *P. sativum* [33, 40]. Not in accord with our view is a report claiming that *G. fujikuroi* converts GA_{13} anhydride (**22**) to GA_4/GA_7 (**23**) and GA_3 (**24**) [41]. The conversion was extremely low and could not be reproduced by others [37]. Another report, also concerning *G. fujikuroi*, claims the incorporation of GA_{14}- and GA_{13}-7-aldehydes (**25**, **26**) into GA_3 [42], which would mean that the conversion to C_{19}-GAs takes place via the C-7 aldehyde with C-20 at the carboxylic acid stage. Although the situation in *G. fujikuroi* theoretically could differ from that in *C. maxima* and *P. sativum*, a conversion via GA_{13}-7-aldehyde now seems less likely. Bearder and Sponsel have discussed possible mechanisms for the loss

of C-20 [43]. The fact that C-20 is lost at the aldehyde stage limits their number.

The microsomal 13-hydroxylating enzymes require NADPH and oxygen (Table 3) and thus are probably of the cytochrome P_{450}-containing kind of mono-oxygenases known for several microsomal steps in GA biosynthesis [6, 7, 44]. It has previously been reported that Fe^{2+}, ATP and PEP stimulate 13-hydroxylation in the pea system in the presence of NADPH [20]. We also found a clear stimulation by ATP and PEP when the low speed supernatant was used as an enzyme source but only to a very small extent when washed microsomes were used (Table 3). This effect is probably due to side reactions similar to the ones reported in ref. [6] occurring in the crude system.

The soluble enzymes of the cell-free system require Fe^{2+}, α-ketoglutaric acid and ascorbate for full activity. The same cofactors are required for the corresponding part of the pathway in the cell-free system from *C. maxima* [13]. Both the *C. maxima* and the pea systems contain enough endogenous α-ketoglutarate for this factor not to be limiting even after dialysis. The requirements are typical for certain soluble dioxygenases extensively discussed in ref. [13]. A GA-2β-hydroxylating system from cotyledons of germinating *P. vulgaris*, which has previously been shown to require Fe^{2+} and ascorbate or NADPH for activity [45] has recently been shown to be of the same kind [Smith, V. A. and MacMillan, J., unpublished work].

The conversion of MVA to C_{19}-GAs has now been shown in three cell-free systems: the one from endosperm of *C. maxima*, the present system from pea embryos and a system originating from suspensors of *Phaseolus coccineus* [46–48]. The pea system is well suited for the preparation of the GAs shown in Scheme 1 which are isotopically labeled. However, since the part of the pathway leading from MVA to GA_{12} is less active in the pea system, we use GA_{12} prepared with the *C. maxima* system as starting material. On the other hand, the conversion of C_{20}-GAs to C_{19}-GAs is better studied in the pea system, since it is weakly represented in the *C. maxima* system. Since the 13-hydroxylating activity is microsomal, the 13-deoxy- and the 13-hydroxy pathways can be studied separately by using the high speed supernatant and either GA_{12} or GA_{53} as a substrate. Particularly clean data are obtained with the high speed supernatant in conjunction with GA_{12} since there are no endogenous 13-deoxy-GAs to obscure the results. In the 13-hydroxy pathway, large amounts of endogenous GA_{20} and GA_{29} dilute the label of the products unless substrates of very high sp. act. are used. The endogenous GAs can be removed by gel filtration but this leads to loss of activity. Most of the activity was found in the cotyledons rather than in the seed coats, which is also known to be true for the synthesis of ent-kaurene [49].

The system from suspensors of *P. coccineus* appears to be very active, since it converts 7β-hydroxy-ent-kaurenoic acid all the way to GA_5, GA_1 and GA_8 even without the addition of Fe^{2+} [48]. This high activity may be explained by the very specific tissue used—the suspensors are painstakingly dissected from 5–8 mm long seeds. The unequivocal demonstration that the GAs identified in the *P. coccineus* system are truly products of the precursor is still outstanding.

It is clear from this discussion that the cell-free system from peas can become instrumental in the study of many general problems of GA biosynthesis. Its significance is further stressed by the fact that the 13-hydroxylated GAs obtained or parts of them have been identified in many plants in addition to *P. sativum*, e.g. refs. [39, 50–53].

EXPERIMENTAL

Preparation of cell-free extracts. Pisum sativum L. cv 'Grosser Schnabel mit Gedrücktem Korn' was field grown in the summers of 1980 and 1981. Flowers were marked at anthesis and immature seeds were harvested at defined times 15–30 days later. Immature seeds were cut with a razor blade and the embryos separated from the seed coats. The embryos were homogenized in KPi buffer (0.05 M, pH 8.0, 1:1 w/v) with a mortar and pestle for 10 min. The homogenate was filtered through gauze and centrifuged at 2000 *g* for 5 min. The supernatant was concd with dry Sephadex (G-25 fine, 3:1 v/w) for 5 min at 2°, then centrifuged on sintered glass at 1000 rpm for 2 min [28]. The concentrate (2–3-fold), referred to as S-2, was stored in liquid N_2. The high speed supernatant, referred to as S-200, was prepared from S-2 by centrifugation at 200 000 *g* for 1 hr. Microsomal fractions were prepared by centrifugation of S-2 at 15 000 *g* for 5 min to remove larger particles and subsequent centrifugation of the supernatant at 200 000 *g* for 1 hr. The pellet was washed twice with KPi buffer (0.05 M, pH 8.0) and was finally suspended in KPi buffer (0.05 M, pH 7.0) to give 1/3 of the original vol. of the homogenate. This preparation was called P-200.

Protein determination. The preparations used contained 47–53 mg protein/ml as estimated with the Lowry method [54] applied to TCA ppts. However, the protein concn was found to be a less reliable reference value than the vol. of the enzyme extracts as prepared by the standard method. Individual protein concns, therefore, are not given.

Purification of S-200 by gel filtration. Lyophilized S-200 (8 ml) was dissolved in H_2O (2 ml) and filtered over a Sephadex G-25 column (1.6 × 30 cm) equilibrated and eluted with KPi buffer (0.05 M, pH 7.5) containing 2.5 mM $MgCl_2$. The effluent was monitored at 280 and 254 nm. The protein fractions (15 ml) were collected, lyophilized and stored at −14°. Before use, each portion was dissolved in H_2O (4 ml), resulting in a Lowry protein concn of *ca* 50 mg/ml.

Substrates. [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} were prepared from [2-^{14}C]MVA by using a cell-free system from the endosperm of *C. maxima* as described in ref. [10]. Other substrates were the products of incubations with the pea system as described in this paper. The open lactones of [^{14}C]GA_{15} and [^{14}C]GA_{44} were prepared by hydrolysis of [^{14}C]GA_{15} and [^{14}C]GA_{44} with 0.5 M KOH (30 μl) at 100° for 2 hr in sealed tubes. GA_{25} anhydride was prepared from GA_{25} by heating at 200° for 1 hr under N_2 [34]. The purity of all substrates was checked and the sp. act. determined by GC/MS [32].

Incubation. No standard size of incubation mixtures and no standard concn of substrates were used, since these parameters were varied according to activity and available amount of enzyme as well as to the specific radioactivity and available amounts of substrates. This ensured reliable identification of all products. The amount of enzyme preparation given for each expt also represents the total vol. of the incubation mixture.

Extraction of products. After incubation, the mixtures were acidified to pH 3.0, Me_2CO (1:1) was added and the products were extracted × 3 with EtOAc (same vol. as Me_2CO). Combined EtOAc extracts were washed with little H_2O and dried under N_2 flow.

TLC and RC. TLC was on Si gel. Solvent systems were: (1) $CHCl_3$–EtOAc–HOAc, in (1a) (70:30:1), in (1b) (50:50:1) in (1c) (40:60:1), in (1d) (25:75:1); (2) petrol

(40–60°)–EtOAc–HOAc (80:25:1). R_f-values of non-13-hydroxy-GAs were in (1a): GA_9 and GA_{15} (0.66), GA_{12} (0.53), GA_{24} (0.47), GA_{51} (0.15); and in solvent system (2) (×3 developed): GA_9 and GA_{12} (0.50), GA_{15} (0.39), GA_{24} (0.23). R_f-values of 13-hydroxy-GAs were in (1c): GA_{20} (0.50), GA_{53} (0.41), GA_{44} (0.37), GA_{19} and GA_{29} (0.12); in solvent system (1b) (developed twice): GA_{53} (0.50), GA_{44} (0.42); and in solvent system (1d) (developed twice): GA_{19} (0.50), GA_{29} (0.40). Prep. TLC was also on 0.25 mm layers but washed with Me_2CO–MeOH (1:1) before use. The plates were scanned for radioactivity and radioactive zones were counted by liquid scintillation with or without prior elution from the gel. Counting efficiency of dissolved samples was 88% as measured with an int. standard. When Si gel adsorbed samples were counted, interference by Si gel was corrected for by comparing dissolved and adsorbed standards of individual compounds.

Separation of products in specific incubations. Table 1. The products of [^{14}C]GA_{12} (42 Ci/mol) gave three peaks in solvent system (1a) corresponding to GA_{24}, GA_{12} and GA_9/GA_{15}. Rechromatography of the latter in (2) yielded GA_{15} but not GA_9. The products of [^{14}C]GA_{12} (118 Ci/mol) gave three radioactive zones in solvent system (1b), corresponding to GA_{51}, GA_{24} and GA_9/GA_{12}/GA_{15}. Rechromatography in solvent system (2) separated GA_{15} and GA_9/GA_{12}, the latter were separated in (1a). Incubation of GA_{15}-open lactone gave four peaks in solvent system (2), which were identified as GA_{51}, GA_{24}, GA_{15} and GA_9. [^{14}C]GA_{24} gave two products in solvent system (1a), which were identified as GA_{51} and GA_9. GA_9 gave a single product in solvent (1c), which was identified as GA_{51}.

Table 4. Solvent system (1c) was used to purify GA_{53} resulting from GA_{12} and to purify GA_{20} resulting from GA_9-open lactone. Solvent system (1b) was used to purify GA_{44} resulting from GA_{15}-open lactone and (1d) was used for GA_{19} resulting from GA_{24}.

Table 5. Products from GA_{53} were separated in solvent system (1c), yielding two peaks. The slower moving peak on rechromatography in (1d) separated into GA_{29} and GA_{19}. The faster moving peak was separated by (1b) into GA_{44}, GA_{53} and GA_{20}. Products from GA_{44}-open lactone were separated in (1c), giving GA_{19}/GA_{29}, GA_{44} and GA_{20}. Solvent system (1d) resolved GA_{29} and GA_{19}. Products from GA_{19} were separated by (1d) into GA_{29} and GA_{20}. There was no more GA_{19} left over. Products from GA_{20} were separated by (1d), showing a small peak of GA_{20} and a large one of GA_{29}.

GC/MS. Methylated (CH_2N_2) and trimethylsilylated (MSTFA 80°, 30 min) samples were injected (260°) into a fused Si capillary column (WCOT, OV-101, 25 m × 0.25 mm) using the Grob splitless injection method. The column was maintained at 50° for 1 min, then programmed at 15°/min to 200° and at 4°/min to 260°. The He flow rate was 2 ml/min. The split (50:1) was opened 0.5 min after injection. The column effluent was led into the ion source at 290°. Electron energy was 70 eV, emission current 0.21 mA. Mass peak intensities for the determination of sp. act. were measured by scanning over a limited mass range with an integration time of 20 msec/ion. Mass chromatograms were generated for each ion and the peak areas were determined.

Reference compounds and spectra. GA_{12}-aldehyde, GA_9, GA_{12}, GA_{15}, GA_{20}, GA_{24} and GA_{53} were identified by comparison of their MS with those of authentic compounds supplied by Professor J. MacMillan, Bristol. GA_{44} and GA_{51} were identified by comparison with spectra also sent by MacMillan. GA_{19} and GA_{29} were identified by comparison with published spectra, found in refs. [33] and [21], respectively.

Determination of GA_9 and GA_{51} in Table 2. The extracted products were purified by TLC in solvent system (1b) followed by rechromatography in solvent systems (2) (GA_9) and (1b) (GA_{51}). One-tenth was used for radio-counting, the rest for GC/MS. The following ions were used with mass fragmentography to calculate the sp. act.: GA_9Me (*m/z* 298, 304, 306); GA_{51} MeTMS (*m/z* 284, 290, 292). The large amount of GA_9 formed in the incubation with GA_{24} diluted the sp. act. to below a measurable level. GA_9 was, therefore, estimated by comparison of relative mass units of *m/z* 298. Meaningful statistical treatment of the data was not possible and also unnecessary. All values given in Table 2 are adjusted for recovery losses.

Reproducibility. All expts, except the one reported in Table 2, were repeated at least once. The conversions comprising the main pathway (Scheme 1) were done numerous times with qualitatively identical results.

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