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Partial Purification and Characterization of the Gibberellin A_{20} 3β-Hydroxylase from Seeds of *Phaseolus vulgaris*[1]

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ABSTRACT

The GA_{20} 3β-hydroxylase present in immature seeds of *Phaseolus vulgaris* has been partially purified and characterized. The physical characteristics of the enzyme are similar to those of the GA 2β-hydroxylases present in mature and immature seeds of *Pisum sativum*. It is acid-labile, hydrophobic, and of M_r 45,000. The enzyme catalyzes the synthesis of GA_1, GA_5, and GA_{29} from GA_{20}. Activity is dependent upon the presence of Fe^{2+}, ascorbate, 2-oxoglutarate, and oxygen. 2-Oxoglutarate does not function as a cosubstrate; in the presence of the enzyme, succinate is not a reaction product.

Gibberellin A_1 is the endogenous gibberellin (GA) that induces stem elongation in maize (27) and pea (15). In addition to GA_1, GA_5, and GA_{29} are also proven metabolic products derived from GA_{20} (Scheme I) in maize vegetative tissue (8, 9) and in *Phaseolus vulgaris* seed tissue (2, 16). In the latter case it has been tentatively suggested that a single enzyme, the GA_{20} 3β-hydroxylase, may catalyze the synthesis of all three compounds (2). This possibility is confirmed in the present communication, concerned mainly with the characteristics of the GA_{20} 3β-hydroxylase present in immature seeds of *P. vulgaris*. In view of the physiological significance of the GA_{20} 3β-hydroxylase in producing GA_1 and also, indirectly, GA_3 in vegetative tissue (9), possible metabolic control mechanisms are also discussed.

MATERIALS AND METHODS

Labeled GA Substrates

[1β,2β,3β-$^3H_3$]GA_{20} (2.02 TBq mmol^{-1}) was custom-synthesised by Amersham International plc (Amersham, U.K.) using methods described by Albone *et al.* (3). [17-^{13}C,$^3H_2$]GA_{20} (1.27 GBq mmol^{-1}) was synthesized by Dr. C. L. Willis (15). [1β,2β-$^3H_2$]GA_1 (1.11 Bq mmol^{-1}) was obtained from Amersham International plc (Amersham, U.K.) and [2,3-$^3H_2$]GA_9 (1.72 TBq mmol^{-1}) was a gift from Dr. A. Crozier (University of Glasgow, U.K.).

Plant Material

Bean plants (*Phaseolus vulgaris* cv Canadian Wonder) were grown from seed supplied by Nutting and Thoday, Long Stanton, Cambridge, U.K., and maintained in constant-environment growth cabinets (Conviron 510H; Controlled Environments, Winnipeg, Canada) under conditions detailed by Albone *et al.* (2). Flowering commenced 5 weeks after germination. Immature beans were harvested on d 23 postanthesis and dissected. Isolated seed cotyledons were stored in liquid N_2 before extraction.

Enzyme Preparation

Unless otherwise stated, all operations were carried out at 4°C. The cotyledons from seed harvested at 23 d after anthesis were pulverised in liquid N_2 using a mortar and pestle, warmed to 4°C, then homogenised in 0.1 M Tris HCl (pH 7.6), containing 0.25 M sucrose and 10 mM DTE. The homogenate was filtered through a double layer of muslin, then centrifuged at 18,000*g* for 40 min at 4°C. LSS[2] was decanted and pumped immediately onto a column (30 × 2.2 cm) of DEAE-cellulose (Whatman DE-52; Whatman International Ltd., Maidstone, Kent) preequilibrated and eluted with 0.05 M Tris HCl (pH 7.6) containing 5 mM DTE and 10% glycerol at a flow rate of 20 mL h^{-1}. Fractions (12 mL) were collected and assayed against [1β,2β,3β-$^3H_3$]GA_{20}. The 3β-hydroxylase was not retained by the anion exchange column; those fractions that contained activity were pooled and the protein precipitated with ammonium sulfate (70% w/v) added as a saturated solution in 0.25 M Tris HCl (pH 7.6). The precipitated protein was recovered by centrifugation at 18,000*g* for 30 min at 4°C, dissolved in 25 mM potassium phosphate (pH 7.0) containing 10 mM DTE and 10% glycerol, and dialyzed against the same buffer. This material was then eluted through a column (25 × 2.2 cm) of CM-cellulose (Whatman CM-52) with 25 mM potassium phosphate (pH 7.0), 2.5 mM DTE, 10% glycerol at a flow rate of 16 mL h^{-1}. The 3β-hydroxylase activity was again recovered in the column flow-through and concentrated, as described above, by $(NH_4)_2SO_4$ precipitation. The precipitated protein was then redissolved in 0.25 M Tris HCl (pH 7.6), 5 mM DTE, 10% glycerol for chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). Unless otherwise stated this preparation was used for characterising the enzyme.

Gel Filtration Chromatography and Estimation of Mol Wt

A column (90 × 2.2 cm) of Sephadex G-100 was equilibrated with 0.25 M Tris HCl (pH 7.6) containing 5 mM DTE

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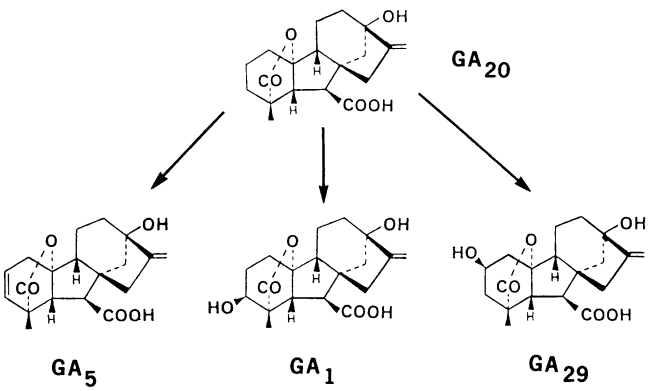
[2] Abbreviations: LSS, low speed spin supernatant; THO, tritiated water.



Scheme 1. Metabolic fate of GA_{20}.

and 10% glycerol, and calibrated with bovine serum albumin (M_r 68,000), ovalbumin (M_r 45,000), lactoglobulin (M_r 38,000), and lactalbumin (M_r 14,500). A sample (5 mL) of the concentrated CM-cellulose enzyme was loaded onto the column and eluted with equilibration buffer at a flow rate of 5.0 mL h^{-1}. Fractions (2.5 mL) were collected and assayed against [1β,2β,3β-$^3H_3$]GA_{20}. Those that contained 3β-hydroxylase activity were pooled and concentrated using an ultrafiltration membrane. A typical elution profile is shown in Figure 1.

Enzyme Assays

The assay protocol was similar to that described previously (23) except that the incubation buffer was 0.1 M Tris HCl (pH 7.6) and catalase was omitted. The GA_{20} 3β-hydroxylase activity was measured routinely at 25°C by determining the rate of liberation of THO from [1β,2β,3β-$^3H_3$]GA_{20} (0.01 μM). Since the ratio of radioactivity at positions 1β, 2β, and 3β in this substrate was estimated to be 1:2:2, respectively, by NMR and mass spectrometry (30) the assumed specific activity of the THO liberated is 8.08×10^{14} Bq $mmol^{-1}$.

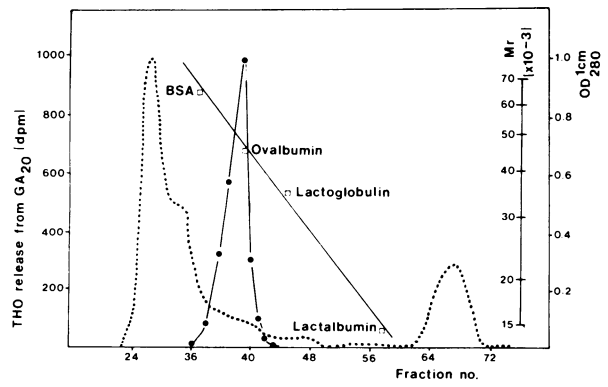


Figure 1. GA_{20} 3β-hydroxylase purification, Sephadex G-100 elution profile.

Product Analysis

The products formed during incubation of GA_{20} with the 3β-hydroxylase enzyme preparation were analyzed by reverse-phase HPLC using a stainless-steel column (25 × 0.8 cm) packed with ODS Hypersil (5 μm) (Shandon Southern Products, Runcorn, Cheshire, U.K.), fitted to an LDC HPLC apparatus (Riviera Beach, Florida, U.S.A.). Samples were prepared by adding ice-cold ethanol (2 volumes) to the incubation mixtures and, after 16 h at −20°C, recovering the ethanolic supernatants by centrifugation. These were reduced to dryness under vacuum in silylated vials and then redissolved in aqueous methanol (30% v/v in 0.5% phosphoric acid). Reaction product samples (90 μL) were injected onto the column and eluted with an exponential gradient of aqueous methanol (30–70% v/v in 0.5% H_3PO_4) over 20 min at a flow rate of 1.2 mL min^{-1}. Under these conditions the elution volumes of authentic samples of GA_1, GA_8, and GA_{29} were 14.4, 7.2, and 9.6 mL, respectively, but GA_{20} and GA_5 were inseparable (elution volume 27.5 mL). To achieve separation of GA_5 and GA_{20}, the column was run isocratically (30% aqueous methanol in 0.5% H_3PO_4) at a flow rate of 1 mL min^{-1}. The elution volume of GA_5 and GA_{20} was 38 and 45 mL, respectively.

Protein Estimation

The protein content of the various enzyme preparations was estimated by the Biuret method (10) or by measuring peptide absorption at 225 nm ($A_{225}^{1\%/rcm}$ 1 mg mL^{-1} = 9.17 [12]).

Polyacrylamide Gel Electrophoresis

The basic procedure described by Laemmli (18) was followed. Enzyme preparations were run either on 'native' slab gels (5% w/v acrylamide) or on SDS-slab gels (12% w/v acrylamide) using Tris-Glycine (pH 8.3) ± SDS (0.1%) as running buffer.

[$^{18}O_2$]-Labeling Experiments

[$^{18}O_2$]Oxygen (100 mL; 1.99 atom per molecule) was supplied by Amersham International plc. The flask was connected to two round bottom flasks (25 mL) containing stirrer bars, capped with suba seals and sitting on ice. Final reaction volumes were 1.5 mL. The enzyme (ammonium sulfate precipitated LSS preparation) and buffer (0.1 M Tris HCl [pH

Table I. *Enzyme Purification*

Purification Step	Total Protein	Specific activity	Total Units[a]	Recovery
LSS	3186	0.044	140	100
DEAE-cellulose	851	0.125	106	75
CM-cellulose	570	0.025	14.25	10
G-100	25	0.20	5	3.5

[a] 1 unit = 1×10^{-12} mol h^{-1}.

Table II. *Stability of the DEAE-Cellulose Enzyme Preparation*

Enzyme Treatment	Enzyme Activity	Activity
	pmol h^{-1} *mg*$^{-1}$	%
Control (thawed ex liq. N_2)	0.124	100
16 h at 4°C in 0.25 M Tris HCl (pH 7.6), 5 mM DTE, 10% glycerol	0.112	90
Dialyzed 16 h at 4°C *versus* 0.05 M Tris HCl (pH 7.6), 5 mm DTE	0.108	87
Dialyzed 16 h at 4°C *versus* 0.05 M Mes (pH 6.8), 5 mM DTE	0.066	53

7.6]) were added to both flasks. To one of the flasks, Fe^{2+} (1 mM) and ascorbate (2.5 mM) were also added. While stirring the reaction media on ice, the apparatus was flushed with helium then nitrogen. [17-^{13}C,$^{3}H_2$]GA_{20} (20 μM) and 2-oxoglutarate (1 mM) were injected into the media through the suba seals and the glass seal on the [$^{18}O_2$]oxygen broken with a ball-bearing. The apparatus was then incubated at 25°C for 2 h. Samples were removed for quantitative product analysis by HPLC. The remainders of the reaction media were added to ice-cold ethanol (2 volumes) and centrifuged after 16 h at −20°C. The supernatants were then concentrated under N_2 to remove the ethanol and were acidified to pH 3.0. Subsequent extraction procedures and processing for GC-MS analysis has been described previously (2).

Estimation of Succinate Generation from 2-Oxoglutarate

In all reactions, 2-[^{14}C(U)]oxoglutaric acid (1 mM, 9.3 × 10^{12} Bq mol^{-1}, New England Nuclear) was incubated with or without the enzyme (Sephadex G-100 or post DEAE-cellulose preparations) and its other cofactors in a final volume of 30 μL. Reactions were sampled by removing and spotting aliquots (3 μL) onto plastic backed silica gel 60 TLC sheets (Merck Ltd). Standard solutions of 2-oxoglutarate and succinate were also applied as references. The TLC sheets were developed for 5 h in *n*-butanol-acetic acid-water (120:30:50). They were then dried and cut into strips. These were placed in scintillation vials and counted for radioactivity.

RESULTS

Enzyme Purification

The procedure adopted to purify partially the GA_{20} 3β-hydroxylase achieved only a five-fold increase in specific activity (Table I). The enzyme is similar to the GA_1/GA_{20} 2β-hydroxylase(s) isolated from pea seed cotyledons in that it does not bind either anionic or cationic exchangers (24). In this respect our findings differ from those of Kwak *et al.* (17), who apparently succeeded in binding the enzyme to a DEAE anionic exchanger in 50 mM phosphate buffer (pH 8.0) and eluting it with the same buffer containing approximately 0.15

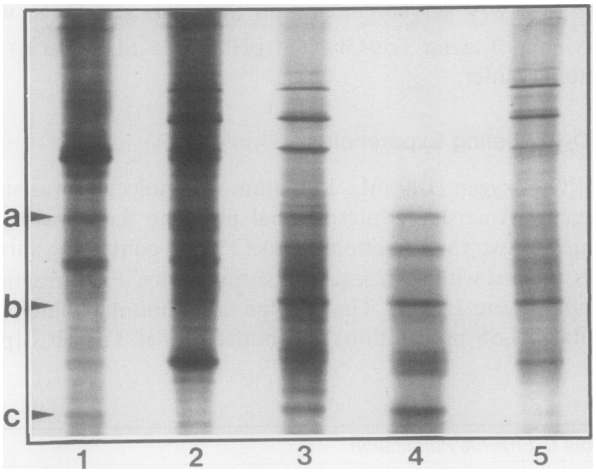


Figure 2. SDS-PAGE analysis of individual fractions comprising the GA_{20} 3β-hydroxylase activity peak after gel filtration on Sephadex G-100 (lanes 1–4). Lane 5 shows the overall protein composition of the Sephadex G-100 enzyme preparation used for experiments described in this paper. Protein standards: (a) BSA (M_r 65,000), (b) ovalbumin (M_r 45,000), (c) lactalbumin (M_r 14,500).

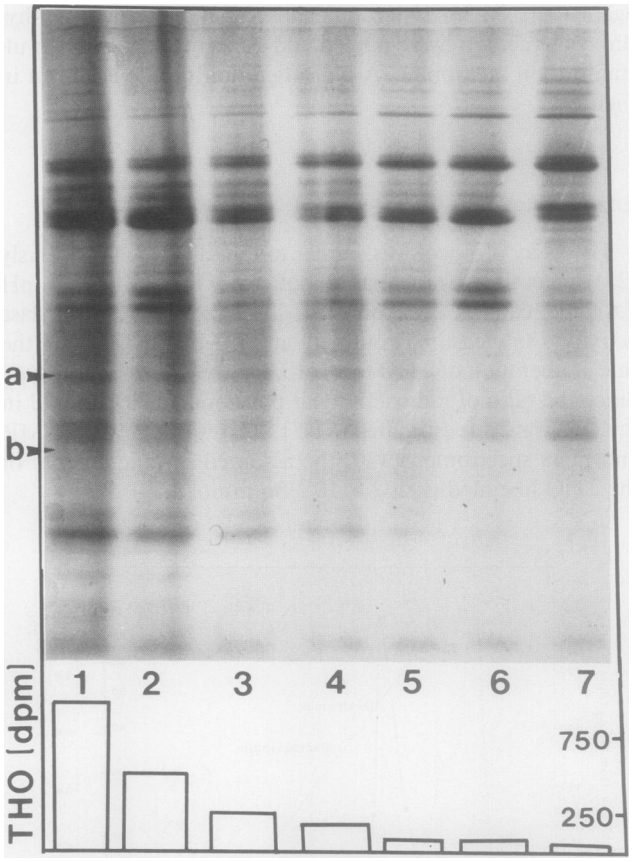


Figure 3. Second dimension SDS-PAGE analysis of the proteins/polypeptides contained in sequential slices of a native polyacrylamide gel in which a DEAE-preparation of the GA_{20} 3β-hydroxylase was run and assayed for activity. Protein standards: (a) ovalbumin corunning with presumed enzyme band; (b) lactoglobulin (M_r 38,000).

Table III. *Cofactor Requirement for the Synthesis of GA_1, GA_5, and GA_{29}*

Cofactors Added	Product Formed		
	GA_1	GA_{29}	GA_5
	pmol		
A. Substrate [1β, 2β, 3β-$^3H_3$]GA_{20}			
Fe/asc	0.14	0.036	ND[a]
Fe/asc/2-oxoglutarate	2.05	0.57	ND
B. Substrate [17-$^3H_2$, ^{13}C]GA_{20}			
Fe/asc	0.38	<0.03	<0.05
Fe/asc/2-oxoglutarate	7.15	0.35	2.15

[a] Not detected.

M NaCl. The impure LSS enzyme preparation loses all activity over a period of 16 h at 4°C, possibly due to protease activity or enzymic tanning reactions. After further purification the enzyme is labile under acidic conditions (Table II) and also in dilute solution. Attempts were made, therefore, to stabilize the enzyme during chromatography on Sephadex G-100 with BSA. However, the protein concentration required to stabilise the enzyme was too high (about 10 mg mL^{-1}) to be useful; in any case, the subsequent separation of the enzyme from the BSA by anion exchange chromatography on DEAE-cellulose incurred massive losses in enzyme activity.

Attempts were also made to purify the enzyme further by hydrophobic chromatography on phenyl-sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Though the enzyme bound tightly to the matrix when applied in buffer (0.2 M potassium phosphate [pH 7.8], 10 mM DTE), active enzyme preparations were not recovered after elution with ethanediol.

The mol wt of the 3β-hydroxylase was estimated to be around 45,000 by gel filtration on Sephadex G-100. Individual fractions comprising the enzyme activity peak in the Sephadex G-100 elution profile were analysed on SDS-polyacrylamide gels. The corresponding specific activity of the enzyme present in these fractions analyzed in lanes 1, 2, 3, and 4 (Fig. 2) were 0, 0.04, 1.02, and 0.464 pmol mg^{-1}. These activities correlate with the density of staining of one particular protein band of similar mobility to ovalbumin (M_r 45,000).

Attempts to analyze the protein complement of various enzyme preparations on 'native' 12% (w/v) acrylamide gels failed; the proteins did not enter the gel. At a concentration of 5% (w/v) acrylamide the proteins comprising the DEAE-cellulose enzyme preparation ran in the gel. On staining with Coomassie blue, however, no discrete bands were visible and the enzyme was located at the top of the gel by assaying sequential slices (0.1 inches) down the gel. Although analysis of these gel sections by second dimension SDS-PAGE showed that they all contained a multiplicity of similar proteins/polypeptides, the protein band of similar M_r to ovalbumin, believed to be the 3β-hydroxylase, was contained in the gel sections that showed enzyme activity (Fig. 3).

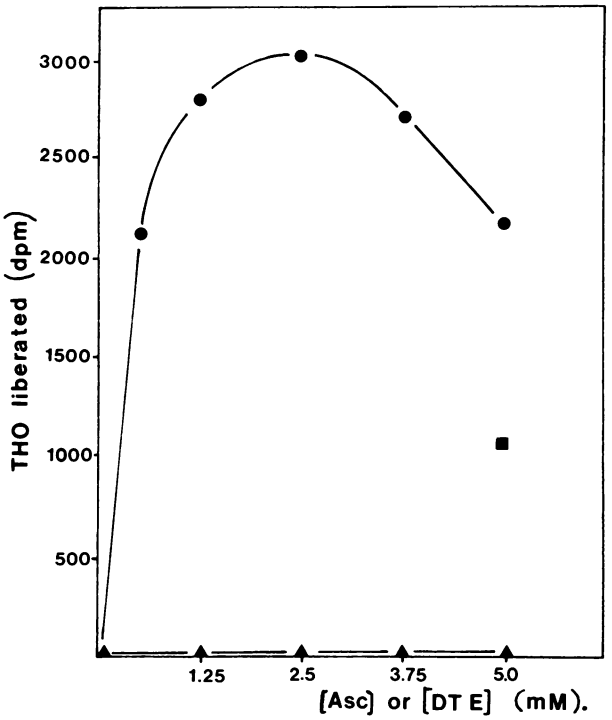


Figure 4. GA_{20} 3β-hydroxylase activity dependence upon ascorbic acid. (●) + ascorbic acid; (▲) + DTE; (■) + ascorbic acid + DTE.

Product Analysis

In addition to catalyzing the formation of GA_1 from GA_{20}, the Sephadex G-100 enzyme preparation also catalyzed GA_{20}

Table IV. *GA_{20} Metabolic Activity Dependence upon Fe^{2+} and Ascorbate (5 mM)*

$[Fe^{2+}]$	Rate THO Liberation	
	+ ascorbate	− ascorbate
mM	*pmol h^{-1}*	
0	ND[a]	ND
0.01	0.183	<0.01
0.10	0.177	ND
1.0	0.191	<0.02

[a] Not detected.

Table V. *GA_{20} Metabolic Activity Dependence upon 2-Oxoglutarate*

2-Oxoglutarate Concentration	Rate THO Liberation
mM	*pmol h^{-1}*
0	0
0.05	0
0.10	0
0.50	0.067
1.00	0.124
2.50	0.123

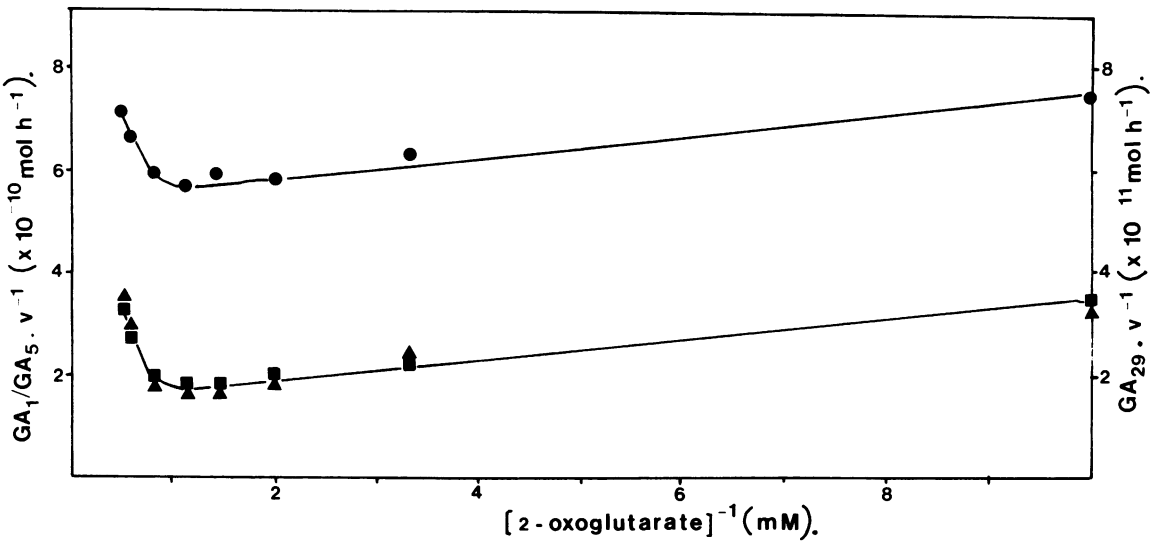


Figure 5. Lineweaver-Burk plots of reaction rate against 2-oxoglutarate concentration for GA_1 (●), GA_5 (▲), and GA_{29} (■) synthesis.

dehydrogenation to GA_5 and GA_{20} 2β-hydroxylation to GA_{29}. However, which of these products was formed depended on the nature of the isotopically labeled substrate. When [1β,2β,3β-$^3H_3$]GA_{20}, with a ratio of 1:2.2 at the 1β, 2β, and 3β positions, respectively, (30), was presented as substrate at high concentration (1.0 μM), only two radio-labeled products, GA_1 and GA_{29}, could be detected (Table IIIA) in the ratio 4:1. The possibility that GA_5 was also formed, but with total loss of label, was unlikely since the calculated molar ratio of labeled GA products to THO was approximately 1:1. Under standard assay conditions the observed radio-labeled products formed from [1β,2β,3β-$^3H_3$]GA_{20} (0.01 μM) were THO (0.106 pmol) GA_1 (0.075 pmol) and GA_{29} (0.022 pmol). By contrast, the major radio-labeled products formed from [17-^{13}C,$^3H_2$] GA_{20} (1.0 μM) were GA_1 and GA_5, formed in a 3:1 molar ratio. In this case the GA_{29} formed was less than 5% of the GA_1 formed (Table IIIB).

Table VI. *Comparison of Succinate and GA Product Yield Obtained after Incubating 2-Oxoglutarate and/or GA_{20} under Varying Conditions*

Incubation Conditions	Succinate Production[a]		Products derived from [^{3}H]GA_{20} (pmol)	
	−ENZ	+ENZ	Estimation by THO assay	Total (GA_1, GA_5, GA_{29}) from [17-^{13}C, $^3H_2$]GA_{20}
			pmol	
Buffer	36	38	0	0
+Fe/Asc	79	5	0.23[b]	ND[c]
+Fe/Asc GA_{20} (20 μM)	93	0	2.25[b]	2.4

[a] No other product was detected. All radioactivity comigrated with either the 2-oxoglutarate itself or with a succinate reference. [b] Adjusted, taking into account the ^3H isotope effect. [c] Not determined.

Enzyme Cofactors

Ferrous Ion

Activity of the Sephadex G-100 enzyme preparation was dependent upon Fe^{2+}. Contrary to the results of Kwak *et al.* (17), the absolute requirement for this metal ion was demonstrable without the addition of chelators such as α,α-bipyridyl or EDTA. Maximal activity was obtained at concentrations of 10 μM Fe^{2+} (Table IV), but significantly higher concentrations (10 mM) were not inhibitory.

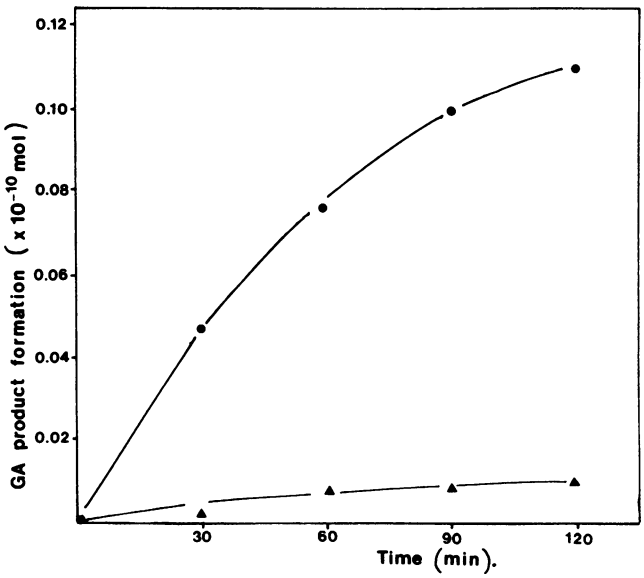


Figure 6. Kinetics of GA_{20} metabolism in absence (▲) and presence (●) of 2-oxoglutarate (0.5 mM).

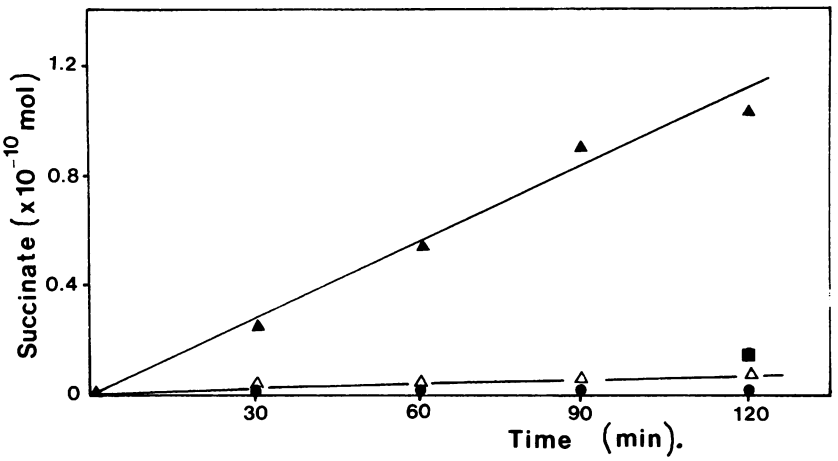


Figure 7. Kinetics of succinate production from 2-oxoglutarate (0.5 mM) in the absence and presence of the GA_{20} 3β-hydroxylase. (▲), − enzyme + cofactors; (△), − enzyme − cofactors; (●), + enzyme + cofactors; (■), equivalent GA product formation in presence of enzyme and cofactors.

Ascorbic Acid

The GA_{20} 3β-hydroxylase activity was totally dependent upon ascorbic acid. The fact that DTE was no substitute (Fig. 4) may suggest a role other than that of maintaining iron as Fe^{2+} in solution.

2-Oxoglutarate

Total dependence of the 3β-hydroxylase activity upon 2-oxoglutarate may be demonstrated under standard assay conditions ([$^3H_3$]GA_{20} substrate concentration 0.01 μM), but only after purification on Sephadex G-100. The results obtained (Table V) indicated that 2-oxoglutarate was an essential cofactor for the GA_{20} 3β-hydroxylase, but the narrow range over which enzyme activity increased from zero to maximal was unexpected in view of its postulated role as cosubstrate. Additionally, the same enzyme preparation exhibited partial activity when assayed at a higher GA_{20} substrate concentration (1.0 μM) in the absence of 2-oxoglutarate (Table III). With respect to the synthesis of GA_1, GA_5, and GA_{29} from [17-^{13}C,$^3H_2$]GA_{20}, double reciprocal plots of formation rate *versus* 2-oxoglutarate concentration were parallel (Fig. 5). These data are consistent with an hypothesis that the three reactions may be catalyzed by the same protein. They did not, however, conform to Michaelis-Menten kinetics.

Since succinate is the postulated reaction product formed from 2-oxoglutarate dependent dioxygenases, the stoichiometric ratios of succinate to the GA reaction products were analyzed in two series of reactions. In the first series, the incubation media contained 2-[^{14}C(U)]-oxoglutaric acid (0.5 mM) and cold GA_{20} (20 μM). In the second series, cold 2-oxoglutarate (0.5 mM) and radio-labeled GA_{20} (20 μM) was incubated under otherwise identical conditions in the presence or absence of the Sephadex G-100 enzyme preparation. The results obtained (Table VI) show that in the presence of the enzyme, Fe^{2+} and ascorbate plus or minus the GA_{20} substrate, little or no succinate was generated. In the absence of the enzyme, however, the added cofactors accelerated 2-oxoglutarate decomposition to succinate, quantitatively far exceeding the amount of GAs produced in the enzyme catalyzed reaction.

In an attempt to obtain greater activities with respect to GA_{20} metabolism, similar experiments were carried out using a post-DEAE enzyme preparation. Under the experimental conditions, however, it was not possible to raise significantly the rate of GA product formation relative to the spontaneous rate of succinate generation. After 2 h incubation with the 3β-hydroxylase and its essential cofactors (2-oxoglutarate concentration 0.5 mM), 11 pmol [^{3}H]GA products were formed from [^{3}H]GA_{20} (20 μM) but again, succinate was not produced (Fig. 6). In the absence of the enzyme, approximately 100 pmol succinate was generated from 2-oxoglutarate (0.5 mM) incubated with Fe^{2+}, ascorbate and GA_{20} (Fig. 7).

Table VII. *Quantification of the Products Formed from [17-^{13}C,$^3H_2$] GA_{20} under N_2, Air and ^{18}O*

GA Product	Incubation Atmosphere			
	N_2	Air	^{18}O (complete medium)	^{18}O (−Fe/Asc)
			nmol	
GA_1	0	0.282	0.456	0.05
GA_5	0	0.080	0.126	0.014
GA_{29}	0	0.010	0.017	0.002

Table VIII. *Incorporation of ^{18}O Into the Products Formed from [17-^{13}C,$^3H_2$]GA_{20} by the 3β-Hydroxylase*

Product Formed	Isotopic Composition of ^{18}O and ^{16}O			
	Complete reaction mix		Reaction mix −Fe^{2+} and ascorbate	
	^{18}O	^{16}O	^{18}O	^{16}O
			%	
GA_1	76.2	23.8	75.5	24.5
GA_{29}	76.0	24.0	36.5	63.5

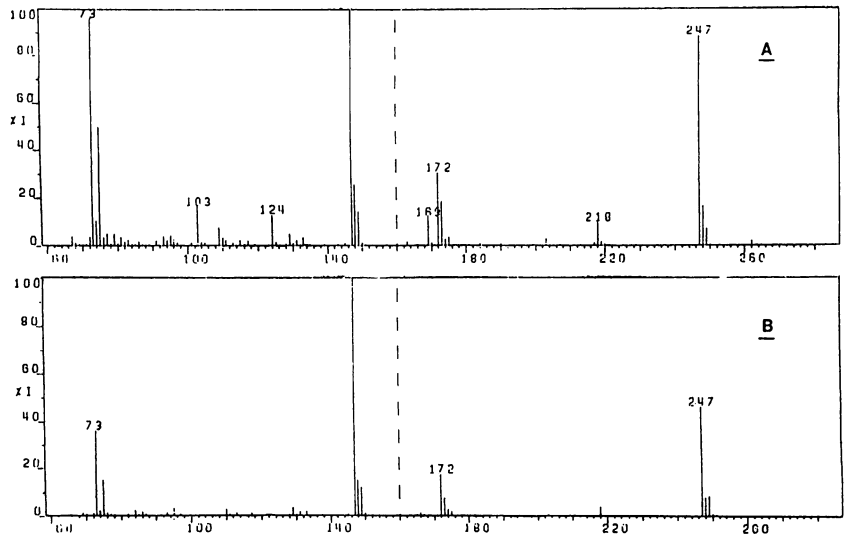


Figure 8. GC-MS analysis of succinate generated in the GA_{20} 3β-hydroxylase incubation medium in the presence of $^{18}O_2$. A, Time 0; B, after 2 h incubation.

^{18}O-Labeling Experiments

Gibberellin Metabolites

HPLC analysis of the reaction products formed from [17-^{13}C,^{3}H$_2$]GA_{20} incubated with the GA_{20} 3β-hydroxylase (post-DEAE preparation) under N_2, air or pure oxygen ($^{18}O_2$) in otherwise identical conditions showed that oxygen availability was rate limiting with respect to enzyme activity and the ratio of the products formed (GA_1:GA_5:GA_{29}) was not altered by changing the concentration of oxygen available (Table VII).

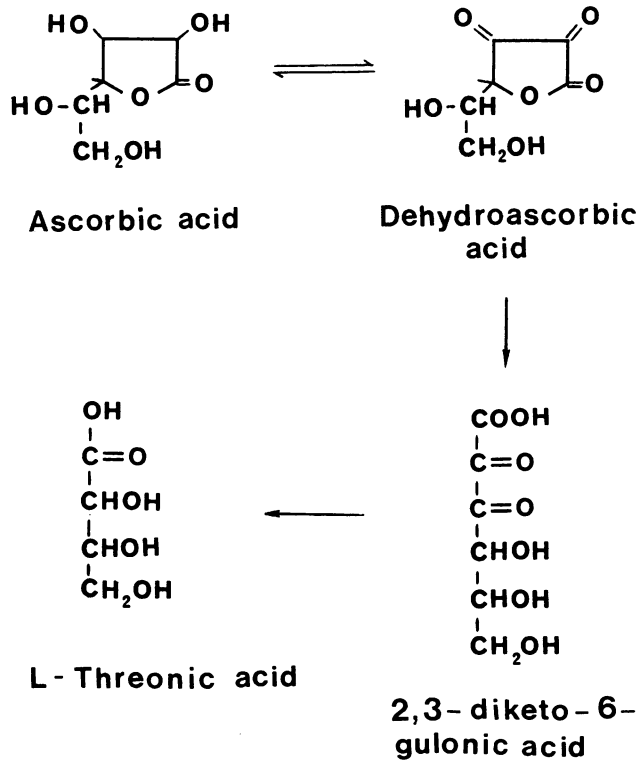
The [17-^{13}C,^{3}H$_2$]GA_{20} reaction products formed by the 3β-hydroxylase under [$^{18}O_2$]oxygen in the presence or absence of Fe^{2+} and ascorbate were purified by immunoaffinity chromatography on MAC 136 Sepharose (25) and analyzed by GC-MS. Full scan spectra of GA_1, GA_5, and GA_{29} were obtained. The isotopic ratios of ^{18}O:^{16}O in GA_1 formed in the presence or absence of Fe^{2+} and ascorbate and in GA_{29} formed in complete reaction media were similar. The GA_{29} formed in the absence of Fe^{2+} and ascorbate contained a significantly greater abundance of ^{16}O than ^{18}O (Table VIII). In this case apparently, where oxygen is limited through inability to form an Fe^{2+}/ascorbate/oxygen complex, the 2β-hydroxyl group may be derived from water.

Fate of 2-Oxoglutarate

The sum total of the GA reaction products formed by the 3β-hydroxylase (post-DEAE preparation) under [$^{18}O_2$] oxygen in the presence of Fe^{2+} and ascorbate was 6.5 nmol. Thus, for 2-oxoglutarate to function as cosubstrate, the reaction mixture should have contained a similar quantity of [$^{18}O_2$] succinate at the end of the incubation period. Samples were prepared from the incubation media after GA extraction using ethyl acetate and submitted for GC-MS analysis. The small peak of [^{18}O] succinate (M − 15 = 249) generated during the reaction period did not account for the expected 860 ng of product. Unlabeled succinate (M − 15 = 247) was present in samples taken from the reaction medium at the beginning and end of the incubation period (Fig. 8). This was probably derived from 2-oxoglutarate during workup or when derivatising the sample for GC-MS. It was not a contaminant of the 2-oxoglutarate as supplied (crystalline free acid, Sigma Chemical Co.).

Ascorbic Acid

Although only trace amounts of [^{18}O]succinate were detected in samples taken from the 3β-hydroxylase reaction medium, other ^{18}O-labeled compounds, subsequently proven



Scheme 2. Fe^{2+}-Catalyzed Degradation of Ascorbic Acid.

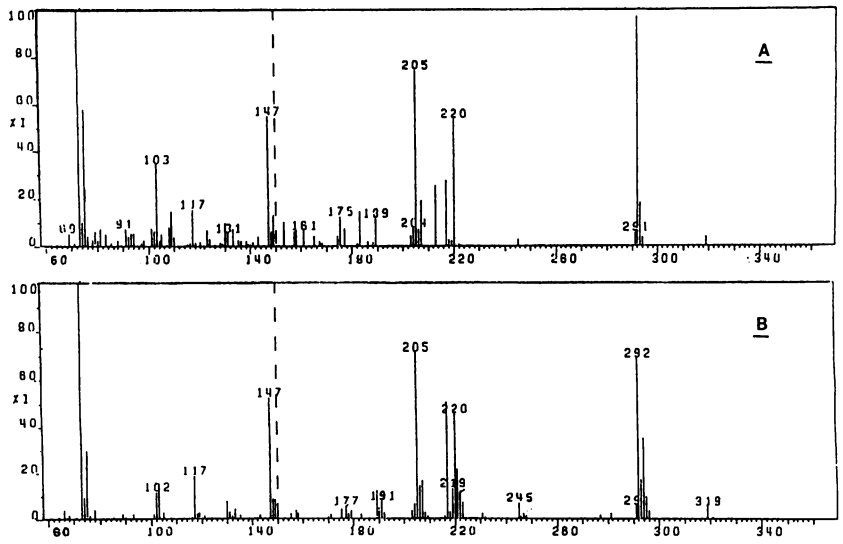


Figure 9. GC-MS analysis of threonic acid generated from ascorbate in the GA_{20} 3β-hydroxylase incubation medium in the presence of $^{18}O_2$. A, Time 0; B, after 2 h incubation at 25°C.

to be degradation products of ascorbic acid, were relatively abundant. Of these, [^{18}O]threonic acid ($M^+ - 132 = 294$) was the major catabolite, derived as shown in Scheme 2 (Fig. 9).

The nonenzymatic decomposition of ascorbate in water was catalysed by Fe^{2+}. After 2 h at 25°C in the presence of [$^{18}O_2$]oxygen, the major compound remaining was threonic acid with an ^{18}O:^{16}O isotopic composition of 1:1.7. When a solution containing 2-oxoglutarate as well as ascorbate and Fe^{2+} was incubated for 2 h at 25°C in an atmosphere of [$^{18}O_2$] oxygen, GC-MS analysis showed that, in this case, the ascorbate remained largely intact (Fig. 10) and the small amount of threonic acid formed had an ^{18}O:^{16}O isotopic composition of 1:2.0. In this case also, [^{18}O]succinate was produced. Thus, while 2-oxoglutarate may protect the ascorbic acid from decomposition, it is itself degraded. As shown by Figure 11 and Table IX, 2-oxoglutarate degradation occurs to similar extents in water and water containing Fe^{2+}. Only in the latter case, however, is succinate produced in significant quantities.

Inhibition Studies

GAs

Within a concentration range of 0.001 to 1.0 μM, GA_1, GA_3, GA_6, and GA_{29} did not affect the metabolism of [1β,2β,3β-$^3H_3$]GA_{20} (0.01 μM) by Sephadex G-100 enzyme preparations. However, GA_5 and GA_9 are both activity inhibitors. At concentrations of 1.0 and 0.2 μM, respectively, THO liberation from [1β,2β,3β-$^3H_3$]GA_{20} (0.01 μM) was inhibited by 50%.

Gibberellin A_5 is postulated to be a reaction product of the GA_{20} 3β-hydroxylase. In agreement with this possibility it has been shown that GA_5 equally suppresses the synthesis of GA_1, GA_5, and GA_{29} from [17-^{13}C,$^3H_2$]GA_{20}.

Gibberellin A_9 has been shown to be a competitive substrate. The rate of [2,3-$^3H_2$]GA_9 metabolism is approximately eight times slower than [1β,2β,3β-$^3H_3$]GA_{20} metabolism at substrate concentrations of 0.01 μM (relative rates 0.02 pmol h^{-1} mg^{-1} and 0.166 pmol h^{-1} mg^{-1}, respectively). The main product formed from [2,3,$^3H_2$]GA_9 was GA_4. In a quantitative analysis of the enzyme reaction products by HPLC, it was estimated that 0.48 pmol THO and 0.476 pmol GA_4 were produced from this substrate.

Immunological

A monoclonal antibody, raised against the Sephadex G-100 enzyme preparation, has been selected by ELISA and by inhibition of the enzyme catalysed THO liberation from [1β,2β,3β-$^3H_3$]GA_{20} (Semenenko, V. A. Smith, unpublished work). The GA products formed from [17-^{13}C,$^3H_2$]GA_{20} incubated with enzyme in the presence or absence of the antibody under otherwise identical conditions were analyzed by HPLC. In the presence of the antibody, the calculated inhibition of GA_1 synthesis was 76%, of GA_5 synthesis 78%, and of GA_{29} synthesis 75%.

Kinetics

A Sephadex G-100 enzyme preparation was used to determine the apparent K_m for [1β,2β,3β-$^3H_3$]GA_{20} metabolism. As indicated earlier, the observed radiolabeled reaction products, GA_1 and GA_{29} were formed in a constant ratio. Thus, by measuring the linear rate of THO liberation from this substrate, the overall K_m for GA_{20} in these reactions was estimated to be 1.5 ± ·047 μM and the V_{max} 8.7 nmol h^{-1} mg^{-1} from Eadie-Hofstee plots of v against $v/[S]$. The reactions are, however, subject to primary isotope effects since the tritium labels are at the sites of catalytic attack. The magnitude of the kinetic tritium isotope effect upon GA_{20} 3β-hydroxylation was estimated to be 3.5 from comparative measurements of the rate of GA_1 synthesis from [17-$^3H_2$,^{13}C]GA_{20} (0.5 μM) and [1β,2β,3β-$^3H_3$]GA_{20} (0.5 μM). These data are shown in Figure 12.

The fact that primary isotope effects were measurable suggested a kinetic method of testing the hypothesis that GA_1, GA_5, and GA_{29} synthesis are catalyzed by the same enzyme. The kinetic parameters for product formation from [17-^{13}C,$^3H_2$]GA_{20} and [2β-D,17-^{13}C,$^3H_2$]GA_{20} (7.33×10^8 Bq $mmol^{-1}$; Willis and Birch, unpublished work) were compared.

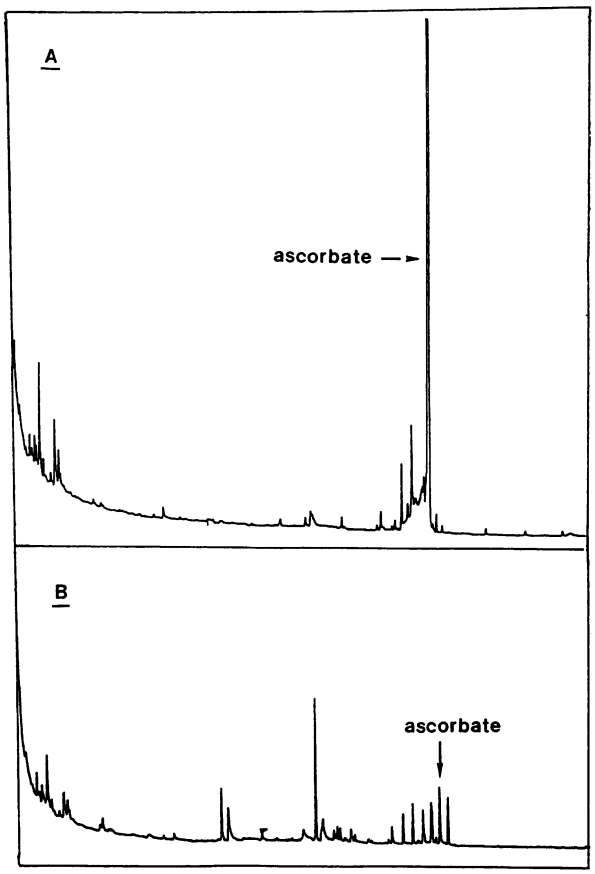


Figure 10. Capillary GC chromatograms of ascorbic acid samples incubated for 2 h at 25°C. A, In water containing Fe^{2+} and 2-oxoglutarate; B, in water containing Fe^{2+}.

These substrates were incubated under standard conditions at varying concentration with an LSS enzyme preparation (ammonium sulphate precipitated protein fraction). The reaction products were purified and quantified by HPLC.

The kinetic data pertaining to the metabolism of [17-^{13}C,$^{3}H_2$]GA_{20} and [2β-D,17-^{13}C,$^{3}H_2$]GA_{20} are presented in Table X. As expected, no isotope effect was apparent on V_{max}/K_m for GA_1 synthesis (Table XI). Gibberellin A_5 and GA_{29} synthesis were both subject to positive isotope effects on V_{max}/K_m, indicating that cleavage of the 2β-carbon-hydrogen (deuterium) bond is the first irreversible step in the formation of GA_5 and GA_{29}. Although there was a corresponding positive isotope effect on the V_{max} for GA_5 synthesis, the isotope effect on V_{max} for GA_1 and GA_{29} synthesis, was negative. This latter data provide conclusive evidence that GA_1, GA_5, and GA_{29} are divergent products of the same enzyme.

The identity and isotopic composition of the products found from [2β-D,17-^{13}C,$^{3}H_2$]GA_{20} was confirmed by GC-MS (Table XII). In addition to the stable isotope-labeled GA_1, GA_5, and GA_{29}, epi-GA_1 and epi-GA_{29} were also detected, albeit in very low abundance. It is considered probable that the enzyme exhibits a degree of nonselectivity with respect to the stereochemistry of proton abstraction. However, the possibility remains that the epimers were derived from GA_1 and GA_{29} either enzymically or during sample preparation. The latter option is less likely, particularly in the case of epi-GA_{29}.

DISCUSSION

The GA_{20} 3β-hydroxylase isolated from immature seeds of *Phaseolus vulgaris* is distinct from the GA_1 2β-hydroxylase isolated previously (23) from mature seeds of *P. vulgaris*. It is, however, very similar in its physical characteristics to the GA_{20} 2β-hydroxylase isolated from mature and immature seeds of *Pisum sativum* (24) and from epicotyls of *P. sativum* (V. A. Smith, unpublished work). The enzyme is a hydrophobic protein of M_r 45,000 that requires DTE for stability. It is also extremely labile at acidic pH and in dilute solution. The K_m for the enzyme is 1.54 μM with respect to [1β,2β,3β-$^{3}H_3$]GA_{20} and activity is not subject to product inhibition by GA_1. The scope for this relatively small monomeric protein to modulate directly GA_1 concentration *in vivo* would appear to be limited. However, the steady-state concentration of GA_1 is dependent upon the relative rate of its formation by the GA_{20} 3β-hydroxylase and inactivation by 2β-hydroxylation. In *P. vulgaris* seeds, the latter reaction has been shown to proceed at the faster rate (2). Thus, in any particular tissue, the absence or presence of GA_1 may depend upon whether or not the GA_{20} 3β-hydroxylase coexists with the GA_1 and GA_{20} 2β-hydroxylases. It has also been suggested that GA_{20} 3β-hydroxylation may be subject to photoperiodic control in vegetative tissue (5, 26). However, the experiments from which such

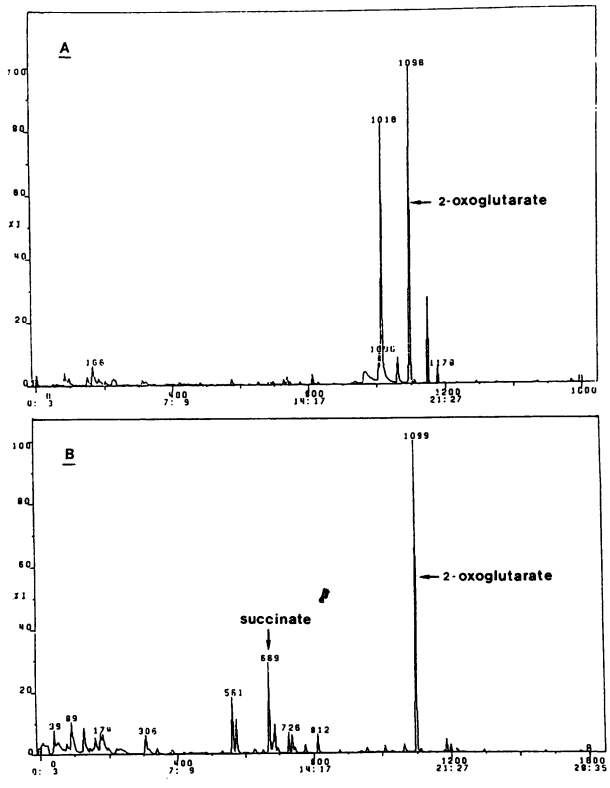


Figure 11. TIC traces of capillary GC-MS chromatograms of 2-oxoglutarate samples incubated in water for 2 h at 25°C in the absence (A) or presence (B) of Fe^{2+}.

Table IX. *Decomposition of 2-Oxoglutarate in Water and Water Containing Fe^{2+}*

Incubation Medium	Total Ion Current	
	2-Oxoglutarate remaining	Succinate produced
H_2O	219,797	5,835
$H_2O + Fe^{2+}$	253,491	143,434

hypotheses were drawn utilized tall and dwarf pea plants, and only in the latter case did the putative phytochrome effect apply. Subsequently, it has been concluded from work with isogenic Le/le pea lines that Le is phenotypically expressed in the dark (21) and the difference in metabolism of $[^3H]GA_{20}$ between Le and le plants grown in the light was also maintained in darkness (22).

The 2-oxoglutarate dependent dioxygenases are a widely distributed group of enzymes for which Fe^{2+} and ascorbic acid serve as cofactors and 2-oxoglutarate as cosubstrate. These enzymes, characteristically stabilized by BSA and catalase (1), catalyse the incorporation of one atom of molecular oxygen into succinic acid, derived by oxidative decarboxylation of 2-oxoglutarate (6, 14) (Scheme 3), and the other into an hydroxyl group of the primary reaction product. A number of authors (13, 17) have reported that the requirements of all soluble GA-hydroxylases, including the GA_{20} 3β-hydroxylase obtained from immature seeds of *P. vulgaris* are typical of the 2-oxoglutarate dependent dioxygenases. However, the characteristics of the GA_{20} 3β-hydroxylase preparation described in this paper did not conform to those expected of the 2-oxoglutarate dependent dioxygenases.

With respect to 2-oxoglutarate, activity dependence was less than absolute for the Sephadex G-100 purified enzyme assayed at saturating GA_{20} concentration, and double reciprocal plots of reaction velocity against 2-oxoglutarate concentration did not obey Michaelis-Menten kinetics. In the presence of the 3β-hydroxylase, succinate was not a detectable reaction product. Indeed 2-oxoglutarate was protected against Fe^{2+}-catalyzed decomposition by the enzyme. These data confirm that 2-oxoglutarate functions as cofactor rather than cosubstrate for the GA_{20} 3β-hydroxylase. Its role has been questioned previously with respect to the GA 2β-hydroxylases. In the case of the GA_1 2β-hydroxylase from mature seeds of *P. vulgaris,* Smith and MacMillan (23) were unable to show that GA_8 and succinic acid were formed in a 1:1 stoichiometric ratio, but found that oxidative decarboxylation of 2-oxoglutarate proceeded in the presence of Fe^{2+} and ascorbate and absence of the enzyme. Additionally, for all the GA_{20} 2β-hydroxylases examined to date, using subsaturating concentrations of $[1\beta,2\beta,3\beta\text{-}^3H_3]GA_{20}$ as substrate, total dependence upon 2-oxoglutarate was demonstrable only after the Sephadex G-100 purification step (23, 24).

Of the other cofactors required by the GA_{20} 3β-hydroxylase from *P. vulgaris,* the addition of reduced iron was essential; chelating agents were not required to eliminate enzyme activity. Thus, uncharacteristically, the association between Fe^{2+} and the enzyme appeared to be weak. For almost all documented 2-oxoglutarate dependent dioxygenases, residual activity, ranging from 10 to 100%, was measurable in the absence of Fe^{2+} (28). In the extreme cases, Fe^{2+} was bound so tightly that chelators such as α,α-bipyridyl failed to abstract protein-bound Fe^{2+} and thus inhibit enzyme activity (20).

A similar situation also existed with respect to the ascorbic acid. For documented 2-oxoglutarate dependent dioxygenases, the requirement for ascorbate is generally less than absolute (7, 19). A postulated role of the compound is to maintain protein-bound iron in a reduced state (7). However, the GA_{20} 3β-hydroxylase activity was found to be totally dependent upon the inclusion of ascorbic acid. Analysis of

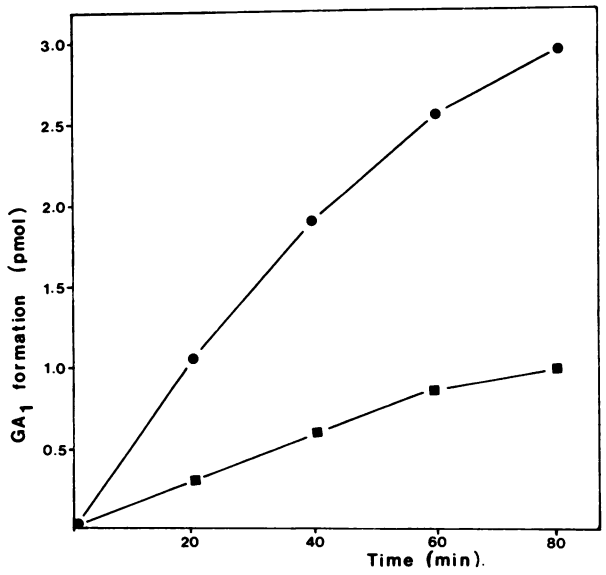


Figure 12. Kinetics of GA_1 formation. (●), Substrate $[17\text{-}^{13}C,^3H_2]GA_{20}$; (■), substrate $[1\beta,2\beta,3\beta\text{-}^3H_3]GA_{20}$.

Table X. *Comparison of Kinetic Parameters for $[17\text{-}^{13}C,\ ^3H_2]GA_{20}$ and $[2\beta\text{-D},\ 17\text{-}^{13}C,\ ^3H_2]GA_{20}$ Metabolism*

GA Product	Kinetic Parameter	Substrate	
		$[17\text{-}^{13}C^3H_2]GA_{20}$	$[2\beta\text{-D},\ 17\text{-}^{13}C^3H_2]GA_{20}$
GA_1	K_m	2.77 ± 0.28 μM	3.57 ± 0.44 μM
	V_{max}	0.175 nmol h^{-1} mg^{-1}	0.30 nmol h^{-1} mg^{-1}
GA_5	K_m	3.79 ± 0.26	13.67 ± 0.92
	V_{max}	0.059 nmol h^{-1} mg^{-1}	0.024 nmol h^{-1} mg^{-1}
GA_{29}	K_m	1.37 ± 0.28	11.64 ± 1.7
	V_{max}	5.5 pmol h^{-1} mg^{-1}	20.84 pmol h^{-1} mg^{-1}

Table XI. *Deuterium Isotope Effects on the Formation of GA_1, GA_5 and GA_{29} from GA_{20}*

Reaction Product	Dv (V_{max})	Dv (V_{max}/K_m)
GA_1	0.6	0.75
GA_5	2.5	9.0
GA_{29}	0.26	2.3

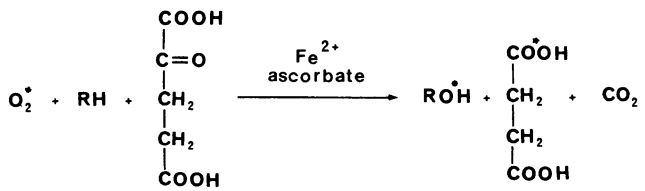
Table XII. *GC-MS Analysis of Products Formed from 2β-D, [17-^{13}C,$^{3}H_2$]GA_{20}*

GA Identified	Recovery[a]	Isotopic Composition				Fit Factor
		M^+	$M^+ + 1$	$M^+ + 2$	$M^+ + 3$	
GA_1	610	4.1	10.8	78.7	5.5	0.9995
epiGA_1	19	3.3	11.8	77.9	6.0	0.9873
GA_5	28	5.4	86.7	7.9	0	0.9946
GA_{20}	1098	1.0	13.3	79.5	5.6	0.9990
GA_{29}	40	24.8	62.7	9.8	1.9	0.9969
epiGA_{29}	18	19.9	49.5	26.3	4.4	0.9774

[a] GA_1/epiGA_1, GA_5, GA_{20}, and GA_{29}/epiGA_{29} recoveries were determined from the radioactivity cochromatographing with appropriate GA standards on HPLC. The proportions of epiGA_1 and epiGA_{29} present in the respective GA_1 and GA_{29} HPLC fractions were calculated after GC-MS by comparison of the TIC areas under the molecular ion peaks.

reaction products formed in the presence of $^{18}O_2$ showed that ^{18}O-labeled threonic acid, an oxidative degradation product of ascorbic acid, was formed in preference to ^{18}O-labeled succinate, the expected product from 2-oxoglutarate. Whether this oxidation was enzyme catalyzed or simply Fe^{2+} catalyzed is unknown, but the possibility remains that the GA_{20} 3β-hydroxylase may utilise ascorbate as a second or alternative oxygen acceptor. It is also possible that a ferrous-ascorbate complex forms part of the catalytic unit as originally proposed by Visser (29). In the absence of the enzyme under anaerobic conditions, a solution of Fe^{2+} and ascorbate was colorless. Under oxygen a blue-colored complex formed. This complex, and the integrity of the ascorbic acid, were apparently stabilized by 2-oxoglutarate and, in this case, ^{18}O-succinate was produced.

Impure preparations of the GA_{20} 3β-hydroxylase also catalyze the formation of GA_5 (2, 16) and GA_{29} (2) from GA_{20}. From observations that all three enzyme activities were parallel throughout seed maturation and that the relative rates of formation of GA_1, GA_5, and GA_{29} were the same, independent of the combination of added cofactors, we previously suggested that all three activities reside in the same catalytic protein (2). Although Kwak *et al.* (17) have recently published a procedure for purifying the GA_{20} 3β-hydroxylase, the authors failed to analyze the reaction products formed at any stage of purification. However, from the present work, the kinetic isotope effects on V_{max} for the enzyme incubated with [2β-D, 17-^{13}C,$^{3}H_2$]GA_{20} as substrate, confirmed that GA_1, GA_5, and GA_{29} are divergent reaction products formed from a common intermediate. Supporting evidence also presented



Scheme 3. Generalized reaction catalyzed by the 2-oxoglutarate dependent dioxygenases.

here include data showing that all three activities have identical cofactor requirements, that double reciprocal plots of reaction velocity against 2-oxoglutarate concentration are parallel for GA_1, GA_5, and GA_{29} synthesis, and that GA_5 and a monoclonal antibody raised against the Sephadex G-100 enzyme both inhibit equally GA_1, GA_5, and GA_{29} synthesis.

The mechanistic implications are under investigation and shall be discussed fully elsewhere. However, GA_5 is a nonhydroxylated reaction product, the formation of which requires 2-oxoglutarate and molecular oxygen. In this, there is an interesting parallel with diacetoxycephalosporin C synthetase which is also a bifunctional enzyme that requires O_2, Fe^{2+}, 2-oxoglutarate, and ascorbate for ring expansion of penicillin N and (subsequent) hydroxylation of the product to deacetylcephalosporin C (4). In the present case, dioxygen clearly plays a role as electron acceptor in the initial, enzymically catalyzed abstraction of the 3β- and 2β-protons from the GA_{20} substrate. The subsequent substrate hydroxylation steps may well be essentially nonenzymic, resulting from the consequent formation of a dioxygen radical anion. The ultimate fate of the individual atoms of dioxygen during the formation of GA_1, GA_5, and GA_{29} remains unknown, but it is concluded that the GA_{20} 3β-hydroxylase is inaccurately classified as a 2-oxoglutarate dependent dioxygenase. Thus, it is proposed that this enzyme, and probably the GA 2β-hydroxylases also, be reclassified as oxidases and referred to as either GA oxidases or hydroxylases.

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