Facile and Economical Preparation of [^{14}C]-Labelled Shikimic Acid

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Summary

A convenient and inexpensive method for the preparation of [^{14}C]-labelled shikimic acid based on the photoassimilation of $^{14}CO_2$ by henbane (*Hyoscyamus niger L.*) leaves in the presence of the herbicide glyphosate is described.

Methanolic extracts were purified by successive anion exchange, paper and thin-layer chromatography to yield [^{14}C]- labelled shikimic acid of 99.5% radiochemical purity, as shown by analytical HPLC. Under the conditions employed, the rate of incorporation of $^{14}CO_2$ into shikimic acid (0.7 - 17.6%) showed a positive correlation with the size of the leaf used in the incubation (3.6 - 146 mg fresh weight), while the specific activity of the acid (6 - 12.7 GBq/mmol) was an inverse function of the leaf size.

Key words: [^{14}C]-Shikimic Acid, Photosynthesis, Glyphosate

Introduction

Shikimic acid is a central intermediate in the shikimate pathway along which the aromatic amino acids and a multitude of other aromatic and alicyclic compounds are biosynthe-

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sized in bacteria, fungi and plants (4, 12). [^{14}C]-Labelled shikimic acid required for investigations of the biosynthesis and metabolism of such compounds has been prepared by several biological and chemical procedures. A number of authors isolated uniformly labelled [^{14}C]-shikimic acid from a mutant of *Escherichia coli* grown in the presence of uniformly labelled [^{14}C]-glucose (6, 7, 9, 13). This procedure has been used for the commercial preparation of [^{14}C]-labelled shikimic acid with a maximum specific activity in the range of 3 GBq/mmol. In spite of its high cost, the obvious advantage of this procedure is the possibility of introducing the radioactive label into individual positions in the shikimic acid molecule by using specifically labelled precursors.
Other investigators used plant tissues which accumulate shikimic acid naturally such as *Ginkgo biloba* leaves. In these cases, $^{14}CO_2$ was the precursor of choice (3, 11). However, to obtain satisfactory incorporation rates (5 to 6%) of $^{14}CO_2$ into shikimic acid several days of photoassimilation were required and specific activities were not higher than 13 MBq/mmol. Chemical syntheses of [^{14}C]-labelled shikimic acid have been described but suffered from a low yield of the natural *D*-enantiomer (2, 8).
We have previously shown that the herbicide glyphosate is a potent inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate synthase and that it causes a drastic accumulation of shikimic acid in plant tissues which do not normally accumulate this acid. (1, 10). Here we report that photoassimilation of $^{14}CO_2$ by appropriate leaf tissue in the presence of glyphosate allows the facile preparation of [^{14}C]-labelled shikimic acid in high yield and with the highest specific radioactivity reported to date.

Results and Discussion

Plant material suitable for the production of [^{14}C]-labelled shikimic acid of high specific activity and in high yield should preferably have a low background level of the acid, but accumulate it rapidly in the presence of glyphosate. Young leaves from henbane (*Hyoscyamus niger*) plants grown under short day conditions fulfilled these requirements ideally. However, leaf material from an ornamental plant, the Madagaskar periwinkle (*Catharanthus roseus*) or from tomato plants (*Lycopersicon esculentum*) were also

found to be suitable. In preliminary experiments, young henbane leaves accumulated almost 2% shikimic acid per dry weight during a 24 h incubation in the light in the presence of 1 mM glyphosate, representing a nearly 100-fold increase in concentration. When leaves of different sizes (fresh weight) were allowed to photoassimilate $^{14}CO_2$ in the presence of glyphosate over a 24 h period, the rate of incorporation of $^{14}CO_2$ into shikimic acid was positively correlated with leaf size, while an inverse relationship between the specific radoactivity of the acid and the leaf size was found (Table I). The highest specific activity of shikimic acid obtained (12.7 GBq/mmol) represents an isotopic enrichment of 79%. Since the $^{14}CO_2$ had an isotopic enrichment of 92%, it is apparent that almost no isotopic dilution of label from $^{14}CO_2$ occurred during its incorporation into shikimic acid. For all practical purposes, a reduction of the specific activity of shikimic acid by 50% is fully compensated by a more than 20-fold increase in yield of the radioactive product (Table I). HPLC-analysis of the purified acid revealed a 99.5% radiochemical purity (Figure 1). Decarboxylation of the product and recovery of the carboxyl group as $BaCO_3$ showed that 13.2% of the total radioactivity resided in the carboxyl group. Uniform distribution of the radiolabel in the shikimic acid may therefore be assumed. Compared with previously published procedures for the preparation of uniformly [^{14}C]-labelled shikimic acid the method presented here appears to be the most efficient and economical.

Table I: Incorporation of $^{14}CO_2$ into shikimic acid by henbane leaves as a function of leaf size.

Leaf Size (mg fresh weight)	Rate of Incorporation into Shikimic Acid (%)	Specific Radioactivity (GBq/mmol)
3.6	0.7	12.7
6.4	2.9	11.5
59.1	5.2	8.6
106.0	12.6	5.5
146.2	17.6	6.0

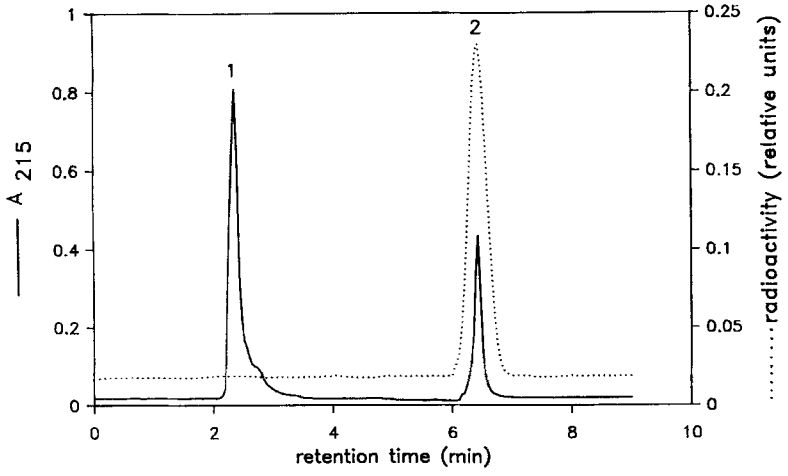

Fig. I: HPLC-analysis of [^{14}C]-labelled shikimic acid. Peak 1 is the solvent peak, peak 2 represents shikimic acid.

Experimental

Plant material: Seeds of *Hyoscyamus niger* L. were a kind gift of Prof. A. Lang, MSU-DOE Plant Research Laboratory, East Lansing, MI, USA. Plants were grown in a greenhouse under short day conditions. Leaves were collected from 4 month old plants.

Incubation of leaflets: A leaf was placed in the centre well of a conical 50 ml flask containing 2 ml 10 mM potassium phosphate buffer, pH 5.5, supplemented with 1 mM glyphosate. The outer ring of the flask was filled with 0.5 ml $Na_2H^{14}CO_3$ (37MBq, 2.035 GBq/mmol, Amersham). The flask was flushed with CO_2-free air and closed with a serum cap. $^{14}CO_2$ was then generated by injecting 2 ml of 60 % perchloric acid into the outer ring. The flask was irradiated for 24 h with light from a 1000 W quartz halogen lamp (light intensity at the leaf surface: 35 klx). After the incubation residual $^{14}CO_2$ was drawn into a KOH trap.

Extraction and purification of shikimic acid: The leaf was extracted with 3 changes of 15 ml refluxing methanol. After filtration of the combined extracts the solvent was evaporated and the residue taken up in 30 ml H_2O. The extract was transferred to a glass column

(100 x 10 mm) containing Amberlite (type IRA 410, 20 to 50 mesh, Serva, Heidelberg), which had been converted to the CO_2^{2-} form by washing with 80 ml 1 N Na_2CO_3 and then H_2O to neutrality. After washing with 25 ml H_2O, organic acids were eluted with 50 ml 1 N $(NH_4)_2CO_3$. The eluate was reduced to dryness at 70°C and the residue taken up in 2 ml H_2O and spotted on Whatman No.3 chromatography paper. The chromatogram was developed (ascending) with isobutanol/ethyl acetate/acetic acid/formic acid/H_2O (35:35:8:2:20). Shikimic acid (Rf 0.35) was eluted with several changes of hot water. The eluate was reduced to dryness, taken up in a small volume of H_2O and chromatographed on cellulose thin-layer plates (Merck, Darmstadt) with benzyl alcohol/butan-3-ol/propan-2-ol/H_2O/formic acid (3:1:1:1:0.12). Shikimic acid (Rf 0.4) was again eluted with hot water. The eluate was reduced to dryness and taken up in 5 ml H_2O.
Shikimic acid was analyzed by HPLC as described previously (5) using a Beckman System Gold instrument equipped with a Berthold radioactivity monitor LB 507 A.

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