Fluorine-Containing Analogues of Intermediates in the Shikimate Pathway†

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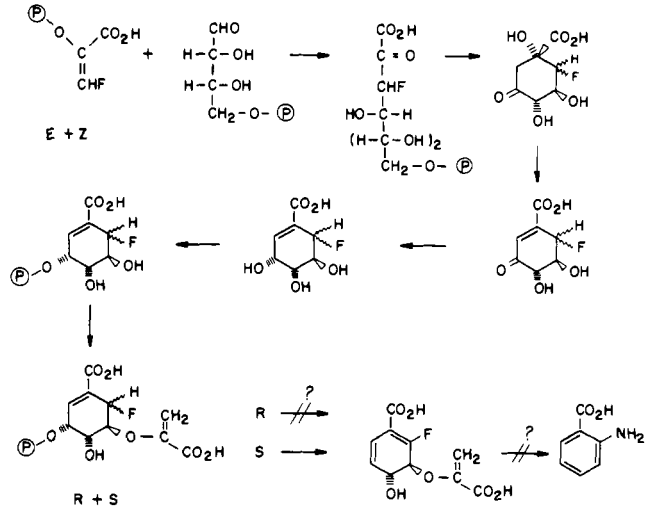
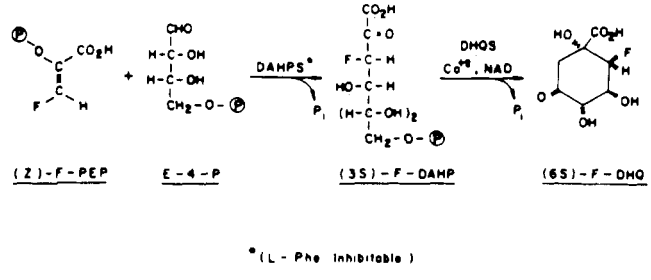
ABSTRACT: The phosphoenolpyruvate analogue (*Z*)-phosphoenol-3-fluoropyruvate is a substrate for phenylalanine-inhibitable 3-deoxy-D-*arabino*-heptulosonic acid-7-phosphate synthase from *Escherichia coli*. In the presence of excess erythrose 4-phosphate, apparent K_M values of 65 and 38 μM were observed for phosphoenol-3-fluoropyruvate and phosphoenolpyruvate, respectively. Because the apparent V_{max} for phosphoenol-3-fluoropyruvate is only 1.17% of that for phosphoenolpyruvate, one can study the former as an inhibitor of 3-deoxy-*arabino*-heptulosonic acid-7-phosphate synthase. Kinetic experiments showed phosphoenol-3-fluoropyruvate to be competitive with respect to phosphoenolpyruvate. Two distinguishable K_i values of 8 and 48 μM were obtained. The product (3*S*)-3-deoxy-3-fluoro-*arabino*-heptulosonic acid 7-phosphate was purified, characterized, and shown to act as a substrate for 5-dehydroquinate synthase. 3-Deoxy-3-fluoro-*arabino*-heptulosonic acid 7-phosphate, in contrast to 3-deoxy-*arabino*-heptulosonic acid 7-phosphate reacts slowly or not at all with reagents specific for 2-keto-3-deoxy sugars and is relatively resistant to oxidative cleavage by sodium periodate. The expected product of periodate oxidation, 3-fluoro-3-formylpyruvate, cannot be detected. This observation was clarified by studies with model compounds.

The unique properties of the carbon–fluorine bond have stimulated interest in the synthesis of fluorinated substrate analogues as particularly versatile probes of enzyme–substrate interaction (Ciba Foundation Symposium, 1972). Stubbe and Kenyon (1972) described the synthesis of the fluorinated substrate analogue F-PEP[1] and compared its properties as a substrate for enolase and pyruvate kinase to that of the normal substrate PEP. These workers demonstrated that F-PEP can act as a substrate for a phosphotransferase and a carbon–oxygen lyase. It is, thus, pertinent to inquire whether other classes of PEP metabolizing enzymes, such as carbon–carbon lyases, are also capable of accepting F-PEP as a substrate in place of PEP. One such lyase is the first enzyme specific for aromatic amino acid biosynthesis, DAHP synthase (Srinivasan and Sprinson, 1959), which catalyzes the condensation of PEP with E-4-P. If F-PEP were a substrate of DAHP synthase, further metabolism of F-DAHP could result in the incorporation of fluorine into every intermediate of the shikimate pathway (Haslam, 1974) according to Scheme I. We describe several features of the interaction of F-PEP with DAHP synthase, and the characterization of the resultant purified reaction product, F-DAHP. The enzymatic conversion of F-DAHP to F-DHQ has also been established (Scheme II).

SCHEME I: Possible Fluorinated Intermediates in the Shikimate Pathway Deriving from F-PEP.



SCHEME II: Fluorinated Aromatic Pathway Intermediates Demonstrated to Date.



Experimental Procedures

Materials

Chemicals. Cyclohexylammonium dihydrogen phosphoenol-3-fluoropyruvate was synthesized according to Stubbe

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[1] Abbreviations used are: PEP, 2-phosphoenolpyruvic acid; F-PEP, 3-fluoro-2-phosphoenolpyruvic acid (unless otherwise specified, the (*Z*)-isomer was used throughout the present study); DAHP, 3-deoxy-*arabino*-heptulosonic acid 7-phosphate; F-DAHP, (3*S*)-3-deoxy-3-fluoro-*arabino*-heptulosonic acid 7-phosphate; E-4-P, erythrose 4-phosphate; DHQ, 3-dehydroquinate; F-DHQ, 6-fluoro-3-dehydroquinate; Mops, 3-(*N*-morpholino)propanesulfonic acid; TBA, thiobarbituric acid; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance.

and Kenyon (1972). In confirmation of Lane and Hurst (1974), we detected a few percent of the E isomer by NMR. This preparation of 94% minimum Z isomer is hereafter referred to as Z-F-PEP; mp 139–140 °C dec.

Anal. Calculated for $C_9H_{17}FNO_6P$: C, 37.90; H, 5.98; N, 4.91; P, 10.53. Found: C, 37.74; H, 5.94; N, 4.99; P, 10.80.

A 60:40 mixture of (Z:E)-F-PEP was prepared by irradiating a solution of the Z isomer (0.4 M in 99.8% D_2O) for 22 h at 2537 Å (Hg lamps) at 35 °C, in a Rayonet photochemical reactor.

Cyclohexylammonium dihydrogen phosphoenolpyruvate was synthesized according to Clark and Kirby (1966), as modified by Stubbe and Kenyon (1972).

Erythrose 4-phosphate was synthesized according to Sieben et al. (1966).

Fluoro oxaloacetate was synthesized by the method of Kun et al. (1959).

3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate was isolated and purified as described by Nasser and Nester (1967). However, a partially purified preparation of DAHP synthase (vide infra) was used as the enzyme source, rather than a crude extract. (D,L)-1-Deoxy-1-fluoroglycerol was furnished by Dr. R. Harrison.

All other chemicals were purchased from commercial sources and used without further purification.

Enzymes. Pyruvate kinase (E.C. 2.7.1.40) and lactate dehydrogenase (E.C. 1.1.1.27) were purchased from Calbiochem and Worthington. Both DAHP synthase and DHQ synthase were partially purified from extracts of *E. coli,* strain *aro*10-2 (*aro*D352, Pittard and Wallace, 1966). The phenylalanine-sensitive DAHP synthase was purified approximately 15-fold to a specific activity of 20.3 units/mg by the method of Herrmann et al. (manuscript in preparation). An enzyme unit is defined as micromoles DAHP formed in 10 min as assayed by the modified (Gollub et al., 1970) method of Warren (1959). DHQ synthase was purified 100-fold to a specific activity of 19.8 units/mg (units defined as micromoles DAHP consumed in 10 min, assayed as for DAHP synthase) by the method of Maitra and Sprinson (personal communication).

Methods

Enzyme Assays. Both DAHP synthase and DHQ synthase were routinely assayed for activity by monitoring the appearance or disappearance, respectively, of the thiobarbituric acid chromogen after periodate cleavage of DAHP (Gollub et al., 1970). Kinetic parameters (Figure 1A,B, Table II) for PEP and F-PEP were determined by the continuous assay procedure of Schoner and Herrmann (1976) at a wavelength of 240 nm using a Gilford 2400-2 spectrophotometer. The extinction coefficient for F-PEP was found to be 580 M^{-1} cm^{-1} at 240 nm and 1540 M^{-1} cm^{-1} at 230 nm in 0.05 M phosphate buffer, pH 6.70. The former value differs considerably from the value of 1280 M^{-1} cm^{-1} reported by stubbe and Kenyon (1972). Although the extinction coefficients of PEP (Wold and Ballou, 1957) and F-PEP (Pilch and Somerville, unpublished observations) are pH dependent, the discrepancy is far greater than expected for a difference of only 0.1 pH unit. Our values for PEP agree well with those of Wold and Ballou (1957). The activity was monitored at 30 °C in a jacketed cell containing 50 μmol of potassium phosphate, pH 6.7, 10 μmol of potassium fluoride, 2 μmol of erythrose 4-phosphate, substrate, and enzyme in a volume of 1.0 ml. Between 15 and 20 enzyme units were used per assay when F-PEP was the substrate, while 0.5–0.75 enzyme unit was used with PEP. The apparent Michaelis constants were determined graphically by measuring the slope and intercept values in Lineweaver–Burk plots, constructed using a linear least-squares regression analysis to obtain the best fit for the data. From three to five separate experiments were conducted in each case. No corrections were made for the presence in some preparations of DAHP synthase of traces of enolase activity.

The conversion of DAHP to DHQ and F-DAHP to F-DHQ was monitored by measuring orthophosphate release, by the procedure of Chen et al. (1956), as modified by Ames (1966). The assay mixture typically contained 25 μmol of Mops, pH 7.4, 1 μmol of NAD, 1 μmol of $CoCl_2$, 1 μmol of DAHP or F-DAHP, and 30 units of DHQ synthase in a volume of 1.0 ml. During incubation at 37 °C, portions were withdrawn at intervals, mixed with an equal volume of 10% trichloroacetic acid, and then assayed for orthophosphate.

Preparation and Purification of F-DAHP. A reaction mixture consisting of 20 mmol of potassium phosphate, pH 6.7, 0.39 mmol of F-PEP, 0.65 mmol of E-4-P, 0.4 mmol of NaEDTA, and 7000 units of DAHP synthase in a volume of 400 ml was prepared. During 4 h of incubation at room temperature, the progress of the reaction was monitored by withdrawing samples which were assayed for F-PEP according to the coupled enzyme assay procedure of Stubbe and Kenyon (1972). After 2 h, the reaction came to a halt. The calculated yield was 84%, based on F-PEP. The reaction mixture was lyophilized and redissolved in 50 ml of H_2O. The pH was adjusted to 8.1 with NH_4OH and the solution was run into a 1.6 × 35 cm AG-1-X8 column, Cl^- form (Bio-Rad). Subsequent steps of elution, concentration, and isolation of Ba-F-DAHP were carried out as described for Ba-DAHP by Nasser and Nester (1967). The elution of F-DAHP was monitored spectrophotometrically at 230 nm. Prior to use, F-DAHP was freed of barium with sodium sulfate or by passage over Dowex 50 (H^+ form) (see Table II). F-DAHP was also prepared chemically from fluoro oxaloacetate and E-4-P by the method of Herrmann and Poling (1975).

Analytical Procedures. Total phosphate and orthophosphate were determined by the method of Ames (1966). The acid phosphatase-dependent release of orthophosphate (Table II) was conducted as follows. In 0.1 M sodium acetate, pH 5.0, various concentrations of F-DAHP and wheat germ acid phosphatase (Calbiochem) at 1 mg/ml were incubated at 37 °C. Portions of the reaction mixture were removed, mixed with an equal volume of 10% trichloroacetic acid, and centrifuged. The level of orthophosphate in the supernatant was determined. The plateau value for inorganic phosphate was taken to represent the F-DAHP concentration of the original solution.

Quantitative periodate oxidation (Figures 2 and 3) was performed by the method of Avigad (1969). The seven carbon sugars (1 mM) were oxidized at 37 °C in 10 mM $NaIO_4$. Glycerols (1 mM) were oxidized at ~5 °C in 2 mM $NaIO_4$.

Ascending thin-layer chromatography was done on Eastman Chromagram cellulose in acetic acid–ethyl acetate–water, 3:3:2. Orthophosphate and phosphate esters were detected by the Hanes and Isherwood (1949) spray. DHQ and F-DHQ were detected by spraying with a solution of 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl. DAHP was also detected using the thiobarbituric acid spray of Millican (1963). The R_f values were as follows: DAHP, 0.46; F-DAHP, 0.48; PEP, 0.64; F-PEP, 0.65; P_i, 0.62; E-4-P, 0.30; DHQ, 0.61; F-DHQ, 0.62.

Total fluorine was determined with a specific ion electrode (Orion Model 96-09) as described by Woodward et al. (1970). Although th precision of this analysis gave values differing no more than 4% relative error (three samples, each preparation),

TABLE I: Analytical Data for F-DAHP.

	F-DAHP (Preparation No. 1)[a]		F-DAHP (Preparation No. 2)[b]	
	mM	Ratio	mM	Ratio
Acid phosphatase dependent release of inorganic phosphate	5.68	1.00	8.23	1.00
Total phosphate	5.31	0.94	8.25	1.00
Total fluorine	4.80	0.84	6.67	0.81

[a] $(Ba)_x$F-DAHP (ca. 20 mg) was dissolved in 1.0 ml of H_2O, 1.0 ml of 0.1 M Na_2SO_4 was added, and $BaSO_4$ was removed by centrifugation. [b] $(Ba)_x$F-DAHP (ca. 50 mg) dissolved in ca. 1.0 ml of H_2O and passed over a 1.5 × 0.25 in. Dowex 50 column. The effluent pH was adjusted to 6.0. Final volume ca. 4.0 ml. 86% recovery of fluorine observed for pure F-PEP control. To calculate ratios, the value obtained for acid phosphatase-dependent release of inorganic phosphate was set equal to 1.00.

TABLE II: Kinetic Parameters, PEP vs. F-PEP.

Enzyme	K_M PEP (μM)	K_M (Z)-F-PEP (μM)	K_i (Z)-F-PEP (μM)	% Relative Rate (PEP = 100%)
DAHP synthase (L-Phe inhibitable from *E. coli*)	38	65	8 (48)	1.2
Pyruvate kinase[a] (rabbit muscle)	26	400		0.23
Pyruvate kinase[b] (yeast)	2100	400		5.0
Enolase[a]	92	20		0.9
DAHP synthase[c] (L-Tyr inhibitable from *E. coli*)	5	+[d]	ND	ND

[a] Stubbe and Kenyon (1972). [b] Blumberg and Stubbe (1975). [c] Schoner and Herrmann (1976). [d] Reaction demonstrated qualitatively.

the fluorine content of F-DAHP was significantly lower than theory (Table I). However, this also proved true for an F-PEP control (legend, Table I) and may be attributable to the presence of phosphate. Woodward et al. (1970) did not analyze any sugar phosphates by this method but did obtain similarly low results for sodium fluoroacetate. If one normalizes the F-DAHP fluorine content to that of F-PEP, one obtains excellent agreement with theory.

Protein concentration was determined by the method of Lowry et al. (1951).

Results and Discussion

Interaction of F-PEP and DAHP Synthase. F-PEP was shown to serve as a substrate for DAHP synthase of *E. coli* by the following criteria. The disappearance of F-PEP from reaction mixtures, measured either directly by spectrophotometry at 240 nm or indirectly using a coupled enzyme assay procedure, was totally dependent on both E-4-P and DAHP synthase. Using the phenylalanine-sensitive isozyme of DAHP synthase, the formation of F-DAHP was strongly inhibited by phenylalanine (data not shown). Upon thin-layer chromatography (see Experimental Procedure), purified, enzymatically synthesized F-DAHP migrated indistinguishably from DAHP. A mixture of fluorinated 3-deoxy-heptulosonic acid phosphates, synthesized chemically (Herrmann and Poling, 1975), migrated at rates indistinguishable from those of enzymatically synthesized DAHP and F-DAHP. The analytical data for purified F-DAHP (Table I) was consistent with the foregoing observations.

A comparison of the kinetic parameters so far observed for F-PEP and PEP is given in Table II. For DAHP synthase, the apparent K_M values differ by less than a factor of 2 (line 1, Table II). This observation is reminiscent of earlier comparisons made between PEP and F-PEP with pyruvate kinase and enolase (Table II). Previous workers noted that the K_M for F-PEP ranged from fivefold lower than the normal substrate to 15-fold higher than that of PEP. To the extent that the K_M for PEP reflects binding, one can assume that F-PEP and PEP are similar in affinity for DAHP synthase. If, for this enzyme, the ionizable acidic groups of PEP and F-PEP play major roles in substrate binding, as suggested for pyruvate kinase (Stubbe and Kenyon, 1972), the inductive effect of fluorine on the carboxylic or phosphoric acid moieties is minimal. One might expect inductive effects rather than steric effects to be important, since the length of the carbon–fluorine bond closely approximates that of the carbon–hydrogen bond (Sharpe, 1972).

Also consistent with previous studies is our finding of a major decrease in V_{max} when F-PEP replaced PEP as a substrate for DAHP synthase (Table II, line 1). At saturating concentrations of F-PEP, the relative rate of formation of F-DAHP was 1.17% of that observed for the conversion of PEP to DAHP. The relative V_{max} difference in our case (85-fold) is intermediae between previously observed V_{max} decreases for F-PEP ranging from 20-fold to over 400-fold (Table II). The mechanistic significance of these observations awaits a more detailed study of the mechanism of DAHP synthase, particularly with respect to rate-determining steps.

In determining the K_i for F-PEP as an inhibitor of the normal DAHP synthase reaction, it was noted that the Dixon plot (Figure 1B) with F-PEP concentrations from 40 to 100 μM was nonlinear with respect to zero F-PEP levels (data points not shown). When the F-PEP concentrations were reduced, the zero F-PEP data became consistent with other inhibited rates determined below 40 μM F-PEP (Figure 1A). Although other interpretations are possible, we favor the notion that the experiment of Figure 1 reflects the existence of two categories of F-PEP binding sites. It is not known whether PEP binds to more than one site on this enzyme. It is interesting to note, however, that 0.5 mM PEP was required to protect homogeneous tyrosine-inhibitable DAHP synthase from irreversible inactivation, even though the K_M for PEP was 5 μM (Schoner and Herrmann, 1976).

The steric fate of the carbon three protons of PEP has been

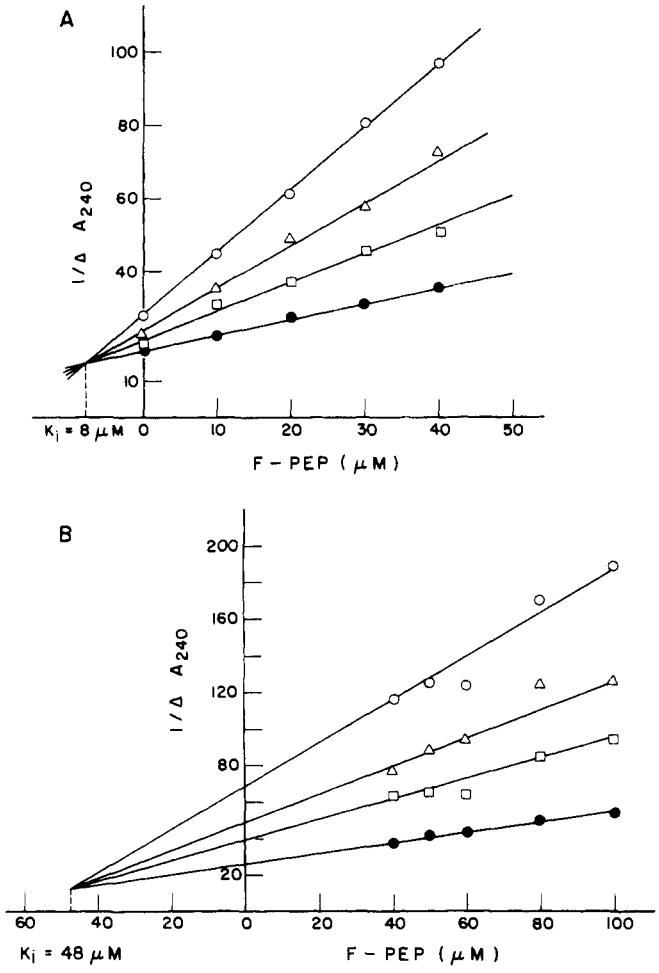

FIGURE 1: Determination of K_i for F-PEP. (A) F-PEP levels below 40 μM; (B) F-PEP levels above 40 μM. Concentrations of PEP (μM): (●), 100; (□), 50; (△), 33; (○), 25.

determined for the DAHP synthase and chorismate synthase reactions (Floss et al., 1972). Since (Z)-F-PEP can be assumed to interact in the same fashion as PEP with substrate sites on DAHP synthase, we have made the stereochemical assignment about C-3 of F-DAHP as depicted in Scheme II. We have photochemically isomerized (Z)-F-PEP to a 60:40 mixture of (Z):(E) isomers as determined by NMR. This mixture is completely converted to F-DAHP as assayed directly at 240 nm, thereby lending credence to the feasibility of Scheme I. the kinetics of this conversion was nonlinear, thereby precluding simple analysis of the data.

Properties of F-DAHP. A convenient and sensitive assay for 2-keto-3-deoxyglyconic acids is based on periodate oxidation to give formylpyruvic acid, which, in turn, reacts with TBA to give an intense red color (Warren, 1959; Chaby et al., 1975). This reaction has been extended to a 2-keto-3-deoxy-fluoro derivative by Taylor et al. (1975), the expected TBA reactive material being 3-fluoro-3-formylpyruvic acid. We have been unable to obtain a similar result with F-DAHP under a range of oxidative conditions. We therefore compared the reaction of periodate with DAHP, F-DAHP, and F-DAH (Figure 2) and with the model compounds (D,L)-1-deoxyglycerol and (D,L)-1-deoxy-1-fluoroglycerol (Figure 3).

Fluorine substitution adjacent to vicinal diols inhibits periodate consumption for both the heptoses and the glycerols. Second-order rate constants calculated for the latter reactions were 125 M^{-1} min^{-1} for 1-deoxyglycerol and 6.26 M^{-1} min^{-1} for 1-deoxy-1-fluoroglycerol, a 20-fold difference. Owing to the greater complexity of reaction with the heptoses, comparable rate constants cannot be determined. A crude comparison of initial rates (tangents to the initial reaction slope) suggests a 13-fold slower reaction for F-DAHP as compared with DAHP. The reaction of periodate with deoxy sugars is known to be complex (Stanek et al., 1963). In particular, the problem of "overoxidation" can hinder the correct interpretation of results. As seen from the course of the DAHP oxidation (Figure 2), there is never stoichiometry between periodate reacted and TBA color (hence, formylpyruvate concentration) formed. Where over-oxidation is accelerated and the initial diol cleavage inhibited, sufficient fluoroformylpyruvate might never accumulate to levels detectable by reaction with TBA. This would explain the anomolous results with F-DAHP. Since Taylor et al. (1975) have studied the nonphosphorylated compound 3-deoxy-3-fluoro-D-gluconic acid, we examined the effect of phosphate removal upon the periodate reaction (Chaby et al., 1975) (F-DAH, Figure 2). Although the rate of periodate consumption was increased, no TBA reactive substance was detected. It is likely that oxidation at carbons 5 through 7 explains the increased reaction rate. Gantt et al. (1964) described the synthesis and properties of 3-fluoro-*N*-acetylneuraminic acid, a compound closely related to F-DAHP. They describe a periodate–TBA reaction product for *N*-acetylneuraminic acid but not its fluorinated derivative. However, we cannot reconcile our results with those of Taylor et al. (1975).

F-DAHP exhibits somewhat anomolous behavior with other reagents used in sugar chemistry. Diphenylamine, the deoxy sugar specific reagent of Chaby et al. (1975) and the phenol–sulfuric acid test of Dubois et al. (1956) both have positive results for DAHP and negative results for F-DAHP. We surmise that the presence of fluorine on carbon three of F-DAHP either reduces reactivity (as with $NaIO_4$) or, perhaps, negatively influences the extinction coefficient of the chromophore formed.

Further proof of authenticity for F-DAHP was obtained by demonstrating the enzymatic conversion of F-DAHP to F-DHQ (Figure 4). Although the formation of F-DHQ was somewhat less efficient than that of DHQ, under identical conditions, the enzyme- and cofactor-dependent liberation of inorganic phosphate proceeded to completion. Analysis by thin-layer chromatography (see Experimental Procedure) of F-DHQ-containing reaction filtrates verified the presence of a 2,4-dinitrophenylhydrazine-reactive substance having chromatographic mobility indistinguishable from that of DHQ. The ^1H NMR spectrum of F-DAHP (not shown) is consistent with the proposed structure, although definitive assignments of specific protons were not attempted.

The conversion of F-DAHP to F-DHQ strengthens the notion that further metabolism (Scheme I) will occur. We have preliminary spectroscopic evidence (data not shown) for conversion of F-DHQ to 6-fluorodehydroshikimate (reaction 3, Scheme 1). The substituents on carbon six of DHQ are not directly involved in further reaction until the chorismate synthase step (Floss et al., 1972). It is our opinion that further study of fluorinated intermediates of the shikimate pathway beyond F-DAHP will not only yield mechanistically significant data but will also aid in the characterization of substrate specificity in aromatic biosynthesis.

Acknowledgments

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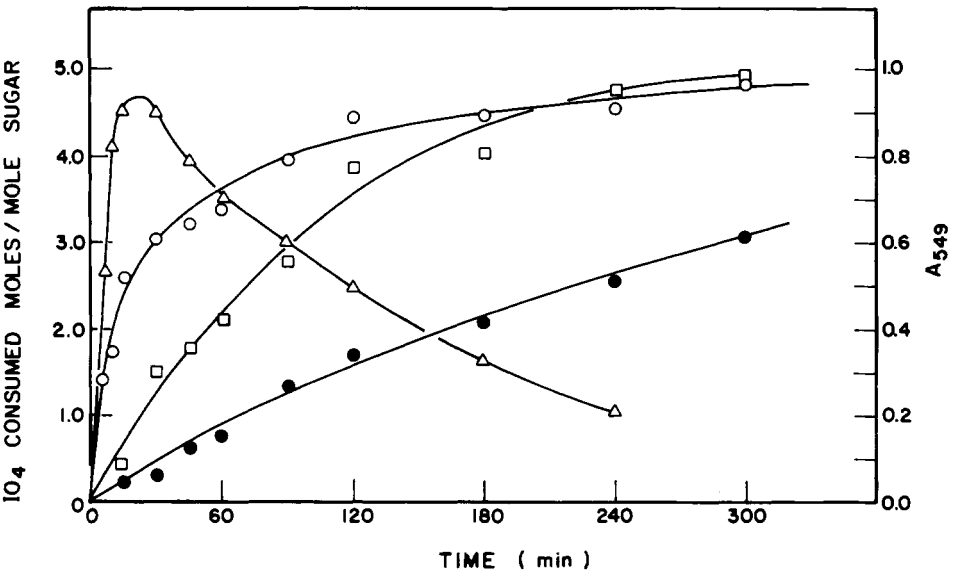

FIGURE 2: Reaction of $NaIO_4$ with DAHP, F-DAHP, and F-DAH: (○), DAHP; (●), F-DAHP; (□), F-DAH; (△), DAHP (A_{549}).

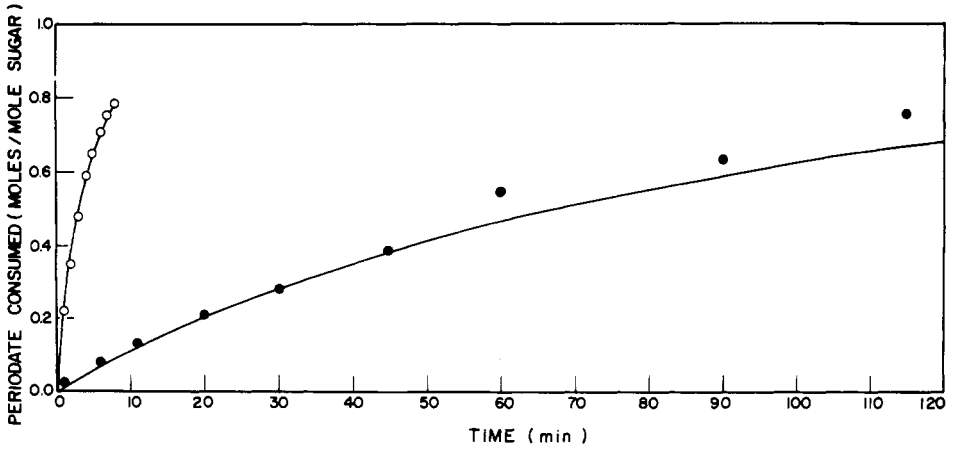

FIGURE 3: Reaction of $NaIO_4$ with propanediols: (○), 1-deoxyglycerol; (●), 1-deoxy-1-fluoroglycerol. The data points are experimental; the curves were calculated from the second-order rate constants.

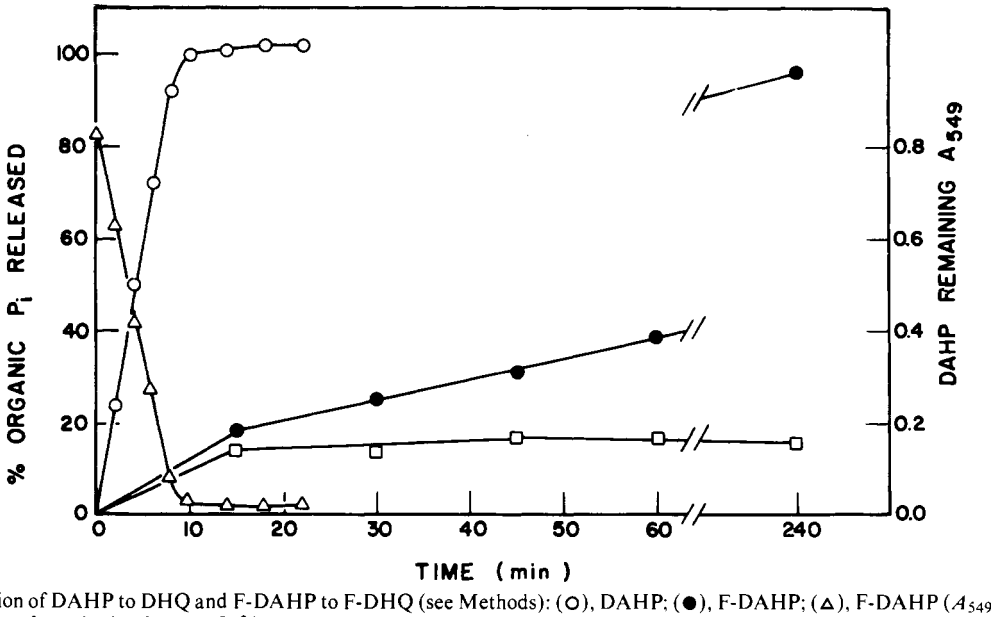

FIGURE 4: Conversion of DAHP to DHQ and F-DAHP to F-DHQ (see Methods): (○), DAHP; (●), F-DAHP; (△), F-DAHP (A_{549}); (□), F-DAHP control in the absence of required cofactors, Co^{2+} and NAD.

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