Direct Observation of the Enzyme–Intermediate Complex of 5-Enolpyruvylshikimate-3-phosphate Synthase by ^{13}C NMR Spectroscopy†

Paul N. Barlow, Richard J. Appleyard, Ben J. O. Wilson, and Jeremy N. S. Evans*
Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.
Received June 1, 1989; Revised Manuscript Received August 2, 1989

ABSTRACT: The interaction of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, 4,5-dideoxyshikimate 3-phosphate (ddS3P), and [2-^{13}C]- and [3-^{13}C]phosphoenolpyruvate (PEP) has been examined by ^{13}C NMR spectroscopy. Although no resonances due to a dead-end intermediate complex could be detected, an enzyme active site specific formation of pyruvate was observed. The interaction of EPSP synthase with shikimate 3-phosphate (S3P) and [2-^{13}C]- or [3-^{13}C]PEP has been examined by ^{13}C NMR spectroscopy. With [2-^{13}C]PEP, in addition to the resonances due to [2-^{13}C]PEP and [8-^{13}C]EPSP, new resonances appeared at 164.8, 110.9, and 107.2 ppm. The resonance at 164.8 ppm has been assigned to enzyme-bound EPSP. The resonance at 110.9 ppm has been assigned to C-8 of an enzyme-free tetrahedral intermediate of the sort originally proposed by Levin and Sprinson [Levin, J. G., & Sprinson, D. B. (1964) *J. Biol. Chem. 239*, 1142–1150] and recently independently observed by Anderson et al. [Anderson, K. S., Sikorski, J. A., Benesi, A. J., & Johnson, K. A. (1988) *J. Am. Chem. Soc. 110*, 6577–6579]. The resonance at 107.2 ppm has been assigned to an enzyme-bound intermediate whose structure is closely related to that of the tetrahedral intermediate. With [3-^{13}C]PEP, new resonances appeared at 88.9, 26.2, 25.5, and 24.5 ppm. The resonance at 88.9 ppm has been assigned to enzyme-bound EPSP. The resonance at 26.2 ppm, which was found to correlate with 1.48 ppm by isotope-edited multiple quantum coherence ^1H NMR spectroscopy, has been assigned to the methyl group of 4-hydroxy-4-methylketoglutarate. The resonance at 25.5 ppm, which was found to correlate with a ^1H resonance at 1.16 ppm, could be substantially reduced by preincubating the enzyme with the inhibitor glyphosate and could be isolated free from the protein by ultrafiltration. This has been interpreted in terms of the same enzyme-free tetrahedral intermediate that gave rise to the resonance at 110.9 ppm with [2-^{13}C]PEP and proposed by Levin and Sprinson. No ^1H correlation has been detected for the ^{13}C resonance at 24.5 ppm due to its large line width (100 Hz), but it has been interpreted as arising from the same enzyme-bound intermediate which gave rise to the resonance at 107.2 ppm with [2-^{13}C]PEP.

5-Enolpyruvylshikimate-3-phosphate (EPSP)[1] synthase (EC 2.5.1.19) catalyzes the penultimate step in the biosynthetic pathway in plants and bacteria that leads to chorismic acid, the common precursor for the three aromatic amino acids phenylalanine, tyrosine, and tryptophan. EPSP (**4**) is formed from shikimate 3-phosphate (S3P, **1**) and phosphoenolpyruvate (PEP, **2**) (see Scheme I). The enzyme as isolated from higher plants and bacteria (Lewendon & Coggins, 1983; Mousdale & Coggins, 1984) is a monomer with molecular weight (M_r) of 46 000 of known sequence (Duncan et al., 1984a). The cloned *Escherichia coli* gene has been used to generate an overproducing strain by Duncan et al. (1984b) and Rogers et al. (1983), and the bacterial enzyme is available in milligram quantities. Furthermore, EPSP synthase is the primary site of action of the herbicide glyphosate, or *N*-(phosphonomethyl)glycine. This is a broad-spectrum postemergence herbicide with worldwide applications in agriculture and horticulture.

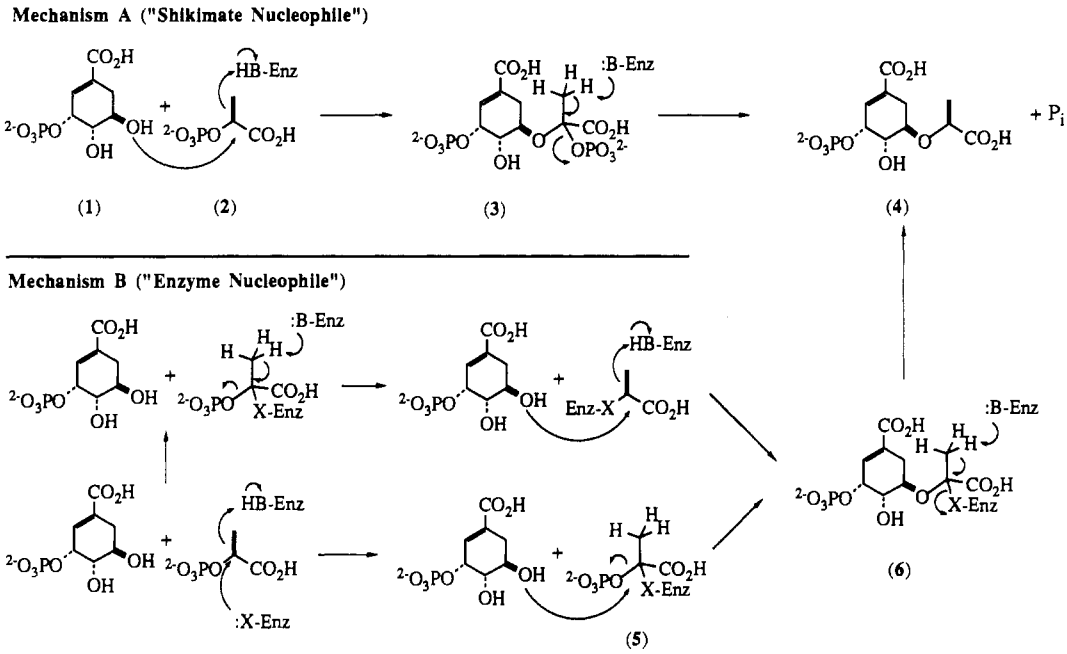
Although EPSP synthase has been the subject of detailed investigation, there remain many important questions as to its mechanism. Early work by Sprinson and co-workers (Bondinell et al., 1971) established that incubation of the enzyme with S3P and PEP in tritiated water gave tritium-labeled EPSP. This was seen as evidence for an unprecedented reaction of PEP involving an addition–elimination mechanism, in which nucleophilic attack at C-2 with concomitant protonation at C-3 yields a transient intermediate from which the phosphate is eliminated. Elegant work by the group of Knowles (Grimshaw et al., 1982, 1984) with regiospecifically deuterated PEP confirmed that C-3 of the enolpyruvyl moiety transiently becomes a methyl group, whose rotation is rapid with respect to the steps leading to and from its formation. Tracer studies by Bondinell et al. (1971) suggested that the ester oxygen attached to C-2 of PEP is recovered quantitatively in inorganic phosphate and not in EPSP. In addition, work by Anton et al. (1983) showed that the bridging oxygen of [^{18}O]PEP is not scrambled in the complex of 4,5-dideoxyshikimate 3-phosphate (ddS3P) and EPSP synthase. Thus it appears that EPSP synthase catalyzes the transfer of an enolpyruvyl moiety with C–O cleavage in the presence of S3P, but there is no such cleavage in the presence of ddS3P. Furthermore, the nature of the nucleophile that is proposed to attack at C-2 of PEP is open to question. Anton et al. (1983) have shown that incubation of ddS3P and PEP with EPSP synthase in tritiated water resulted in incorporation of

† Supported by the Research Corporation Trust, Dow Agricultural Co. (U.S.A.), and the Science & Engineering Research Council.
* Author to whom correspondence should be addressed at the Biochemistry/Biophysics Program, Washington State University, Pullman, WA 99164.

[1] Abbreviations: COSY, correlated spectroscopy; ddS3P, 4,5-dideoxyshikimic acid 3-phosphate; EPSP, 5-enolpyruvylshikimic acid 3-phosphate; glyphosate, *N*-(phosphonomethyl)glycine; HMKG, 4-hydroxy-4-methylketoglutarate (or parapyruvate); HMQC, heteronuclear multiple quantum coherence; LW, line width; S3P, shikimic acid 3-phosphate; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; PNPP, *p*-nitrophenyl phosphate; UDP-NAG, uridine-5′-diphospho-5-(*N*-acetyl-2-amino-2-deoxyglucose).

0006-2960/89/0428-7985$01.50/0

Scheme I



tritium into PEP, thereby suggesting that the 5-hydroxyl of S3P is not the nucleophile. The only other known enzyme that catalyzes the transfer of a PEP moiety in an ostensibly similar manner is UDP-NAG-pyruvyl transferase, which substitutes the UDP-NAG unit for the phosphate group of PEP (Strominger, 1958). This reaction has been shown (Cassidy & Kahan, 1973; Zemell & Anwar, 1975) to involve an enzyme nucleophile, since treatment of the enzyme with [^{14}C]PEP resulted in protein labeled with radioactivity. The label was released upon treatment with UDP-NAG.

Two contrasting mechanisms have been proposed for the EPSP synthase reaction, according to what is thought to be the nucleophilic group that attacks C-2 of PEP. While both require the transient formation of an intermediate with a methyl group at C-3 and with tetrahedral geometry at the C-2 position, the "shikimate nucleophile" mechanism (Levin & Sprinson, 1964; Grimshaw et al., 1982) requires attack on C-2 by the 5-hydroxyl of S3P (Scheme IA) while the "enzyme nucleophile" mechanism (Ganem, 1978; Anton et al., 1983) requires attack on C-2 by a nucleophilic group on the enzyme (Scheme IB). Evidence for the shikimate nucleophile mechanism has been provided by the work of Bondinell et al. and Grimshaw et al. already described. The involvement of a concerted reaction with a carbocation-like transition state has also been proposed (Grimshaw et al., 1982). Evidence for the enzyme nucleophile mechanism has been provided by Anton et al. (1983), and the precedence of UDP-NAG-pyruvyl transferase has been presented (Ganem, 1978) in support of this mechanism. However, recent work by Anderson et al. (1988a,b) has provided evidence for the shikimate nucleophile mechanism, by isolation and characterization of an enzyme-free species under rapid chemical quench conditions whose structure is consistent with that of **3**. It should, however, be noted that the mechanism of EPSP synthase cannot be considered to be settled until the enzyme-bound species has been detected and characterized, since intermediate **3** could be formed from intermediate **6** as a result of nucleophilic attack by inorganic phosphate. Thus to date, the conflict between these two mechanisms has not been resolved completely.

In this paper we examine the complexes derived from 4,5-dideoxy-S3P, S3P, [^{13}C]PEP, and EPSP synthase, using ^{13}C and isotope-edited ^{1}H NMR spectroscopy.

Materials and Methods

Chemicals and Enzymes. All chemicals and enzymes, including PEP, NADH, ADP, lysozyme, lactate dehydrogenase, and pyruvate kinase, were purchased from Sigma (Poole, U.K.) except where stated otherwise. Deoxyribonuclease was purchased from Boehringer-Mannheim. S3P was purified from cultures of *Klebsiella pneumoniae* according to known methods (Bondinell et al., 1971) with slight modifications. 4,5-Dideoxyshikimate 3-phosphate was synthesized according to the method of Anton et al. (1983). Potassium [2-^{13}C]- and [3-^{13}C]phosphoenolpyruvate and sodium [3-^{13}C]pyruvate were purchased from MSD Isotopes (Canada). All enzyme manipulations other than assays were carried out at 4 °C.

Growth of Cells. E. coli AB2829/pKD501, a generous gift from Prof. J. R. Coggins, was grown with aeration to late logarithmic phase in an LH 2000 25-L fermenter in minimal salts medium (Vogel & Bonner, 1956) containing 0.4% (w/v) glucose and 1 mM thiamin hydrochloride. The cells were concentrated to ca. 2 L with a Sartorius Sartocon II tangential-flow concentrator and harvested by centrifugation in a Beckmann J2-21 centrifuge. Cells were stored for periods of up to 2 months at −70 °C.

Enzyme Assay and Protein Determination. EPSP synthase activity was routinely assayed for release of phosphate (Van Veldhoven & Mannaerts, 1987) or in the reverse direction by method 1 of Lewendon and Coggins (1983). Protein was determined by the method of Bradford (1976).

Assay of Phosphatase Activity. Phosphatase activity was measured in the water-jacketted cell holder of a Pye-Unicam PU-8800 double-beam UV/visible spectrophotometer, by monitoring the hydrolysis of *p*-nitrophenyl phosphate (PNPP, 20 μL, 100 mg mL^{-1}) at 405 nm in buffer (700 μL, 0.4 mM $ZnSO_4$, 10 mM $MgSO_4$, 100 mM acetate, pH 7.0) in the presence of enzyme (20–50 μL, 18 μM).

Enzyme Purification. EPSP synthase was purified by a modification of established methods (Duncan et al., 1984; Padgette et al., 1987), as outlined elsewhere (Appleyard et al., 1989). Enzyme preparations were found to retain up to 95% of enzymatic activity for several weeks at 4 °C and for

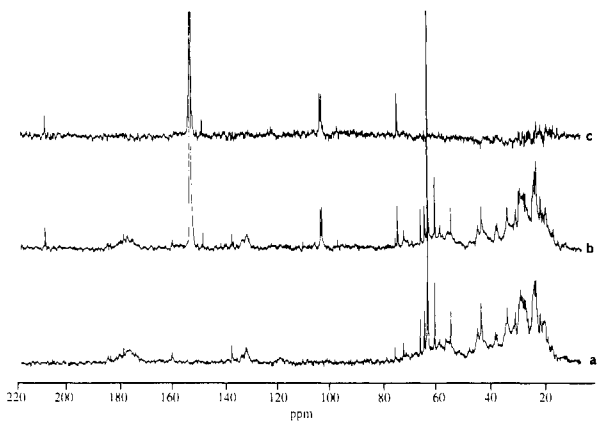


FIGURE 1: ^{1}H-Decoupled ^{13}C NMR spectra of EPSP synthase plus ddS3P and [2-^{13}C]PEP. (a) EPSP synthase (1.4 mM), 20 mM Tris-HCl, pH 7.6, 0.4 mM DTT, 0.4 mM EDTA, 10 mM KF, and 10 mM ddS3P in 450 μL; (b) same plus [2-^{13}C]PEP (50 μL, 200 mM in 100 mM Tris-HCl, pH 8.0), final concentration = 20 mM; (c) difference spectrum, (b) − (a). All spectra were obtained with the following: number of scans = 46000, recycle time = 1.3 s, pulse width = 6 μs, size = 32K data points, and FID's Fourier transformed with line broadening = 20 Hz.

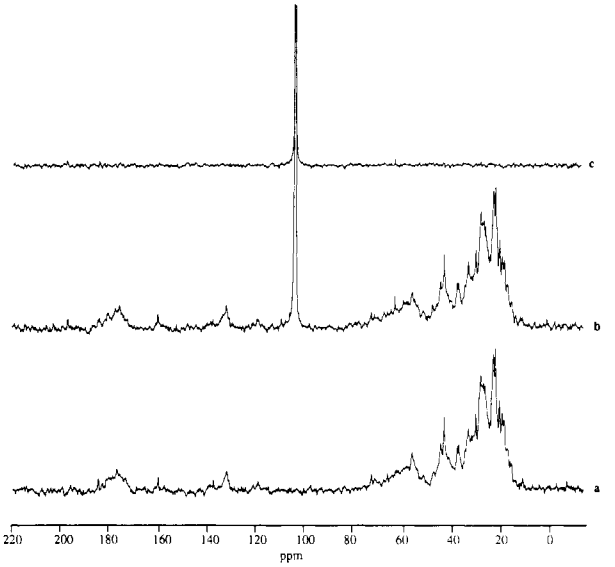


FIGURE 2: ^{1}H-Decoupled ^{13}C NMR spectra of EPSP synthase plus ddS3P and [3-^{13}C]PEP. (a) EPSP synthase (1.4 mM), 20 mM Tris-HCl, pH 7.6, 0.4 mM DTT, 0.4 mM EDTA, 10 mM KF, and 10 mM ddS3P in 450 μL; (b) same plus [3-^{13}C]PEP (20 μL, 244 mM in 100 mM Tris-HCl, pH 8.0), final concentration = 10 mM; (c) difference spectrum, (b) − (a). All spectra were obtained with the following: number of scans = 22000, recycle time = 1.1 s, pulse width = 6 μs, size = 32K data points, and FID's Fourier transformed with line broadening = 20 Hz.

several months if frozen rapidly in liquid nitrogen and stored at −70 °C.

NMR Spectroscopy. High-field Fourier transform (FT) NMR studies of EPSP synthase were performed on a Bruker AM-500 (11.75 T, 500 MHz ^{1}H) NMR spectrometer with home-built modifications for indirect-detection experiments. Deuterium was used for locking the field. Chemical shifts were referenced externally to samples of similar dielectric constant: ^{1}H NMR spectra to sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]-propanoate ([^{2}H]TSP) in D_2O buffer (δ_H = 0.00 ppm) and ^{13}C NMR spectra to dioxane in D_2O buffer (δ_C = 67.4 ppm). Sample temperature was maintained with a Bruker VT-1000 variable-temperature control unit, using boil-off liquid nitrogen. All samples containing EPSP synthase were maintained at 278 K. Heteronuclear NMR was carried out with single-frequency Bruker probes, with the exception of ^{1}H indirect detection, which was carried out with a home-built probe (with ^{13}C outside decoupling coil). Proton decoupling was achieved by gated broad-band irradiation, Waltz-16 being used to minimize dielectric heating.

Sample Preparation. The purified EPSP synthase was thawed and washed by cycles of dilution and concentration by ultrafiltration to ca. 0.45 mL with either (i) 10 mM Tris-HCl, 0.4 mM DTT, and 0.4 mM EDTA, in 10% (v/v) D_2O/H_2O, pH 7.8, or (ii) 100 mM potassium phosphate, 0.4 mM DTT and 0.4 mM EDTA, in 10% (v/v) D_2O/H_2O, pH 7.8.

Results

NMR Studies of EPSP Synthase with 4,5-Dideoxy-S3P and [2-^{13}C]PEP. At the time we started this research, the kinetic profile for EPSP synthase had not been determined. It was therefore appropriate to initiate our attempt to detect the enzyme–intermediate complex by NMR spectroscopy by using ddS3P and [^{13}C]PEP to generate a dead-end complex, as postulated by Anton et al. (1983). Figure 1 shows the proton-decoupled ^{13}C NMR spectra of (a) EPSP synthase (1.4 mM) and ddS3P (10 mM) and (b) the same plus [2-^{13}C]PEP (20 mM) and (c) the difference spectrum, (b) − (a). In addition to the broad natural abundance ^{13}C resonances from the enzyme [methyl, methylene, and methine groups at 20–70 ppm, aromatic groups at 120–140 ppm, and carbonyls at 170–185 ppm (Roberts & Jardetsky, 1981)], Figure 1a exhibits additional sharper resonances from ddS3P [see Anton et al. (1983)], DTT (reduced, 75.6 ppm; oxidized, 72.5 ppm), Tris buffer (64.7 ppm), and EDTA (60.5 and 62.5 ppm). The difference spectrum Figure 1c shows resonances due to natural abundance C-3 of [2-^{13}C]PEP [103.7 ppm (doublet)], due to C-2 of [2-^{13}C]PEP (152.4 ppm), and due to [2-^{13}C]pyruvate (208.2 ppm). The resonance at 74.7 ppm (LW = 28 Hz) is due to C-4 of the aldol product of pyruvate, 4-hydroxy-4-methylketoglutarate (HMKG), or parapyruvate. This is known to form in neutral solutions of pyruvate (Montgomery & Webb, 1956). It has been synthesized (De Jong, 1901) and the ^{13}C NMR spectrum completely assigned [C-1, 169.0 ppm; C-2, 203.6 ppm; C-3, 48.5 ppm; C-4, 73.4 ppm; C-4 methyl, 26.1 ppm; C-5, 181.7 ppm (Dr. N. E. Mackenzie, personal communication)]. It should be noted that C-2 of HMKG is not detected, which is due to the fact that the recycle time is sufficiently short to result in partial saturation of the carbonyl resonances.

The resonance at 148.5 ppm (LW = 32 Hz) has not been assigned. However, it is clear from the line width (Malthouse, 1986) that this species is not enzyme bound. On occasions when the sample was stored for more than 24 h, an additional resonance at 97.5 ppm was detected, which was assigned to hydrated (C-2 *gem*-diol) pyruvate. The control experiment (data not shown) in which EPSP synthase and [2-^{13}C]PEP were examined by ^{1}H-decoupled ^{13}C NMR spectroscopy under identical conditions in the absence of ddS3P confirmed that ddS3P is required for pyruvate formation. However, this pyruvate formation is slow, since the intensity of the resonances increases gradually over the course of several days.

NMR Studies of EPSP Synthase with 4,5-Dideoxy-S3P and [3-^{13}C]PEP. Figure 2 shows the proton-decoupled ^{13}C NMR spectra of (a) EPSP synthase (1.4 mM) and S3P (10 mM) and (b) the same plus [3-^{13}C]PEP (10 mM) and (c) the difference spectrum, (b) − (a). Apart from the resonance due to [3-^{13}C]PEP and a small resonance due to Tris buffer, no additional resonances are detectable. Although resonances consistent with pyruvate are not detected, this can be explained by the fact that this sample required a relatively short accu-

mulation time and was therefore relatively fresh. However, when the sample was examined by the intrinsically more sensitive technique of indirect-detection ^{1}H NMR (vide infra), the methyl resonance of pyruvate was readily detectable (data not shown).

From these experiments, it is clear that PEP is hydrolyzed to pyruvate in the presence of the EPSP synthase preparation. This phosphatase activity might arise from contaminating nonspecific phosphatase enzymes. Alternatively, it might be a nonspecific general protein effect of the EPSP synthase itself. The most interesting possibility is that hydrolysis arises from a mechanistically relevant interaction of PEP with the active site of the EPSP synthase. Experiments were performed to determine whether the EPSP synthase active site has phosphatase activity. *p*-Nitrophenyl phosphate (PNPP) is a good substrate for phosphatases in general, and its hydrolysis is easily monitored spectrophotometrically. It was found that when EPSP synthase (18 μM) is added to a solution of PNPP (10 mM), after a lag period, PNPP hydrolysis occurs at a rate of 0.4 μmol/min. This result merely confirms that the enzyme preparation has a nonspecific phosphatase activity. If this activity is associated with the active site of EPSP synthase, it should be possible to block it by the addition of S3P and glyphosate. It was found that in the presence of 0.8 mM S3P and 1 mM glyphosate the phosphatase activity is reduced by 80%. In another experiment it was found that an equivalent concentration of bovine serum albumin produced no significant hydrolysis of PNPP. Finally, the phosphatase activity of the EPSP synthase preparation was shown not to be inhibited by potassium fluoride. Potassium fluoride is a classical inhibitor of phosphatase enzymes. Therefore, all the evidence points to an active site specific phosphatase activity of EPSP synthase which has not been described previously. Anton et al. (1983) failed to observe the formation of pyruvate by EPSP synthase in the presence of PEP and ddS3P, although they were not using the same enzyme concentrations as was employed in these experiments.

NMR Studies of EPSP Synthase with S3P and [2-^{13}C]-PEP. Recently, elegant work by Anderson et al. (1988a) has established the complete kinetic profile for EPSP synthase. This work suggests, but does not prove, that only one, surprisingly stable, intermediate forms on the reaction pathway. Since the enzyme is freely reversible (with an equilibrium constant of 180 in the forward direction), the implication from Anderson's work is that with enzyme in the presence of a sufficiently high concentration of inorganic phosphate approximately equal concentrations of S3P/PEP and EPSP may be generated. Under these equilibrium conditions, a large fraction (up to 40%) of the enzyme-bound species is present as the intermediate. The amount of enzyme-bound intermediate that can be generated is thus limited by the amount of enzyme available. Figure 3 shows the proton-decoupled ^{13}C NMR spectra of (a) EPSP synthase (3.4 mM) and the same (b) plus S3P (12 mM) and [2-^{13}C]PEP (12 mM) and (c) the difference spectrum, (b) − (a). In addition to the natural abundance ^{13}C resonances from the enzyme as before, Figure 3a exhibits sharp resonances from S3P (C-1, 137.9 ppm; C-2, 133.9 ppm; C-3, 74.9 ppm; C-4, 72.1 ppm; C-5, 70.6 ppm; C-6, 34.6 ppm). Upon addition of [2-^{13}C]PEP, resonances appear from EPSP (C-3, 76.7 ppm; C-4, 75.5 ppm; C-5, 73.3 ppm; C-6, 31.8 ppm), and apart from subtraction artifacts in the protein aliphatic region, additional resonances occur at 107.2, 110.9, 152.4, 156.4, 164.8, and 208.2 ppm. Furthermore, the resonance at 110.9 ppm was found to increase in intensity with time at the expense of the resonance at 107.2 ppm.

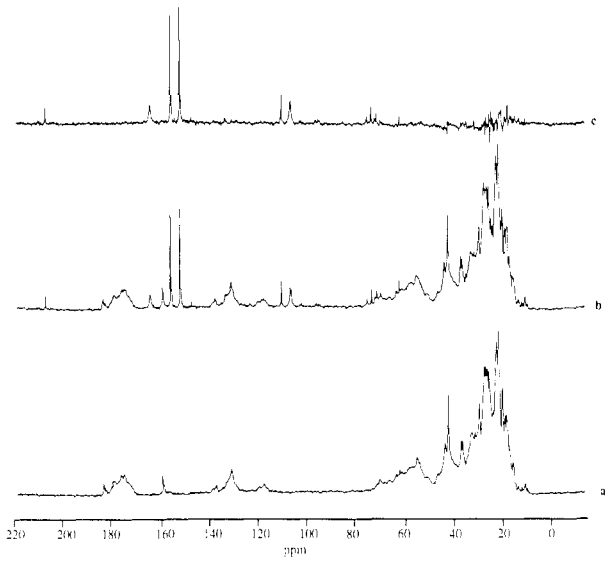


FIGURE 3: ^{1}H-Decoupled ^{13}C NMR spectra of EPSP synthase plus S3P and [2-^{13}C]PEP. (a) EPSP synthase (3.4 mM), 100 mM potassium phosphate, pH 7.6, 0.4 mM DTT, and 12 mM S3P in 450 μL; (b) same plus [2-^{13}C]PEP (21 μL of 267 mM in 100 mM Tris-HCl, pH 8.0), final concentration = 12 mM; (c) difference spectrum, (b) − (a). All spectra were obtained with the following: number of scans = 43 000, recycle time = 1.1 s, pulse width = 8 μs, size = 32K data points, and FID's Fourier transformed with line broadening = 20 Hz.

The assignment of these latter resonances was made by saturation-transfer experiments (Forsén & Hoffman, 1963; Robinson et al., 1984), in which selected resonances are subjected to a frequency-selective 180° pulse with the DANTE pulse train (Morris & Freeman, 1978), and the intensity of the remaining resonances is monitored relative to an off-resonance control. Irradiation at 156.4 ppm ([8-^{13}C]EPSP) resulted in a large attenuation of the resonance at 164.8 ppm. Conversely, when the resonance at 152.4 ppm was irradiated, the resonance at 107.2 ppm was greatly reduced in intensity. The relationship between the resonances at 164.8 and 107.2 ppm was confirmed when each resonance was selectively irradiated in turn, and irradiation of one resulted in a large attenuation of the other resonance. Finally, the relationship between the resonances at 110.9 ppm and at 107.2 ppm was confirmed by attenuation of the resonance at 110.9 ppm upon irradiation at 107.2 ppm. This latter result needs to be regarded with caution, since, although every effort was made to ensure that the side lobes of the DANTE irradiation at 107.2 ppm did not coincide with the resonance at 110.9 ppm, some spillover saturation is possible. To further confirm the assignment of the resonance at 110.9 ppm, the same sample was examined as a lyophilized powder by ^{13}C CP-MAS 2D spin-diffusion spectroscopy (data not shown), and a chemical exchange crosspeak between the resonances at 156.4 ppm (free EPSP) and 110.9 ppm was observed. This type of spectroscopy can only work in the solid state, in which the exchange rates are sufficiently slow. The assignments are summarized in Table I. Attempts to use ^{31}P NMR spectroscopy to detect the phosphate resonance of the group attached to C-8 of the intermediate have failed to date. Presumably this is due to the large anisotropy of ^{31}P, which in the presence of the enzyme would give rise to very large line widths. No $^2J_{CP}$ splitting of the resonance at either 110.9 or 107.2 ppm is observed, since the line widths of both these resonances exceed the expected coupling constants ($^2J_{CP} \sim 8$ Hz).

NMR Studies of EPSP Synthase with S3P and [3-^{13}C]-PEP. Figure 4 shows the proton-decoupled ^{13}C NMR spectra

Table I: Assignments for EPSP Synthase–S3P–[2-^{13}C]- or [3-^{13}C]PEP Complex

with [2-^{13}C]PEP			with [3-^{13}C]PEP				
			chemical shift		LW (Hz)		
chemical shift, δ_C (ppm)	LW (Hz)	assignment	δ_C (ppm)	δ_H (ppm)	^{13}C	^{1}H	assignment
107.2	100	C-8 enzyme-bound intermediate	24.5	?	100	?	C-9 enzyme-bound intermediate
110.9	30	C-8 enzyme-free intermediate	25.5	1.16	50	10	C-9 enzyme-free intermediate
152.4	30	C-2 enzyme-free intermediate	26.2	1.48	30	5	4-methyl-HMKG
156.4	30	C-8 enzyme-free EPSP	28.4	1.05	30	5	C-3 hydrated pyruvate
164.8	130	C-8 enzyme-bound EPSP	29.4	1.95	40	5	C-3 pyruvate
208.2	30	C-2 pyruvate	88.9		240		C-9 enzyme-bound EPSP
			95.7		30		C-9 enzyme-free EPSP
			103.2		30		C-3 enzyme-free PEP

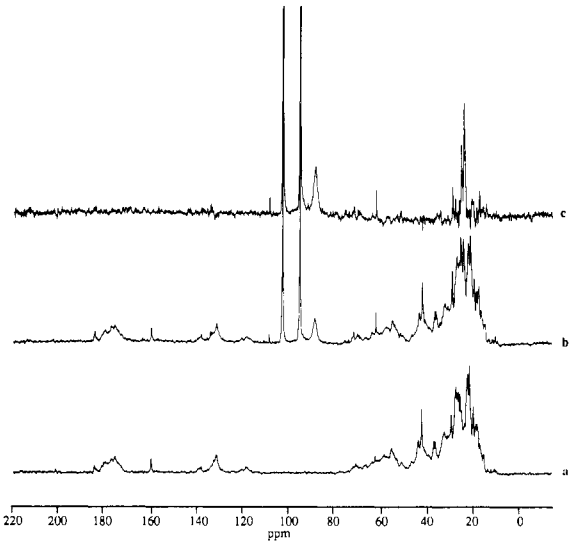


FIGURE 4: ^{1}H-Decoupled ^{13}C NMR spectra of EPSP synthase plus S3P and [3-^{13}C]PEP. (a) EPSP synthase (1.1 mM), 100 mM potassium phosphate, pH 7.6, 0.4 mM DTT, and 4 mM S3P in 450 μL; (b) same plus [3-^{13}C]PEP (8 μL, 244 mM in 100 mM Tris-HCl, pH 8.0), final concentration = 4 mM; (c) difference spectrum, (b) − (a). All spectra were obtained with the following: number of scans = 46000, recycle time = 1.1 s, pulse width = 8 μs, size = 32K data points, and FID's Fourier transformed with line broadening = 20 Hz.

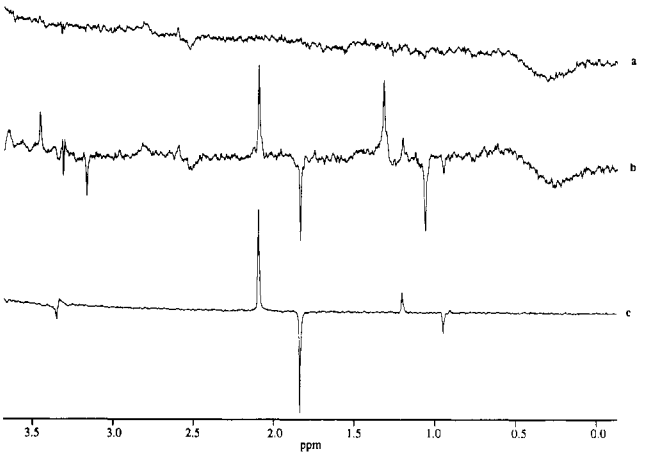


FIGURE 5: Isotope-edited ^{1}H{^{13}C} NMR spectra of EPSP synthase plus S3P and [3-^{13}C]PEP. (a) EPSP synthase (1.1 mM), 100 mM potassium phosphate, pH 7.6, 0.4 mM DTT, and 4 mM S3P in 450 μL; (b) same plus [3-^{13}C]PEP (8 μL, 244 mM in 100 mM Tris-HCl, pH 8.0), final concentration = 4 mM; (c) pure [3-^{13}C]pyruvate (20 mM in 100 mM potassium phosphate, pH 7.6). All spectra were obtained with the following: number of scans = 1280, recycle time = 2.82 s, pulse width (^{1}H) = 18 μs, pulse width (^{13}C) = 22 μs, size = 8K data points, and FID's Fourier transformed with line broadening = 1 Hz.

of (a) EPSP synthase (1.7 mM) and S3P (10 mM) and (b) the same plus [3-^{13}C]PEP (10 mM) and (c) the difference spectrum, (b) − (a). in addition to the natural abundance ^{13}C resonances from the enzyme as before, Figure 4a exhibits sharp resonances from S3P (vide supra). Upon addition of [3-^{13}C]PEP, additional resonances appear due to EPSP (vide supra) and at 24.5, 25.5, 26.2, 28.3, 29.4, 88.9, 95.7, and 103.2 ppm. The resonances at 25.5, 26.2, 28.3, and 29.4 ppm have been assigned by ^{1}H NMR spectroscopy (vide infra). On occasions when the sample is left for several days, the resonance at 25.5 ppm was found to increase at the expense of the resonance at 24.5 ppm and then decrease as the resonance at 26.2 ppm increased.

The relationship between the resonances at 103.2 ([3-^{13}C]PEP), 95.7 ([9-^{13}C]EPSP), 88.9, and 24.5 ppm was examined by saturation-transfer experiments. By analogy with the [2-^{13}C]PEP experiments, irradiation at 95.7 ppm resulted in a large attenuation of the intensity of the resonance at 88.9 ppm. Conversely, when the resonance at 103.2 ppm was irradiated, the resonance at 24.5 ppm was greatly attenuated. The relationship between the resonances at 88.9 and 24.5 ppm was confirmed when each resonance was selectively irradiated in turn, and irradiation of one resulted in a large attenuation of the other resonance. Irradiation of the resonance at 24.5 ppm resulted in attenuation of the resonance at 25.5 ppm, although, as in the case of the saturation-transfer experiments with [2-^{13}C]PEP at 107.2 and 110.9 ppm, this latter result needs to be viewed with caution. The assignments are summarized in Table I.

Isotope-Edited NMR Studies of EPSP Synthase with S3P and [3-^{13}C]PEP. One of the perennial problems in solution-state ^{13}C NMR of enzyme–substrate or enzyme–intermediate complexes is separating the resonances of the isotopically enriched substrate or intermediate from the natural abundance ^{13}C background of the protein. This is particularly true where the detection of an intermediate containing a methyl group is concerned, as has been shown in the preceding section. Although two resonances have been detected in the region of the ^{13}C NMR spectrum consistent with methyl groups potentially arising from the postulated intermediate, there is a possibility that slight changes in the natural abundance ^{13}C spectrum of the protein could give rise to such resonances. Indirect detection of an isotope-edited ^{1}H NMR spectrum has been used recently with great success in detecting isotopically enriched species bound to an enzyme (Fesik et al., 1988) and an enriched protein itself (Westler et al., 1988). We have used the method introduced by Müller (1979) as modified by Cavanaugh and Keeler (1988) (with a further slight modification) for the detection of zero and double quantum coherences in both one and two dimensions. Figure 5 shows the indirect isotope-edited heteronuclear multiple quantum coherence (HMQC) ^{1}H NMR spectra of (a) EPSP synthase (1.1 mM) and S3P (4 mM), (b) the same plus [3-^{13}C]PEP (4 mM), and (c) pure [3-^{13}C]pyruvate.

The protein ^{1}H resonances are essentially removed (Figure

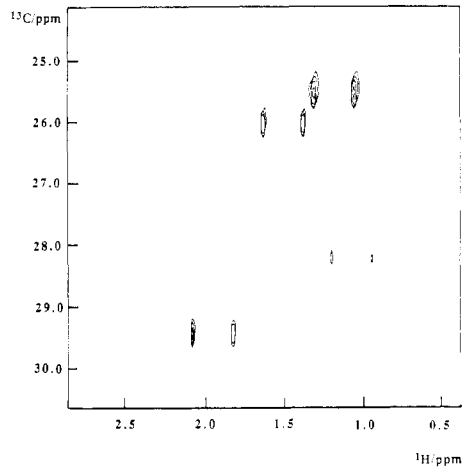


FIGURE 6: 2D HMQC isotope-edited $^{1}H\{^{13}C\}$ COSY spectrum of EPSP synthase plus S3P and [3-^{13}C]PEP. EPSP synthase (1.4 mM), 100 mM potassium phosphate, pH 7.6, 0.4 mM DTT, and 4 mM S3P in 450 μL plus [3-^{13}C]PEP (8 μL, 244 mM in 100 mM Tris-HCl, pH 8.0), final concentration = 4 mM. The spectrum was obtained with the following: number of scans = 1280, recycle time = 2.82 s, pulse width (^{1}H) = 18 μs, pulse width (^{13}C) = 22 μs, size = 1K × 2K data points, and FID's Fourier transformed with a sine-bell weighting function (shifted by $\pi/3$ in the F_2 domain and $\pi/6$ in the F_1 domain).

5a), and four resonances are detectable at 1.05, 1.16, 1.95, and 3.31 ppm. Note that this experiment does not employ broad-band ^{13}C decoupling, so the ^{1}H resonances appear as antiphase doublets. By comparison with [3-^{13}C]pyruvate (Figure 5c), the resonances centered at 1.05 and at 1.95 ppm can be assigned as given in Table I. The additional resonance at 3.31 ppm (LW = 5 Hz) has not been assigned. As the sample aged, it was found that the resonance at 1.16 ppm increased to a maximum and then decreased as a resonance at 1.48 ppm appeared. This latter resonance also increased in intensity as a function of the decrease of the resonance at 1.95 ppm. When a sample of [3-^{13}C]pyruvate was aged for several days, in addition to the resonances shown in Figure 5c, an additional resonance at 1.48 ppm became prominant. In a separate experiment (data not shown), it was confirmed that negligible pyruvate formation occurred with EPSP synthase and [3-^{13}C]PEP in the absence of S3P and that the two additional resonances were also absent. That approximately equal concentrations of [3-^{13}C]PEP and [9-^{13}C]EPSP were present in the sample was confirmed by ^{1}H-decoupled ^{13}C NMR spectroscopy. In another experiment, EPSP synthase was preincubated with S3P and glyphosate (0.5 enzyme equiv). Subsequent incubation with additional S3P and [3-^{13}C]PEP and examination by isotope-edited ^{1}H NMR spectroscopy showed no loss of intensity of the resonance at 3.31 ppm but considerable depletion of intensity at 1.16 ppm.

Figure 6 shows the 2D HMQC double-difference reverse correlated spectrum of EPSP synthase–S3P–[3-^{13}C]PEP complex. In this sample, the resonances in the proton domain occur at 1.05, 1.16, 1.48, and 1.95 ppm. The correlations with ^{13}C resonances are given in Table I. In a separate experiment, a sample of EPSP synthase–S3P–[3-^{13}C]PEP complex was subjected to ultrafiltration, and the enzyme-free filtrate was examined by 2D HMQC double-difference COSY. Only the resonance at 1.16 ppm (correlating with the ^{13}C resonance at 25.5 ppm) was detectable.

Discussion

Origin of Pyruvate Formation. The formation of pyruvate from PEP in our incubations with EPSP synthase in the presence of ddS3P might arise from a nonspecific phosphatase activity (i.e., P–O cleavage) or an active site mediated activity (i.e., C–O cleavage). Anton et al. (1983) reported that free pyruvate did not form in their incubation of PEP with EPSP synthase and ddS3P. The intermediate (**3**) detected by Anderson et al. (1988a,b) (as in Scheme IA) has been shown to be converted to pyruvate in both acid and base—however, these workers did not study the formation of the intermediate under physiological conditions. Under our conditions, high-field ^{13}C NMR has been valuable in identifying a novel phosphatase activity which appears to be associated with EPSP synthase activity. The fact that previous workers in the field failed to detect this can be attributed to the fact that in our experiments EPSP synthase is present at high protein concentrations (1–3 mM). The essential control NMR experiment in which a similar sample of protein was examined in the *absence* of ddS3P, and failed to show pyruvate production, establishes that the formation of pyruvate arises from EPSP synthase activity itself. This was further confirmed in an independent assay of phosphatase activity with the substrate PNPP, which showed that S3P/glyphosate afforded some protection against phosphatase activity. While the hydrolysis of PNPP tends to point to a nonspecific phosphatase activity, the possibility remains that the phosphatase activity, located in the active site, may also play a role in the formation of EPSP from PEP. Further experiments using ^{18}O labeling will be carried out to establish the precise origin of pyruvate formation.

Interpretation of Unassigned Resonances. The unassigned resonance at 148.5 ppm in the ^{13}C NMR spectra of the EPSP synthase–ddS3P–[2-^{13}C]PEP complex is consistent with a fully substituted vinylic carbon as in C-2 of PEP, again free from the enzyme. A more precise characterization of this resonance is under way.

The absence of any additional resonances in the ^{13}C NMR spectra of EPSP synthase–ddS3P–[3-^{13}C]PEP complex suggests that there is no significant accumulation of an intermediate (**5**, Scheme IB) such as proposed by Anton et al. This is consistent with the observation by these workers that tritium was incorporated into PEP in the presence of ddS3P at very low levels relative to V_{max}. It is possible that the tritium incorporation may be explained in terms of a specific EPSP synthase catalyzed equilibrium between PEP and pyruvate in the presence of ddS3P.

Use of saturation-transfer experiments have allowed the interpretation of the unassigned resonances in the ^{13}C NMR spectra of the EPSP synthase–S3P–[2-^{13}C]PEP complex. The resonance at 164.8 ppm is consistent with enzyme-bound [8-^{13}C]EPSP, presumably in the form of the E·EPSP·P_i complex as proposed by Anderson et al. (1988a). The resonance at 107.2 ppm is consistent with an enzyme-bound intermediate which is tetrahedral at C-8 and is attached to two oxygens and a carboxylate (cf. C-2 of pyruvate hydrate, δ_C = 97.5 ppm). This chemical shift is also to be compared with 101.7 ppm reported by Anderson et al. (1988b) for C-8 of intermediate **3**. The line width is consistent with either covalent attachment to the protein or slow exchange of an electrostatically bound species with enzyme-bound EPSP. However, the fact that no intermediates were detected in the experiments with ddS3P suggests that covalent attachment of the intermediate to the protein is highly unlikely. The resonance at 110.9 ppm is consistent with an enzyme-free (on the basis of line width) intermediate of a structure also similar to that of **3**.

The unassigned resonances in the ^{13}C NMR spectra of EPSP synthase–S3P–[3-^{13}C]PEP complex have also been interpreted on the basis of saturation-transfer experiments in

conjunction with HMQC double-difference ^1H NMR spectroscopy. The resonance at 88.9 ppm is consistent with enzyme-bound [9-^{13}C]EPSP, and the resonance at 26.2 ppm is consistent with the 4-methyl group of HMKG, since it correlates with 1.48 ppm in the ^1H domain, and this latter resonance has been detected and assigned in aged samples of [3-^{13}C]pyruvate. The resonance at 25.5 ppm correlates with a ^1H resonance at 1.16 ppm. The former shift is consistent with an intermediate structure involving a methyl group attached to a quaternary carbon (cf. C-3 of pyruvate hydrate, δ_C = 28.3 ppm), and the latter shift is to be compared with 1.25 ppm (corrected for differences in referencing) observed by Anderson et al. (1988b) for intermediate **3**. Since this species has also been isolated free from the protein in this laboratory, it is likely that it corresponds to intermediate **3**. Similar conclusions were reached in independent work by Anderson et al. (1988b), which was published when our research was at an advanced stage. However, their study isolated and characterized a species that arose from quenching the EPSP synthase reaction in neat triethylamine, which therefore represents an indirect and physiologically questionable experiment. Furthermore, it is conceivable that the resonance at 25.5 ppm may correlate with that observed at 110.9 ppm in the same complex with [2-^{13}C]PEP. Experiments to test this hypothesis are in progress.

Finally, the resonance at 24.5 ppm remains to be assigned. Unfortunately, it has not been possible to obtain a ^1H correlation for this resonance. The ^{13}C line width (100 Hz) suggests a ^1H line width > 100 Hz or a T_2 which is shorter than the $1/2J$ delay required for transfer of multiple quantum coherences. This is borne out by the results summarized in Figures 5 and 6, in which no such crosspeaks are detectable. Attempts to use difference decoupling, in which ^1H difference spectra are obtained with and without single-frequency ^{13}C decoupling, produced ambiguous results. However, the ^{13}C line width suggests that the species giving rise to this resonance may correlate with the broad resonance observed at 107.2 ppm in the same complex with [2-^{13}C]PEP. It is, we believe, consistent with an enzyme-bound (or slowly exchanging) intermediate.

Structure of the Intermediate. The data reported in this paper do not yet permit a complete definition of the structure of the enzyme–intermediate complexes of EPSP synthase that we have detected by ^{13}C NMR spectroscopy. It is clear however that the 5-hydroxyl of S3P is required for their detection. Furthermore, *two* species have been detected, both of which have structures closely allied to that of intermediate **3** and that isolated by Anderson et al. The line width of the resonance from one of these species is relatively narrow in the presence of the enzyme and in the presence of a broader resonance of closely similar chemical shift and, furthermore, increases in intensity with time at the expense of the broader resonance. This implies that under these conditions there is a large difference in on and off rates, and the enzyme-free intermediate dissociates slowly from the enzyme and does not reassociate. Experiments are currently under way to establish the complete structures of the two intermediates and their rates of interconversion.

ACKNOWLEDGMENTS

We acknowledge Prof. J. R. Coggins (University of Glasgow) for the gift of recombinant *E. coli*, Dr. A. E. Derome for assistance on various technical aspects of the NMR spectroscopy and many useful discussions, and M. Robertson for modification of the spectrometer and construction of the indirect detection probe. We thank one of the referees for a helpful suggestion regarding presentation of data.

REFERENCES

Alberg, D. G., & Bartlett, P. A. (1989) *J. Am. Chem. Soc. 111*, 2337–2338.
Anderson, K. S., Sikorski, J. A., & Johnson, K. A. (1988a) *Biochemistry 27*, 7395–7406.
Anderson, K. S., Sikorski, J. A., Benesi, A. J., & Johnson, K. A. (1988b) *J. Am. Chem. Soc. 110*, 6577–6579.
Anton, D. L., Hedstrom, L., Fish, S. M., & Abeles, R. H. (1983) *Biochemistry 22*, 5903–5908.
Appleyard, R. J., Barlow, P. N., Wilson, B. J. O., & Evans, J. N. S. (1989) *Arch. Biochem. Biophys.* (submitted for publication).
Bondinell, W. E., Vnek, J., Knowles, P. F., Sprecher, M., & Sprinson, D. B. (1971) *J. Biol. Chem. 246*, 6191–6196.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Cassidy, P. J., & Kahan, F. M. (1973) *Biochemistry 12*, 1364–1374.
Cavanaugh, J., & Keeler, J. (1988) *J. Magn. Reson. 77*, 356.
De Jong, A. W. K. (1901) *Rev. Trav. Chim. Pays-Bas 20*, 81–101.
Duncan, K., Lewendon, A., & Coggins, J. R. (1984a) *FEBS Lett. 170*, 59–63.
Duncan, K., Lewendon, A., & Coggins, J. R. (1984b) *FEBS Lett. 165*, 121–127.
Fesik, S. W., Luly, J. R., Erickson, J. W., & Abad-Zapatero, C. (1988) *Biochemistry 27*, 8297.
Forsén, S., & Hoffman, R. A. (1963) *J. Chem. Phys. 39*, 2829.
Ganem, B. (1978) *Tetrahedron 34*, 3353–3383.
Grimshaw, C. E., Sogo, S. G., & Knowles, J. R. (1982) *J. Biol. Chem. 257*, 596–598.
Grimshaw, C. E., Sogo, S. G., Copley, S. D., & Knowles, J. R. (1984) *J. Am. Chem. Soc. 106*, 2699–2700.
Jardetzky, O., & Roberts, G. C. K. (1981) *NMR in Molecular Biology*, p 232, Academic Press, New York.
Levin, J. G., & Sprinson, D. B. (1964) *J. Biol. Chem. 239*, 1142–1150.
Lewendon, A., & Coggins, J. R. (1983) *Biochem. J. 213*, 187–191.
Malthouse, J. P. G. (1986) *Prog. Nucl. Magn. Reson. Spectrosc. 18*, 1.
Montgomery, C. M., & Webb, J. L. (1956) *J. Biol. Chem. 221*, 359–368.
Morris, G. A., & Freeman, R. (1978) *J. Magn. Reson. 29*, 433.
Mousdale, D. M., & Coggins, J. R. (1984) *Planta 160*, 78–83.
Müller, L. (1979) *J. Am. Chem. Soc. 101*, 4481.
Padgette, S. R., Huynh, Q. K., Bourgmeyer, J., Shah, D. M., Brand, L. A., Biest Re, D., Bishop, B. F., Rogers, S. G., Fraley, R. T., & Kishore, G. M. (1987) *Arch. Biochem. Biophys. 258*, 564–573.
Robinson, G., Chapman, B. E., & Kuchel, P. W. (1984) *Eur. J. Biochem. 143*, 643.
Rogers, S. G., Brand, L. A., Holder, S. B., Sharps, E. S., & Brackin, M. J. (1983) *Appl. Environ. Microbiol. 46*, 37–43.
Strominger, J. L. (1958) *Biochim. Biophys. Acta 30*, 645.
Van Veldhoven, P. P., & Mannaerts, G. P. (1987) *Anal. Biochem. 161*, 45–48.
Vogel, H. J., & Bonner, D. M. (1956) *J. Biol. Chem. 218*, 97.
Westler, W. M., Kainosho, M., Nagao, H., Tomonaga, N., & Markley, J. L. (1988) *J. Am. Chem. Soc. 110*, 4093–4095.
Zemell, R. I., & Anwar, R. A. (1975) *J. Biol. Chem. 250*, 4959–4964.