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# Enzymic Preparation of Specifically $^{14}C$ and $^{3}H$ labelled Shikimic Acids

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SUMMARY

*D-Shikimic acids 1-$^{14}C$, 6-$^{14}C$ and 7-(carboxyl) $^{14}C$ have been biosynthetically prepared with specific activities of about 25 mCi/mmole and with overall yields of 45% based on pyruvate 3,2 or 1-$^{14}C$ as the respective starting materials. A crude cellfree homogenate of* E. coli *83-24 was used to catalyze the condensation of phosphoenolpyruvate-$^{14}C$ with unlabelled erythrose-4-phosphate to give shikimic acid. Phosphoenolpyruvate-$^{14}C$ was synthesized from pyruvate-$^{14}C$ and ATP by the action of phosphoenolpyruvate-synthase. Erythrose-4-phosphate 1,2,3,4-$^{14}C$ and erythrose-4-phosphate-4-T was prepared from glucose-U-$^{14}C$ and glucose-6-T* via *enzymatic phosphorylation to labelled glucose-6-phosphate and subsequent lead tetraacetate oxidation. Condensation of labelled E-4-P with unlabelled PEP gave rise to shikimic acid-2,3,4,5-$^{14}C$ and 2-T.*

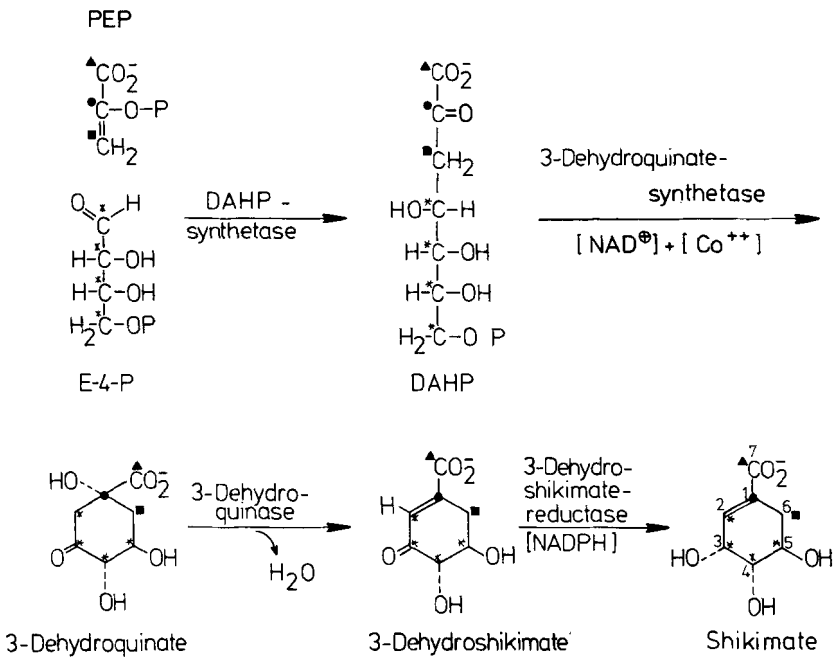
## INTRODUCTION.

Shikimic acid is a precursor of a large number of aromatic compounds in microorganisms and plants and a number of metabolic pathways involving shikimate have been revealed [1, 2, 3]) mainly by use of the labelled cyclite.

Abbreviations used : ATP = adenosinetriphosphate
E-4-P = erythrose-4-phosphate
PEP = phosphoenolpyruvate.

Previous studies have been carried out by using the following labelled $^{14}$C-shikimate species : D-shikimic acid-G-$^{14}$C, which was either derived from glucose-U-$^{14}$C by use of a shikimate-accumulating bacterial mutant [3], or which was prepared by $^{14}CO_2$ fixation in plant tissue [4] and DL-shikimic acid-1,6-$^{14}$C which was chemically synthesized [5]. Tritiated shikimic acids have also been reported : D-shikimic acid-6R-6T and -6S-6T [6] and D-shikimic acid -2-D [7].

Special interest has recently centered on metabolic pathways involving the incorporation of the entire shikimate molecule with the retention of the carboxyl group [3, 8]. To assist in the study of these pathways the biosynthetic preparation of D-shikimic acid specifically labelled with $^{14}$C in the carboxyl (C-7) group was carried out. It had been shown previously that cell-free extracts of *E. coli* 83-24 were able to convert PEP and E-4-P to shikimate [9]. Condensation of correspondingly labelled PEP or E-4-P led to the desired compounds. The reaction sequences used in the preparation were as follows :




The synthesis of labelled PEP 1, 2 or 3-$^{14}$C was carried out using labelled pyruvate and partially purified phosphoenolpyruvate synthase [10]. E-4-P 1,2,3,4-$^{14}$C or 4-T was synthesized from labelled glucose-6-phosphate according to the experimental procedure for the unlabelled compound [11].

EXPERIMENTAL PROCEDURE.

*Phosphoenolpyruvate-$^{14}C$.*

PEP-$^{14}C$ was synthesized from pyruvate-$^{14}C$ using PEP-synthase which had been carried through the first ammonium sulfate precipitation [10] (16 fold purification). The reaction proceeds as follows :

$$CH_3—CO—COOH + ATP + H_2O \rightleftharpoons CH_2 = C(OPO_3H_2)—COOH + AMP + H_3PO_4$$

In all cases pyruvate, 1,2 or 3-$^{14}C$ (Boehringer, Mannheim or Radiochemical Center, Amersham) was converted to PEP without adding carrier. The reaction mixture contained the following ingredients in a total volume of 4 ml : Tris-HCl buffer pH 8.0, 500 μmoles; Mg $Cl_2$, 50 μmoles; ATP (neutralized), 50 μmoles; Na-pyruvate-$^{14}C$ (ca. 25 mCi/mmole) 10 μmoles; PEP-synthase (spec. act. 0.38 U/mg) 2.4 mg protein. The incubation was carried out at 30° C for 15 min and terminated by addition of 1 ml methanol. 1 ml of the mixture was then applied to one sheet of previously washed Whatman 3 MM paper (35 × 40 cm). The chromatogram was developed ascending in methanol : ammonia (25%) : $H_2O$ = 6 : 1 : 3, for 8-10 hours. The chromatogram was afterwards carefully dried and exposed to Agfa X-ray paper overnight at −20°. The zone containing PEP-$^{14}C$ (Rf 0.65) was eluted with distilled water and the volume of the eluate reduced to 0.2 ml. The yield of purified PEP was 78-85% of the labelled pyruvate.

*Erythrose-4-phosphate-$^{14}C$ and T.*

Glucose-U-$^{14}C$ or glucose-6-T was first converted enzymatically to glucose-6-phosphate and this product subsequently oxidized to erythrose-4-phosphate by treatment with lead tetraacetate. Phosphorylation of glucose at the 6-position was carried out using hexokinase and an ATP regenerating system. The reaction mixture contained in a total volume of 4 ml : Tris-HCl buffer pH 7.5, 300 μmoles; EDTA 3 μmoles; $MgCl_2$, 18 μmoles; glucose $^{14}C$ or T 25 μmoles, ATP (neutralized) 20 μmoles; creatinephosphate, 30 μmoles; creatinephosphokinase (18 U/mg), 1 mg; hexokinase (140 U/mg), 0.3 mg. Incubation at 27.5° C for 90 minutes gave a yield of about 90% in glucose-6-phosphate. To purify the glucose-6-phosphate the incubation mixture was diluted with twice its volume of water and applied to a column (4 × 1 cm) of Dowex 1 X 10 200-400 mesh (acetate form). The column was washed with water until the effluent was free of radioactivity (about 50 ml). Glucose-6-phosphate was then eluted with 200 ml of 0.1 N HCl. All fractions containing labelled material were pooled and taken to dryness. The yield of purified glucose-6-phosphate was over 85%. 50 μmoles labelled glucose-6-phosphate were dissolved in 12.5 ml glacial acetic acid. The solution was rapidly stirred at room temperature while 95 μmoles lead tetraacetate dissolved in 2.5 ml

glacial acetic acid containing 0.03 ml 6 N $H_2SO_4$ were added dropwise over a period of 20-30 min. The resulting suspension was filtered through a thin layer of Hyflo-super-cel which was then washed well with water. The filtrates were combined and in order to hydrolyse the formyl groups of the product the solution was taken to dryness on a rotary evaporator, the residue taken up in water and again concentrated. The concentrate was then extracted continuously with ether for 15-20 hours. The aqueous layer was next passed through a column of DOWEX 50 W $\times$ 8, 200-400 mesh, ($H^+$ form) and the column was eluted with water till the eluate contained no radioactivity. The eluate was concentrated and the residual ether evaporated by passing a stream of nitrogen through the chilled aqueous solution. Yield of erythrose-4-phosphate from glucose-6-phosphate : 60-70%.

Labelled E-4-P thus synthesized was used without further purification for the preparation of shikimate.

*D.Shikimic acid-7 (carboxyl)$^{14}$C.*

Shikimic acid was synthesized by condensation of either labelled PEP with unlabelled E-4-P or *vice versa* in essentially the same procedure. In the following, the procedure for the synthesis of D-shikimic acid-7-$^{14}$C is described using PEP-1-$^{14}$C as starting material. The condensation of PEP-1-$^{14}$C with E-4-P to yield shikimic acid-7-$^{14}$C was catalyzed by a cellfree extract of the *Escherichia coli* mutant 83-24 which lacks shikimate kinase.

*Growth of organism.*

*E. coli* 83-24 was grown at 30° C under vigorous aeration for 15 to 20 hours in the following medium containing per liter : $KH_2PO_4$, 13.6 g; $Na_2HPO_4$, 13.4 g; $MgSO_4 \cdot 7H_2O$, 25 mg; $CaCl_2 \cdot 6H_2O$, 22 mg; $(NH_4)_2SO_4$, 2 g; glucose, 4 g (sterilized separately); 50 μmoles each of L-phenylalanine, L-tyrosine and L-tryptophane; 0.5 μmoles each of p-hydroxy benzoic acid, p-amino benzoic acid and 2,3-dihydroxy benzoic acid. Under these conditions the amino acid concentration is growth limiting and gives the highest specific activity of the shikimate pathway enzymes. The bacteria were harvested by centrifugation (0° C, 10,000 g, 10 min) in the log-phase of growth (optical density at 420 nm about 4.0 which corresponds to 3 g bacterial wet mass per liter). After washing with distilled water 5 g portions of the cell mass were stored at —20° for periods up to 4 months without loss of activity.

*Cell free extract.*

5 g of thawed cells were suspended in 20 ml 0.033 M potassium phosphate buffer pH 7.4 and they were broken by use of a sonifier (Branson S 125) at position 4 for 5 minutes. This and subsequent procedures were carried out at

0-4° C. The homogenate was centrifuged for 10 minutes at 27,000 g. The supernatant was dialysed against 0.04 M potassium phosphate buffer pH 7.2 for 12 to 15 hours. The dialysed cellfree extract has a protein content of 15-20 mg/ml and was stored at 0° C in melting ice. Aging of this extract at 0° C increased the activity and highest conversion of PEP to shikimate was observed after 4-6 days storage.

*Enzymatic synthesis.*

The following reactants were incubated in a total volume of 3.5 ml : potassium phosphate buffer pH 7.5, 300 μmoles; $CoCl_2$, 0.3 μmoles; NAD, 3 μmoles, NADPH, 0.8 μmoles; glucose-6-phosphate, 12 μmoles; E-4-P, 10 μmoles; PEP-1-$^{14}C$ (25 mc/mmole) 9 μmoles; glucose-6-phosphate dehydrogenase 1,1 units; *E. coli* 83-24 cellfree extract, 24 mg protein. Incubation proceeded for 2.5 hours at 37° C. Direct chromatographic analysis of the incubation mixture showed a conversion of PEP to shikimate with a yield of 60 to 85%. Figure 1 shows the distribution of radioactivity on a paper chromatogram giving the maximal conversion (86%) of PEP-1-$^{14}C$ to shikimate-carboxyl-$^{14}C$ obtained in this laboratory.

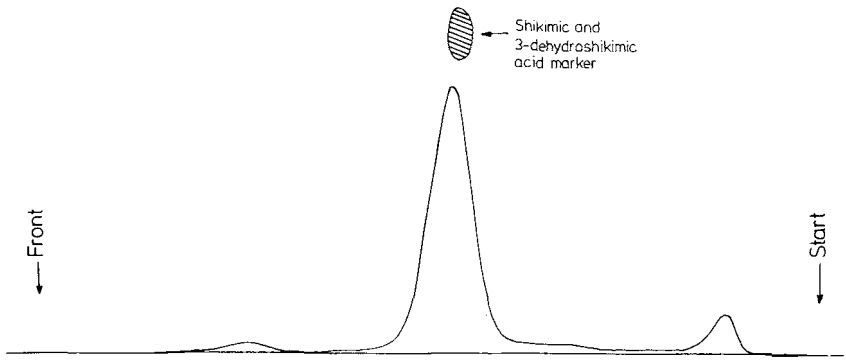



FIG. 1. Radioscan of a chromatographed aliquot of an unpurified incubation mixture showing the products of the condensation of PEP-1-$^{14}C$ with E-4-P. Separation of 7-$^{14}C$-shikimic acid plus 3-dehydroshikimic acid (both Rf 0.50) from minor impurities. The chromatogram was developed using n-butanol : acetic acid : $H_2O$ = 4 : 1 : 5 (organic phase).

*Purification.*

The incubation was stopped by adding 1 ml of 3 M perchloric acid and the precipitated protein was centrifuged off. The protein was washed several times by centrifugation until no more radioactivity could be removed. The combined supernatants were adjusted to pH 5 with 3 N KOH and passed through a column (3 × 1 cm) of DOWEX 1 × 10 (acetate form). The column

was washed with 50 ml $H_2O$ and then the shikimic acid was eluted with 2.5 N acetic acid till no further radioactivity came off. The eluate was taken to incipient dryness in *vacuo* and the residue taken up in 80% ethanol. Further purification was achieved by paper chromatography. The alcoholic solution was banded on washed Whatman 3 paper and chromatographed ascending in *n*-butanol : acetic acid : $H_2O$ = 4 : 1 : 5 (organic phase). By radioautography overnight 2 major bands were detected on the chromatogram, one at Rf 0.48 containing shikimic and 3-dehydroshikimic acid; and another unknown compound at Rf 0.73. The band at Rf 0.48 was eluted and rechromatographed using ethanol : *iso*amylalcohol : 1 N acetic acid = 2 : 1 : 1 as solvent system. This solvent separates shikimic acid (Rf 0.65; 85-90%) from 3-dehydroshikimic acid (Rf 0.40; 10-15%). The compounds were eluted from the paper with distilled water and the eluates kept frozen at −20° C. The yield of purified shikimic acid from PEP was 45-65%; the specific activity, 25 mc/mmole. Radiochemical purity determined by dilution analysis was better than 99.9%.

The same procedure has been applied for the synthesis of shikimic acid 1 and 6-$^{14}$C, using as a starting material PEP-2 or 3-$^{14}$C. Shikimic acid 2,3,4,5-$^{14}$C and 2-T have been obtained from E-4-P-U-$^{14}$C and 4-T respectively.

*Degradation.*

In order to determine the distribution of radioactivity in the shikimic acid species derived from specifically labelled PEP-$^{14}$C, the shikimic acid was subjected to partial degradation. The specific activity of $C_7$ of shikimic acid was determined after thermal decarboxylation catalyzed by quinoline and copper chromite [12].

For the ring C-atoms $C_1$ and $C_6$, shikimate was treated with osmiumtetroxide followed by periodate oxidation [13]. $C_6$ is obtained by this method as iodoform while $C_1$ and $C_7$ are obtained in this way as oxalic acid and the specific activity of $C_1$ can be calculated by difference.

The result are shown in the table.

TABLE. Percent distribution of radioactivity in carbon atoms 7, 1 and 6 of shikimic acid.

| Shikimic acid | $C_7$ as $CO_2$ | $C_1 + C_7$ as oxalate | $C_1$ by difference | $C_6$ as $CHI_3$ | $C_1+C_6+C_7$ |
|---|---|---|---|---|---|
| 7-$^{14}$C | 100 | 97 | 0 | 0 | 100 |
| 1-$^{14}$C | 1 | 103 | 102 | 2 | 105 |
| 6-$^{14}$C | 2 | 2 | 0 | 97 | 99 |

As can be seen from these figures, the label is exclusively in the required position. (The traces of radioactivity detected at other positions are probably the product of chemical randomization during degradation.) This method therefore permits a specific and convenient synthesis of the naturally occurring form of shikimic acid with high specific radioactivities. With glucose labelled specifically in carbon atoms 3, 4 or 5 becoming available all positions of the shikimate molecule could now be specifically labelled.

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