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THE FORMATION OF SHIKIMATE-3-PHOSPHATE IN CELL-FREE PREPARATIONS OF *SORGHUM**

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Key Word Index—*Sorghum bicolor*; Gramineae; biosynthesis; aromatic compounds; shikimic acid pathway; shikimate kinase; shikimate-3-phosphate.

Abstract—Shikimate-3-phosphate was formed by cell-free preparations of etiolated shoots and green stems of *Sorghum bicolor* but not of roots of etiolated seedlings. Shikimate-3-phosphate was purified and identified by GC–MS and NMR spectrometry. The reaction was ATP-dependent and products other than the 3-phosphate were not observed.

INTRODUCTION

Shikimate-3-phosphate (S-3-P) is an obligatory intermediate in the shikimic acid pathway of aromatic amino acid biosynthesis in microorganisms [1]. Subsequent to the isolation and identification of S-3-P by Weiss and Mingioli [2], shikimate kinase (EC 2.7.1.71 ATP: shikimate 3-phosphotransferase†), the enzyme responsible for its formation, has been reported to be present in cell-free extracts of a number of bacteria [3–6], fungi [7], algae [8] and a moss [8]. Although radiotracer studies have established that shikimate is converted into aromatic compounds in higher plants [1], the presence of shikimate kinase has not been conclusively shown. Indeed, it has been suggested that S-3-P is not an intermediate in that conversion [1] or does not arise directly from shikimate [9].

In addition to the utilization of shikimate in aromatic amino acid biosynthesis, higher plants are known to accumulate free shikimate [10] and shikimate depsides [11, 12]. Additional routes for shikimate metabolism have been proposed including dehydration to give quinate [9] and direct incorporation into quinones [13]. Thus the potential formation of positional isomers of shikimate-P (S-P) as an adjunct to transport or further metabolism should not be ignored when the phosphorylation of shikimate is investigated, nor should the presence of shikimate kinase be assumed to verify its role in the shikimic acid pathway in higher plants.

This paper reports the detection of shikimate kinase in cell-free extracts of etiolated shoots and green, lignifying stems of *Sorghum bicolor* (L.) Moench cv Sordan 70A, the purification of a reaction product, and its subsequent identification as S-3-P by ^{1}H-NMR spectrometry and GC–MS.

RESULTS AND DISCUSSION

ATP-dependent shikimate kinase activity was detected in aqueous preparations of both etiolated shoots and mature stems of *Sorghum*, but not of roots of etiolated seedlings (Table 1). The activities in Table 1 represent minimum values as no attempt was made to determine the optimum conditions for shikimate kinase activity or minimize interaction with phosphatase present in both extracts (unpublished observations) beyond the inclusion of F^- and Pi in the reaction solution, the omission of which decreased by 50% the apparent activity of the shoot preparation. While the Ba^{2+} precipitation method of estimating shikimate kinase activity is sensitive to the conversion of about 1% of the substrate, it is not specific for the formation of S-3-P as other phosphate esters, dicarboxylic acids or CO_3^{2-} derived from shikimate will also precipitate, necessitating characterization of the reaction products.

Results of anion-exchange chromatography and PC of the reaction product from an enzyme preparation from etiolated shoots revealed only one radioactive component which co-chromatographed with S-3-P isolated from the culture fluid of *Klebsiella pneumoniae* A170-40 [14], and suggested that the product derived from shikimate was

Table 1. Shikimate kinase activity in preparations of *Sorghum* organs

Organ	Shikimate kinase activity (nkat/mg protein)	Shikimate kinase activity (nkat/g fr. wt)
Shoots from etiolated seedlings	0.13	0.63
Roots from etiolated seedlings	0	0
Green stems	0.11	0.065

*Part 5 in the series 'Regulation of aromatic amino acid biosynthesis in higher plants.' Part 4 was Gilchrist, D. G., and T. Kosuge (1975) *Arch. Biochem. Biophys.* **171**, 36.

†Although the name of this enzyme is ATP: shikimate-5-phosphotransferase in the Recommendations for Enzyme Nomenclature (1972) of the IUPAC and IUB, the product is shikimate-3-phosphate when numbered according to the IUPAC Nomenclature of Organic Chemistry, Sect. C (1965), a convention which is followed in this paper.

Table 2. Analyses of the products liberated by phosphatase treatment† of S-3-P produced by a preparation of etiolated *Sorghum* shoots

Analytical method	Result
Expt. 1	
Periodate oxidation–thiobarbituric acid condensation	*0.66* mol shikimate equiv. wt*
Phosphomolybdovanadate complex	*0.75* mol Pi equiv. wt
Expt. 2	
GLC of TMSi derivatives	*0.75* mol shikimate equiv. wt *0.77* mol Pi equiv. wt

*Based upon a formula of Ba_3(S-3-P)$_2$.5H_2O, equivalent wt = 500 g.

structurally similar to S-3-P. However, it is not known whether these chromatographic systems can resolve the positional isomers of S-P.

A purified S-P sample isolated from a preparative-scale reaction mixture containing a shikimate kinase preparation from etiolated shoots contained no detectable free shikimate, Pi, or pentose and did not exhibit an absorption maximum at 260 nm. The S-P content by weight was 70% of that expected for Ba_3(S-3-P)$_2$.5H_2O [2], the remainder probably consisting of $BaCO_3$, $Ba(OAc)_2$ and H_2O. Treatment of the preparation with alkaline phosphatase released shikimate and Pi in a molar ratio of 1.0 to 1.2 based upon spectrophotometric analytical procedures (Experiment 1, Table 2) while the products released in a similar experiment corresponded to shikimate and Pi in a molar ratio of 1.0 to 1.0 when analyzed by GLC (Experiment 2, Table 2). The results of both experiments indicated that the product is a monophosphate ester of shikimate.

The position of the esterified phosphate was determined from the ^{1}H-NMR spectra of shikimate and this S-P preparation (Table 3), signals being assigned according to Hall [15]. Two differences are apparent between the spectra: the signals from H-2 in the S-P sample are shifted upfield relative to those in shikimate, probably an effect of the proximity of the introduced P group, and the signals from H-3 in the S-P sample are shifted relatively downfield, merging with the HOD peak, an effect of the substitution of the more electron withdrawing P group for OH on C-3. As the chemical shifts and forms of the signals from the other protons in both spectra are essentially identical, P must be esterified to C-3 of shikimate and the phosphorylated compound must be S-3-P. In addition, esters other than S-3-P must be absent. Similar chemical shifts were observed by Bondinell *et al.* [16] in the ^{1}H-NMR spectrum of 5-enolpyruvyl-shikimate-3-P.

Table 3. ^{1}H-NMR spectra* of shikimate and S-3-P

Proton	Chemical shift (δ)	
	Shikimate	S-3-P
H-2	6.83 (*m*)	6.46 (*m*)
H-3	4.46 (*m*)	4.7–4.85†
H-4	3.79 (*q*)	3.78 (*q*)
H-5	4.05 (*m*)	4.05 (*m*)
H-6a	2.22 (*m*)	2.17 (*m*)
H-6e	2.76 (*m*)	2.73 (*m*)

*Spectra were obtained at 99.5 MHz in D_2O with 2,2,3,3-tetradeutero-3-trimethylsilylpropanoic acid, sodium salt, as an internal standard. Hence, no signals were observed from OH, COOH or OPO_3H_2. †Signal was obscured by HOD signal.

By GLC and GC–MS, S-3-P produced by the etiolated shoot preparation was compared with both the less pure product isolated from a reaction solution containing an enzyme preparation from mature stems and a S-3-P sample isolated from the culture fluid of *K. pneumoniae* A170-40. GLC of the TMSi derivative of S-3-P produced by the preparation from etiolated shoots revealed only one peak with relative retention times of 0.924 and 1.55 on columns A and B, respectively. Compounds with the same retention times were also detected by GLC of TMSi derivatives of the product of the *Sorghum* stem preparation and the S-3-P sample isolated from *K. pneumoniae* culture fluid. The mass spectrum of TMSi-S-3-P produced by the etiolated shoot preparation (Table 4) was essentially identical to the spectra of the compound from the two other sources and contained ions characteristic of phosphate esters of *m/e* 211, 227, 243, 299, 315, and 387 [17, 18] as well as an abundant ion of *m/e* 204 which may consist of C-4 and C-5 and their associated O-TMSi groups, a fragment frequently observed in the spectrum of other compounds containing vicinal hydroxyl groups [19].

TMSi-S-3-P was unstable during silylation or GLC and repeated GLC-analyses of a given sample produced variable peak sizes, thus making this procedure undesirable for quantitative analysis. Similar instability has been observed for TMSi-phosphoglyceric acids and

Table 4. The 70 eV mass spectrum of TMSi-S-3-P

m/e	Relative intensity*	*m/e*	Relative intensity	*m/e*	Relative intensity
73	650	167	14.6	283	11.6
74	80	169	21.5	299	49.0
75	120	179	7.5	300	14.3
91	15.6	181	10.0	301	9.6
93	32.3	189	6.3	302	2.4
94	7.3	191	44.4	307	2.9
95	12.4	193	35.3	309	4.8
103	20.3	194	7.4	314	4.8
105	21.7	195	15.0	315	8.7
119	7.4	204	80.7	316	2.2
121	17.7	205	20.3	317	2.4
131	12.0	206	12.0	319	t†
133	38.5	207	12.1	346	t
135	16.4	211	27.1	353	t
141	7.4	225	8.6	372	3.6
147	100	227	14.5	373	3.6
148	19.1	243	14.3	387	2.2
149	19.5	255	9.6	406	3.5
151	16.7	267	17.9	435	2.9
165	8.4	282	7.9	487	5.6

*While *m/e* 73 was the largest peak in the spectrum, it was disregarded in assigning the base peak (*m/e* 147) because of its general irreproducibility. Only those peaks with relative intensity greater than 7% are indicated in the region *m/e* 73–299. †t = trace, generally <2%.

TMSi-6-phosphoaldonic acids [18]. In addition, a second peak which exhibited relative retention times of 0.883 and 1.11 on columns A and B, respectively, was occasionally observed. The occurrence of this compound was unpredictable and was not prevented by altering the silylation conditions and reagents. Its mass spectrum (unpublished) contained ions characteristic of TMSi-P esters [17, 18] and was distinguished from that of TMSi-S-3-P by substantial increases in the relative abundances of ions of *m/e* 225, 328 and 372 and a decrease in the abundance of the ion of *m/e* 204.

The identification of S-3-P as the product of the ATP-dependent reaction with shikimate catalyzed by extracts of *Sorghum* shoots and stems establish the presence of shikimate kinase in these organs but provides no conclusive evidence for the role of the enzyme in the shikimic acid pathway. However, the shikimate kinase activity observed in crude extracts of etiolated shoots (Table 1) is sufficient to allow the synthesis within 3–6 hr of the quantity of dhurrin, a major product of tyrosine metabolism [20], present in other cultivars of *S. bicolor* [21]. Shikimate kinase may function in green stem tissue in the synthesis of the aromatic precursors of lignin as has been suggested for the shikimate: NADP oxido-reductase observed in cell-free preparations of stem tissue of other lignifying grasses [22]. The inability to detect shikimate kinase in root preparations deserves further investigation. It will be of interest to determine if this is due to inactivation or low concentration of the enzyme, or if the enzyme is truly absent in roots of etiolated seedlings. Roots of other species have been shown to contain other enzymes associated with the shikimic acid pathway [23–25].

EXPERIMENTAL

Plant material. For the production of etiolated seedlings, seeds of *Sorghum bicolor* (L.) Moench cv Sordan 70A (Northrup-King) were immersed in aerated H_2O for 24 hr and then spread between 2 layers of cheesecloth tightly stretched across wire racks in covered plastic boxes. The bottoms of the boxes were filled with H_2O to within 0.5 cm of the seed layer and the boxes kept 48 hr in darkness at 25–30°. Etiolated shoots were separated from roots by excision with a razor blade and immediately placed in liquid N_2. Roots were similarly treated. Green stems were taken from greenhouse-grown plants at the time of emergence of the panicle. The portions of the stems between the second node and a point *ca* 10 cm below the tip were stripped of leaves and sheaths and cut into sections which were immediately frozen in liquid N_2. Frozen tissue from all sources was ground to a powder with liquid N_2 in a stainless-steel blender and stored at −15°.

Preparation of shikimate kinase. Frozen *Sorghum* powder was mixed with an equal quantity of buffer (w/v) containing K-Pi (0.1 M, ph 6.8), sucrose (0.4 M), EDTA (5 mM), $MgCl_2$ (1 mM), Na ascorbate (20 mM) and 2-mercaptoethanol (0.1%) at room temp. and stirred at room temp. until the temp. of the extract reached 2°. All further operations with the enzyme were performed at 0–5°. The extract was squeezed through 4 layers of cheesecloth and centrifuged at 20000 *g* for 20 min. Floating material was removed with suction and the supernatant fraction was filtered through one layer of Miracloth (Chicopee Mills). The filtrate was assayed for shikimate kinase activity or treated with $(NH_4)_2SO_4$ as indicated below for the two shikimate kinase preparations.

Estimation of shikimate kinase activity. Shikimate kinase was assayed by a procedure modified from Nakatsukasa and Nester [26]. The reaction soln contained shikimate-[U-^{14}C] (1 mM, 0.1 Ci/mol), ATP (4 mM), $MgCl_2$ (10 mM), K-Pi (10 mM), NaF (10 mM), PIPES buffer (50 mM N pH 6.8) and 50 µl of enzyme preparation in a total vol. of 200 µl. For control reactions, ATP was omitted. The reaction was initiated by addition of the enzyme preparation. After 30 min at 30° the reaction was terminated by addition of 50 µl of TCA (1.25 M) and material was precipitated by the addition of 50µl of NaOH (1.25 M) containing $(NH_4)_2SO_4$ (50 mM), 50µl of $Ba(OAc)_2$ (2 M) and 1.75 ml of 95% EtOH, mixing after each addition. After chilling for *ca* 10 min, the suspension was vacuum-filtered through a glass-fibre filter (Whatman GF/C, 25 mm dia.), the filter rinsed 2× with *ca* 1 ml of 50% EtOH and dried with Me_2CO prior to liquid scintillation counting in a standard toluene cocktail.

Preparation of S-3-P. (a) *Etiolated shoots.* The supernatant fraction obtained from 280 g frozen etiolated shoot powder was treated with solid $(NH_4)_2SO_4$ and the fraction that precipitated between 10 and 50% of satn was resuspended in 170 ml of K-Pi (0.1 M, pH 6.8). This shikimate kinase preparation (43 nkat) was incubated for 30 min at 30° with shikimate (0.4 mM), ATP (4 mM), $MgCl_2$ (10 mM), NaF (10 mM) and PIPES buffer (40 mM, pH 6.8) in a final vol. of 1.7 l. The reaction was stopped by boiling, the pH adjusted to 3 with HCl, and the suspension filtered through Celite (Johns-Manville). The filtrate was applied to a coconut-charcoal column (40 ml, 50–200 mesh, Fisher) and S-3-P was eluted and isolated as the Ba^{2+} salt as described by Knowles and Sprinson [14] except that elution was done with aq. EtOH adjusted to pH 10 with NH_4OH and was continued with an additional 2 l 40% EtOH. The vacuum-dried Ba^{2+} salt was dissolved in 50 ml of H_2O, the soln filtered and S-3-P reprecipitated with 5 vol. of cold MeOH. The ppt. was collected by centrifugation, vacuum-dried, dissolved in 20 ml HOAc (5 mM) and applied to a column of anion-exchange resin AG1-X8 (Cl^-, 9.5 ml, Bio-Rad). The column was developed with NH_4Cl (0.15 M, pH 7.0). After shikimate eluted from the column, the fractions with an A_{205}/A_{260} greater than 1.4 were pooled and lyophilized. The residue was dissolved in 10 ml H_2O and chromatographed in 1-ml portions on a polyacrylamide gel P-2 column (1.5 × 24 cm, 200–400 mesh, Bio-Rad) that was equilibrated and developed with NH_4Cl (0.15 M, pH 7.0). In spite of its lower MW S-3-P eluted before the adenylates. Fractions with an A_{205}/A_{260} greater than 50 eluting before the adenylates were pooled and concentrated, and S-3-P was precipitated as the Ba^{2+} salt [14], yielding 25 mg of white powder. (b) *Green stems.* The supernatant fraction obtained from 600 g of frozen stem powder was treated with solid $(NH_4)_2SO_4$. The fraction that precipitated between 20 and 70% of satn was resuspended in 50 ml of K-Pi (50 mM, pH 6.8) and dialyzed for 16 hr against K-Pi (0.1 M, pH 6.8) containing 2-mercaptoethanol (0.1%) and Na ascorbate (10 mM). This shikimate kinase preparation (12 nkat) was incubated for 30 min at 30° with shikimate-[U-^{14}C] (0.4 mM, 1 mCi/mol), ATP (4 mM), $MgCl_2$ (10 mM), NaF (10 mM), and PIPES buffer (40 mM, pH 6.8) in a final vol. of 550 ml. After boiling and filtration through Celite, the soln was applied directly to a column of AG1-X8 (HCO_3^-, 108 ml) and S-3-P eluted with a linear gradient prepared from 500 ml each of $NaHCO_3$ (1 M) and H_2O. Pooled fractions comprising the second peak of radioactivity were stirred under vacuum with AG50W-X8 (H^+, 25 ml). The suspension was filtered and the filtrate lyophilized. The residue was dissolved in 20 ml of H_2O, and the soln passed through a column of Cation-exchange resin AG50W-X8 (NH_4^+, 25 ml, Bio-Rad). The column effluent was lyophilized and the residue dissolved in 4 ml H_2O. Aliquots (100 µl) were lyophilized in preparation for GLC and GC–MS. (c) Klebsiella pneumoniae A170-40. S-3-P was also isolated from the culture fluid of *Klebsiella pneumoniae* A170-40 [14].

PC and anion-exchange chromatography. After incubation with the supernatant fraction obtained from etiolated shoots, the reaction soln was amended with S-3-P isolated from *K. pneumoniae* culture fluid [14] and subjected to PC in *n*-PrOH–NH_4OH–H_2O (6:3:1) or anion-exchange chromatography as described for the purification of S-3-P. Compounds on the chromatogram were located by autoradiography followed by an acid molybdate spray [27] while the anion-exchange column effluent was monitored for A_{205} and radioactivity.

GLC and GC–MS. GLC of TMSi derivatives was on either (A) 3% OV-101 + 4.5% OV-210 or (B) 5% OV-225. both in 2 mm × 1 m glass columns. N_2 was the carrier at *ca* 20 ml/min. The injection block was at 250° with the FID at 275°. TMSi-S-3-P was chromatographed at 220° on column (A) or 180° on column (B) while TMSi-shikimate and TMSi-Pi were chromatographed on column (B) at 130° and 80° respectively. Retention times were calculated relative to TMSi–Fru-6-P [18] chromatographed under identical conditions. Pi was silylated as the NH_4^+ salt while shikimate and S-3-P were silylated as either the NH_4^+ salts or free acids [17, 18] after passage through a small column of AG50W-X8 and lyophilization, if necessary. Silylation was generally with BSTFA (Pierce) and equal vol. of Py or DMF at 80° for 15 min or room temp. for 16 hr, but occasionally BSTFA was replaced by N-(trimethylsilyl)-imidazole (Pierce) [18] or amended with TMCS (Pierce) [18] with equivalent results. GLC peak areas were determined by planimetry and were proportional to concentration for both TMSi–shikimate and TMSi–Pi. GC–MS was performed at 70 eV with a source temp. of 60° and a 2 mm × 1 m glass column containing OV-17.

Other analytical procedures. Fourier-transform ^{1}H-NMR spectra were measured at 99.5 MHz in D_2O relative to 2,2,3,3,-tetradeutero-3-trimethylsilylpropanoic acid, sodium salt. S-3-P produced by the preparation from shoots was hydrolyzed with alkaline phosphatase (Sigma type V) in $(NH_4)_2CO_3$ (50 mM, pH 10) at 36° for 2 hr Aliquots were used directly in spectrophotometric analyses or lyophilized prior to GLC. Free shikimate was estimated by the IO_4^-–thiobarbituric acid method [28], Pi as a phosphomolybdovanadate complex [29], total shikimate (including S-3-P which reacts like shikimate, unpublished observation) after condensation with *p*-hydroxybenzaldehyde [30], pentose by the orcinol method [31] and protein by the biuret method [32] after precipitation with TCA.

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