& Patchett, A. A. (1982) *Biochem. Biophys. Res. Commun. 109*, 1303-1309.
Olins, G. M., Spear, K. L., Siegel, N. R., & Zurcher-Neely, H. A. (1987) *Biochim. Biophys. Acta 901*, 97-100.
Painter, R. G., Dukes, R., Sullivan, J., Carter, R., Erdos, E. G., & Johnson, A. R. (1988) *J. Biol. Chem. 263*, 9456-9461.
Pozsgay, M., Michaud, C., Liebman, M., & Orlowski, M. (1986) *Biochemistry 25*, 1292-1299.
Roques, B. P., & Fournie-Zaluski, M. C. (1986) *NIDA Res. Monogr. 70*, 128-154.
Roques, B. P., Fournie-Zaluski, M. C., Florentin, D., Waksman, G., Sassi, A., Chaillet, P., Collado, H., & Costentin, J. (1982) *Life Sci. 31*, 1749-1752.
Rupreht, J., Ukponmwan, O. E., Admiraal, P. V., & Dzoljic, M. R. (1983) *Neurosci. Lett. 41*, 331-335.
Schwartz, J. C., Costentin, J., & Lecomte, J. M. (1985) *Trends Pharmacol. Sci. 6*, 472-476.
Shipp, M. A., Vijayaraghavan, J., Schmidt, E. V., Masteller, E. L., D'Adamio, L., Hersh, L. B., & Reinherz, E. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 297-301.
Sidler, W., Niederer, E., Suter, F., & Zuber, H. (1986) *Hoppe-Seyler's Z. Physiol. Chem. 367*, 643-657.
Vijayaraghavan, J., Scicli, G., Carretero, O., & Hersh, L. B. (1990) *J. Biol. Chem.* (in press).
Zoller, M. J., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487-6500.

Phosphatidylinositol-Specific Phospholipase C from *Bacillus cereus* Combines Intrinsic Phosphotransferase and Cyclic Phosphodiesterase Activities: A ^{31}P NMR Study†

Johannes J. Volwerk,* M. S. Shashidhar, Andreas Kuppe, and O. Hayes Griffith*
Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403
Received December 12, 1989; Revised Manuscript Received May 14, 1990

ABSTRACT: The inositol phosphate products formed during the cleavage of phosphatidylinositol by phosphatidylinositol-specific phospholipase C from *Bacillus cereus* were analyzed by ^{31}P NMR. ^{31}P NMR spectroscopy can distinguish between the inositol phosphate species and phosphatidylinositol. Chemical shift values (with reference to phosphoric acid) observed are −0.41, 3.62, 4.45, and 16.30 ppm for phosphatidylinositol, *myo*-inositol 1-monophosphate, *myo*-inositol 2-monophosphate, and *myo*-inositol 1,2-cyclic monophosphate, respectively. It is shown that under a variety of experimental conditions this phospholipase C cleaves phosphatidylinositol via an intramolecular phosphotransfer reaction producing diacylglycerol and D-*myo*-inositol 1,2-cyclic monophosphate. We also report the new and unexpected observation that the phosphatidylinositol-specific phospholipase C from *B. cereus* is able to hydrolyze the inositol cyclic phosphate to form D-*myo*-inositol 1-monophosphate. The enzyme, therefore, possesses phosphotransferase and cyclic phosphodiesterase activities. The second reaction requires thousandfold higher enzyme concentrations to be observed by ^{31}P NMR. This reaction was shown to be regiospecific in that only the 1-phosphate was produced and stereospecific in that only D-*myo*-inositol 1,2-cyclic monophosphate was hydrolyzed. Inhibition with a monoclonal antibody specific for the *B. cereus* phospholipase C showed that the cyclic phosphodiesterase activity is intrinsic to the bacterial enzyme. We propose a two-step mechanism for the phosphatidylinositol-specific phospholipase C from *B. cereus* involving sequential phosphotransferase and cyclic phosphodiesterase activities. This mechanism bears a resemblance to the well-known two-step mechanism of pancreatic ribonuclease, RNase A.

Phosphatidylinositol-specific phospholipase C (PI-PLC)[1] (EC 3.1.4.10) catalyzes cleavage of the membrane lipid phosphatidylinositol (PtdIns), producing a membrane-soluble product, diacylglycerol, and a water-soluble product, inositol phosphate. In mammalian cells, PI-PLC occurs as a family of proteins with limited structural homology (Rhee et al., 1989). These enzymes are believed to play a key role in the PtdIns-dependent pathway of transmembrane signal transduction of calcium-mobilizing hormones and growth factors (Berridge, 1986; Berridge & Irvine, 1989). Because of the importance of PtdIns-dependent signaling for understanding cellular processes such as metabolism, secretion, contraction, proliferation, neural activity, and associated diseases, interest in these enzymes has increased rapidly in recent years and a number of eukaryotic enzymes have now been cloned and sequenced [reviewed by Rhee et al. (1989)].

An enzyme with similar high specificity for inositol phospholipids also has been purified from the culture media of several microorganisms including *Staphylococcus aureus*

†This work was supported by U.S. Public Health Service Grants GM 25698 and GM 27137.

[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; PtdIns, phosphatidylinositol; Ins(1)P, *myo*-inositol 1-monophosphate; Ins(2)P, *myo*-inositol 2-monophosphate; Ins(1:2cyc)P, *myo*-inositol 1,2-cyclic monophosphate (as used here, the abbreviation Ins refers to either the D- or L-enantiomer of the *myo*-inositol phosphates; for clarity and because of the nature of the work described, enantiomers or racemic mixtures of the inositol phosphates are specified by the prefixed D-, L-, or D,L-, respectively); BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid (disodium salt); Tris, tris(hydroxymethyl)aminomethane; d, doublet (NMR).

0006-2960/90/0429-8056$02.50/0

(Low, 1981), *Bacillus cereus* and *Bacillus thuringiensis* (Ikezawa & Taguchi, 1981; Volwerk et al., 1989a,b), and *Clostridium novyi* (Taguchi & Ikezawa, 1978). The bacterial PI-PLCs, which can be isolated in milligram quantities, are important tools in the study of a new class of membrane proteins that are attached to the membrane via a PI-PLC-sensitive glycosyl-PtdIns anchor (Ferguson & Williams, 1988). The PI-PLCs from *B. thuringiensis* (Henner et al., 1988) and *B. cereus* (Kuppe et al., 1989) have recently been cloned and sequenced, and their amino acid sequences are nearly identical. These PI-PLCs show some homology, over a limited section of the polypeptide sequence, with a region of the glycosyl-PtdIns-specific phospholipase C from *Trypanosoma brucei*, recently sequenced by Hereld et al. (1988), and also with a short region conserved among most of the mammalian PI-PLCs, suggesting that this section may contain functionally important residues (Kuppe et al., 1989).

An important step toward understanding the catalytic mechanism of these phospholipases is the study of their mode of action, which includes analysis of the products formed under different experimental conditions. The mode of action of the mammalian PI-PLCs appears to involve formation of cyclic and noncyclic inositol phosphates simultaneously at molar ratios that are dependent on the pH, enzyme subtype, and other conditions (Dawson et al., 1971; Majerus et al., 1986; Kim et al., 1989; Lin et al., 1990). Cleavage of the glycosylphosphatidylinositol membrane anchor of the variable surface glycoprotein (VSG) of *T. brucei* by an endogenous phospholipase C has been reported to result in the formation of a *myo*-inositol cyclic monophosphate (Ferguson et al., 1985). Previous work on bacterial PI-PLCs suggests that cyclic inositol phosphate is the main inositol phosphate product formed upon cleavage of PtdIns (Ikezawa et al., 1976; Taguchi et al., 1980; Lin & Tsai, 1989; Lin et al., 1990), but this has not been examined systematically over a range of experimental conditions.

We report here an analysis of the inositol phosphate products formed upon cleavage of PtdIns by PI-PLC from *B. cereus*. It is shown that this enzyme combines two activities: a phosphotransferase activity, producing 1,2-diacyl-*sn*-glycerol and D-*myo*-inositol 1,2-cyclic monophosphate from phosphatidylinositol, and a cyclic phosphodiesterase activity, producing D-*myo*-inositol 1-monophosphate through hydrolysis of the cyclic phosphate.

Experimental Procedures

Materials. Pure PI-PLC was prepared from the culture supernatant of *B. cereus* as described earlier (Volwerk et al., 1989b). Appropriate dilutions of enzyme were prepared in 0.1% BSA (pH 7.5). Quantities of enzyme are based on the Bradford protein determination (Bradford, 1976) using BSA (fraction V, United States Biochemical Corp.) as the standard. Monoclonal antibodies A72-24 and L26-7, specific for PI-PLC from *B. cereus*, were produced and characterized as will be reported elsewhere (A. Kuppe, K. K. Hedberg, J. J. Volwerk, and O. H. Griffith, unpublished results). Sodium deoxycholate was from Calbiochem. PtdIns (bovine brain) was from Avanti Polar-Lipids Inc. D,L-Ins(1)P, Ins(2)P, D,L-Ins(1:2cyc)P (all as the cyclohexylammonium salts), and Triton X-100 were obtained from Sigma. Butylphosphonic acid was from Aldrich. All other reagents and materials were of the highest grade available.

Preparation of D-*Ins(1:2cyc)P.* D-Ins(1:2cyc)P was prepared through PI-PLC-catalyzed cleavage of PtdIns, which occurs naturally as the D-*myo*-inositol 1-monophosphate derivative. PtdIns (66 mg, 0.075 mmol) was dissolved in 3 mL of 20 mM Tris-HCl (pH 7.5) containing 20 mg/mL sodium deoxycholate. PI-PLC (0.22 μg) was added, and the mixture was incubated at 37 °C. Progress of the reaction was followed by thin-layer chromatography (HETLC-GHL, Analtech) using ether/hexane (1:1 v/v) and chloroform/methanol/water (65:25:4 v/v) as the solvents. After a 5-h incubation the reaction was nearly complete, and the reaction mixture was extracted by adding 3 mL each of methanol and chloroform followed by vigorous stirring. After brief centrifugation, the aqueous layer was collected and reextracted once with chloroform. The mixture was then lyophilized and redissolved in 1 mL of water. This solution was used directly as the stock of D-Ins(1:2cyc)P, containing approximately 75 mM. TLC indicated complete removal of the diacylglycerol, and ^{31}P NMR showed that Ins(1:2cyc)P was the sole phosphorus-containing compound present.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were recorded on a Nicolet NT-360 instrument equipped with a temperature control unit, using 12-mm NMR tubes fitted with a vortex plug. The temperature of the samples (4 mL total volume) was maintained at 35 °C throughout the experiments. Spectra were accumulated without proton decoupling with a 28-μs, 90° pulse, a recycle delay of 2.68 s, and a 3000-Hz sweep width with 8K data points. A line broadening of 2 Hz was used in processing the data. All chemical shift values are with reference to phosphoric acid (85%) as the external standard. All line widths were calculated by assuming a Lorentzian line shape. To obtain progress curves for the PI-PLC-catalyzed conversion of PtdIns or Ins(1:2cyc)P, the percentages substrate [PtdIns or Ins(1:2cyc)P] remaining were calculated by dividing the integrated intensity of the substrate peak by the sum of the integrated intensities of the substrate and product peaks in each spectrum. The systematic error in the estimation of the percentage substrate remaining due to averaging of the spectra over the accumulation period used (see legend to Figure 2) is not greater than 6%.

The action of PI-PLC on PtdIns was studied as follows: PtdIns (20 mg, final concentration 5.65 mM) was dissolved in 4 mL of D_2O/H_2O (1:1 v/v) containing detergent (sodium deoxycholate or Triton X-100, final concentration 12.5 mM) and buffer. In one experiment detergent was omitted and unilamellar vesicles of PtdIns were used, prepared by dialysis from octyl glucoside (Mimms et al., 1981). The buffers used were 50 mM Tris/acetate (pH 5.5, 7.5, or 8.5) and 50 mM sodium borate/HCl (pH 7.5). The reaction was initiated by the addition of 1–10 μL of PI-PLC (0.3–30 ng) in 0.1% BSA (pH 7.5). The mixture was then quickly transferred to an NMR tube and placed in the probe of the instrument. In most cases the reaction mixture was kept in the probe of the NT-360 instrument for the duration of the experiment, while spectra were recorded at various time intervals. Dependent on the rate of conversion of the PtdIns, 300–700 scans were accumulated per time point. In the absence of PI-PLC, there was no detectable conversion of PtdIns under these conditions.

The PI-PLC-catalyzed hydrolysis of Ins(1:2cyc)P was studied by ^{31}P NMR essentially as described above for PtdIns. Samples contained 2–3 mg of Ins(1:2cyc)P and the buffers described above. The reaction was initiated by addition of 20–30 μg of PI–PLC. Generally, 500 scans were accumulated per time point. Ins(1:2cyc)P was completely stable in the absence of PI-PLC under all experimental conditions employed here.

The effect of monoclonal antibodies A72-24 and L26-7 on the hydrolysis of D-Ins(1:2cyc)P was evaluated as follows: PI-PLC (28 μg) was preincubated with 2.4 mL of undiluted

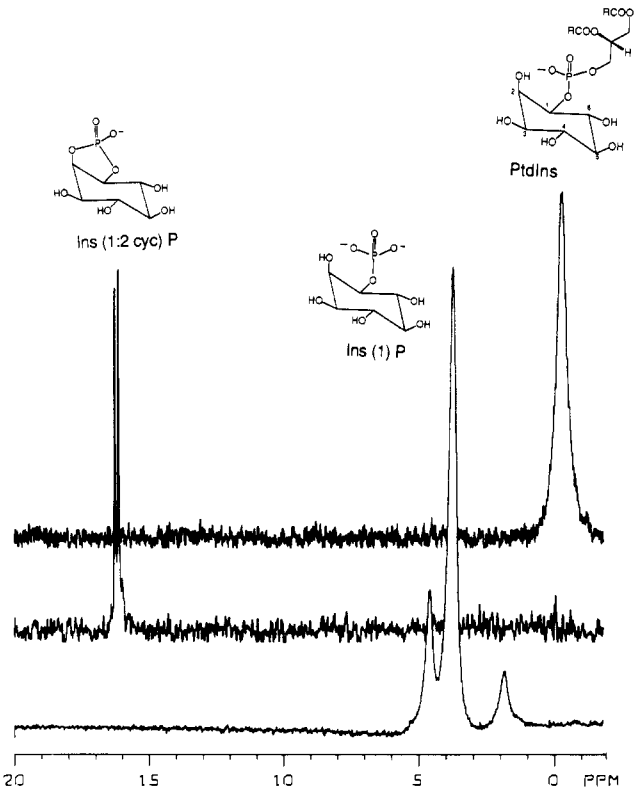


FIGURE 1: ^{31}P NMR spectra (146.18 MHz) of authentic samples of PtdIns (top; −0.411 ppm), D,L-Ins(1:2cyc)P (middle; 16.304 ppm), and D,L-Ins(1)P (bottom; 3.623 ppm). Spectra were recorded at 35 °C under the following conditions: PtdIns, 5.65 mM in 50 mM sodium borate/HCl (pH 7.5) containing 12.5 mM sodium deoxycholate and 50% D_2O (by volume); D,L-Ins(1:2cyc)P, 2.20 mM in 50 mM Tris/acetate (pH 7.5) containing 12.5 mM Triton X-100 and 50% D_2O; D,L-Ins(1)P, 5.47 mM in the same buffer as for D,L-Ins(1:2cyc)P.

hybridoma supernatant and 1.4 mL of 200 mM Tris/acetate (pH 7.5, in D_2O) for 30 min at room temperature. The antibody concentrations in the hybridoma supernatants were estimated at 10–20 μg/mL by using subtype-specific secondary antibodies (A. Kuppe, K. K. Hedberg, J. J. Volwerk, and O. H. Griffith, unpublished results). To initiate the reaction, 0.2 mL of the D-Ins(1:2cyc)P stock solution was added, the mixture was quickly transferred to an NMR tube, and ^{31}P NMR spectra were recorded as a function of time as described above. Samples containing antibodies showed an additional peak in the ^{31}P NMR spectrum at about 0 ppm, probably due to inorganic phosphate present in the hybridoma supernatant.

Polarimetry. Optical rotations were measured on a Perkin-Elmer 141 polarimeter equipped with a thermostated cell (10 cm path length) at 35 °C.

Results

Reference Spectra. ^{31}P NMR spectra of authentic samples of the key inositol derivatives encountered in this study are shown in Figure 1. Analysis of these and other spectra gave the following NMR parameters for these compounds: PtdIns, δ −0.411 ppm (broad); D,L-Ins(1)P, δ 3.623 ppm (d) J_{POCH} = 8 Hz; D,L-Ins(1:2cyc)P, δ 16.304 ppm (d of d) J_{POCH1} = 20 Hz, J_{POCH2} = 3 Hz. These values agree well with those reported earlier for Ins(1)P and Ins(1:2cyc)P at pH 3.5 (Duthu et al., 1988), taking the effect of pH on the chemical shift of Ins(1)P into account (see below).

PtdIns gave a similar broad peak (line width 25–35 Hz) in the presence of sodium deoxycholate or Triton X-100 at the same detergent/lipid molar ratio of about 2. Small changes in the exact chemical shift values of the ^{31}P signals of the individual compounds due to differences in the conditions of the experiment (for example, buffer composition, pH, and presence or absence of detergent) generally did not exceed about 0.2 ppm. Therefore, use of phosphoric acid as the external standard was not rigorously required once the chemical shift values of these compounds had been firmly established. The peak of Ins(1)P was shifted upfield by about 3 ppm at pH 5.5, probably due to a pH effect involving the second ionization of the phosphate group. The commercial sample of D,L-Ins(1)P showed two additional peaks, one upfield and one downfield from the main peak at 3.6 ppm (Figure 1). The downfield peak at 4.45 ppm was identified as Ins(2)P by comparison with an authentic sample (not shown); the upfield peak was not further identified. These results show that PtdIns and the inositol phosphates can easily be distinguished by ^{31}P NMR spectroscopy. Thus, the phosphate-containing products of the PI-PLC-catalyzed cleavage of PtdIns can be identified directly and unambiguously.

Cleavage of PtdIns. The PI-PLC-catalyzed cleavage of PtdIns was examined by ^{31}P NMR under various conditions. An example of the data obtained is given in Figure 2B, where selected ^{31}P NMR spectra of the reaction mixture containing PtdIns solubilized in sodium deoxycholate, recorded at the indicated time intervals following the addition of enzyme, are presented in the form of a series of stacked plots. The complete reaction progress curve for this experiment is presented in Figure 2A. Comparison with the reference spectra in Figure 1 shows that under the conditions of this experiment Ins(1:2cyc)P is the only product detected by ^{31}P NMR, even after prolonged incubations.

During these and similar experiments on the cleavage of PtdIns we noted that during the reaction the initially clear solution became turbid due to phase separation and formation of a microemulsion by the diacylglycerol produced. We therefore examined the possibility that part of the remaining PtdIns was solubilized in the microemulsion and not detected by ^{31}P NMR due to exceptional broadening of the signal. This would not affect the main conclusions regarding the identity of the products formed but could alter the apparent shape of the progress curve. To test this possibility, a solution of PtdIns in deoxycholate-containing buffer, as used in the experiment of Figure 2, and a stable emulsion of an equimolar amount 1,2-dioleoylglycerol in the same buffer, prepared by bath sonication, were mixed and equilibrated in different proportions corresponding to 25, 57, and 70% cleavage of the PtdIns, respectively. The PtdIns solution contained in addition an approximately equimolar amount of water-soluble butylphosphonic acid (sodium salt) which served as an internal standard. ^{31}P NMR spectra of the mixtures were then recorded, and the ratios of the integrals of the PtdIns and butylphosphonate signals and the line widths of the PtdIns peaks were measured. The ratios of the integrals were constant within the experimental error. There was a very slight broadening of the PtdIns line width from 25 ± 2 Hz in the absence of diacylglycerol to 29 ± 2 Hz at 57 and 70% cleavage of PtdIns. These results indicate that there is no problem of loss of the PtdIns signal due to solubilization in the diacylglycerol emulsion. This was further confirmed by measuring the line widths of the PtdIns and Ins(1:2cyc)P signals in all spectra collected for the experiment shown in Figure 2. Here the line widths were found to be approximately constant at 31 ± 5 Hz for the PtdIns peak and 3.1 ± 0.5 Hz for each peak of the Ins(1:2cyc)P doublet. These data, together with the data in Figure 2, show that under these conditions cleavage of PtdIns proceeds essentially to completion with Ins(1:2cyc)P

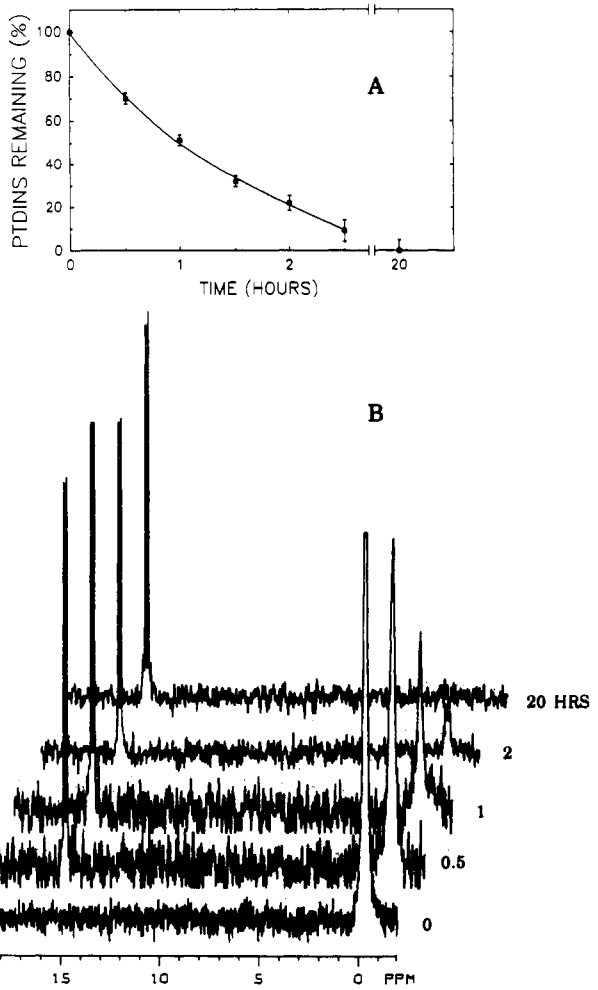


FIGURE 2: (A) Reaction progress curve and (B) stacked plot of selected 146.18-MHz ^{31}P NMR spectra for the cleavage of PtdIns by PI-PLC from *B. cereus*. Spectra were recorded at various time intervals following addition of enzyme, and the precentage of substrate remaining was estimated from the peak areas obtained by integration. The numbers to the right in (B) indicate the times (hours) at which each accumulation period of 300 scans (about 13 min) ended. Each spectrum is offset to the right by 1.37 ppm with respect to the previous one; the x axis scale applies directly to the $t = 0$ spectrum only. To obtain optimal clarity in this representation and because of limitations in the plotting routine, the peak for PtdIns in the $t = 0$ spectrum in the stacked plot (B) appears truncated. The sample contained 5.65 mM PtdIns and 7 ng/mL PI-PLC in 50 mM sodium borate/HCl (pH 7.5), 12.5 mM sodium deoxycholate, and 50% D_2O (by volume).

being the sole inositol phosphate product formed.

In the experiment shown in Figure 2, the reaction proceeded at pH 7.5 using sodium borate/HCl buffer in the presence of sodium deoxycholate and an enzyme concentration of 7.5 ng/mL. These conditions are similar to those used in the standard activity assay of the phospholipase (Ikezawa & Taguchi, 1981; Volwerk et al., 1989b). However, the same results, i.e., Ins(1:2cyc)P being the only inositol phosphate detected by ^{31}P NMR, were obtained under a variety of other conditions. For example, when the PI-PLC concentration was reduced 100-fold, the reaction proceeded very slowly and took several days to approach completion. Even after these long incubations Ins(1:2cyc)P was the only product visible by ^{31}P NMR. In another series of experiments Triton X-100 rather than deoxycholate was used to solubilize the PtdIns, permitting experiments over a wide range of pH, while avoiding precipitation of deoxycholic acid at acidic pH. In the presence of Triton X-100 experiments were done at pH 5.5, 7.5, and 8.5 with Tris/acetate buffers and with PI-PLC concentrations varying from 0.075 to 7.5 ng/mL. In the absence of detergents, when unilamellar vesicles prepared by dialysis from octyl glucoside were used, cleavage of PtdIns was very slow, proceeding to only about 5% completion in 24 h. Under all these conditions, PI-PLC-catalyzed cleavage of PtdIns proceeded with formation of Ins(1:2cyc)P exclusively.

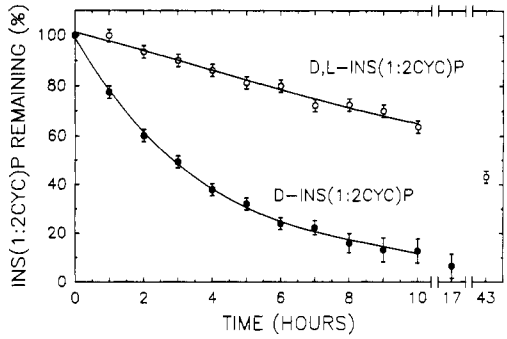


FIGURE 3: Reaction progress curves for the hydrolysis of D- and D,L-Ins(1:2cyc)P catalyzed by PI-PLC from *B. cereus*. The percentages substrate remaining were estimated from the integrated ^{31}P NMR spectra. The samples contained 2.2 mM Ins(1:2cyc)P, 37.5 mM sodium deoxycholate, and 7 μg/mL PI-PLC in 50 mM Tris/acetate (pH 7.5) and 50% D_2O (by volume), except that detergent was absent in the D,L-Ins(1:2cyc)P sample.

Hydrolysis of Ins(1:2cyc)P. In all experiments described above, the product, Ins(1:2cyc)P, formed upon PI-PLC-catalyzed cleavage of PtdIns, was stable and did not undergo further conversion, even when incubations were continued long after all PtdIns had disappeared. However, at that point, addition of more enzyme to concentrations of 5–10 μg/mL initiated hydrolysis of Ins(1:2cyc)P to Ins(1)P, which was readily observed by ^{31}P NMR. Similarly, addition of this much enzyme to a sample of PtdIns in detergent resulted in very rapid conversion of the PtdIns to diacylglycerol and Ins(1:2cyc)P and subsequent hydrolysis of the latter to Ins(1)P at a much slower rate.

The PI-PLC-catalyzed hydrolysis of Ins(1:2cyc)P was further studied by using commercially obtained D,L-Ins-(1:2cyc)P and by using D-Ins(1:2cyc)P prepared from PtdIns as described under Experimental Procedures. ^{31}P NMR spectra of reaction mixtures containing Ins(1:2cyc)P and enzyme in the presence or absence of detergent were recorded as a function of time (data not shown). The line widths of the ^{31}P signals remained constant in these experiments at approximately 3.4 Hz for each peak of the doublet of D-Ins-(1:2cyc)P, and at 23 Hz for the unresolved doublet of D-Ins(1)P. Comparison of these NMR spectra with the reference spectra in Figure 1 showed that conversion of the cyclic phosphate occurred with concomitant formation of Ins(1)P exclusively. In none of these and subsequent experiments in which enzymatic hydrolysis of Ins(1:2cyc)P was studied (see below) did we observe formation of the 2-isomer, Ins(2)P, within the limits of detection (<5%). However, acid hydrolysis (1 M HCl, 90 °C, 10 min) of the cyclic phosphate produced a mixture of Ins(1)P and Ins(2)P (not shown) in which the latter, minor component was readily detected by ^{31}P NMR. Thus, the hydrolysis of Ins(1:2cyc)P by PI-PLC from *B. cereus* is regiospecific in that only Ins(1)P is formed.

Reaction progress curves for the hydrolysis of D-Ins(1:2cyc)P (filled circles) and for the hydrolysis of D,L-Ins(1:2cyc)P (open circles) are shown in Figure 3. Clearly, the hydrolysis of the racemate proceeds much slower than that of the D-enantiomer under otherwise similar conditions. Furthermore, the progress curve for the D,L mixture levels off at approximately 50% hydrolysis. This was observed in all experiments involving the D,L-cyclic phosphate and suggests that the PI-PLC-catalyzed

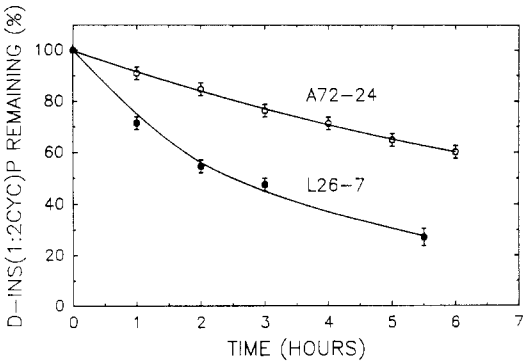


FIGURE 4: Reaction progress curves for the PI-PLC-catalyzed hydrolysis of D-Ins(1:2cyc)P in the presence of the inhibitory monoclonal antibody, A72-24, and the noninhibitory monoclonal antibody, L26-7. The percentages substrate remaining were estimated from the integrated ^{31}P NMR spectra. Experimental details of these experiments are described under Experimental Procedures.

hydrolysis of Ins(1:2cyc)P is stereospecific with the L-enantiomer hydrolyzed much slower than the D-enantiomer or not at all. The much slower rate of hydrolysis of the D-enantiomer in the presence of the L-enantiomer suggests that the latter may act as an inhibitor.

Further evidence for the stereospecific hydrolysis of Ins(1:2cyc)P was obtained by polarimetry. A solution (1.1 mL) of D,L-Ins(1:2cyc)P (9.1 mg/mL) in 50 mM Tris/acetate (pH 7.5) was incubated with 56 μg of PI-PLC at 35 °C in the cell of the polarimeter, and the change in the optical rotation was followed as a function of time. The optical rotation changed with time from the initial value of zero and reached a plateau of $+0.12 \pm 0.01°$ after a 4-h incubation. This value is close to that expected (+0.104°) for an equimolar mixture of L-Ins(1:2cyc)P ($[\alpha]^{23}_D = +17°$, $c = 1$, H_2O) (Watanabe et al., 1988) and D-Ins(1)P ($[\alpha]^{23}_D = +3.8°$, $c = 3.1$, H_2O, pH = 9) (Molotkovsky & Bergelson, 1971) resulting from the stereospecific hydrolysis of D,L-Ins(1:2cyc)P. A similar sample without added PI-PLC served as a control and showed no change in optical rotation.

The hydrolysis of Ins(1:2cyc)P was further examined by ^{31}P NMR under varying conditions. The presence of diacylglycerol and/or detergents was not required and did not significantly affect the conversion of the cyclic inositol phosphate into Ins(1)P. The reaction was observed at pH 5.5, 7.5, and 8.5. At pH 7.5 the rate of hydrolysis of D- or D,L-Ins(1:2cyc)P was unaffected by the presence of 1 or 10 μM $CaCl_2$ or 10 mM EDTA in the reaction medium, indicating that the reaction does not depend on the presence of divalent cations (use of higher Ca^{2+} concentrations was complicated by some precipitation in the samples). Under all these conditions, hydrolysis of the D,L-Ins(1:2cyc)P leveled off at 50–60%, while hydrolysis of the D-enantiomer was essentially complete. Furthermore, Ins(1)P was the only product detected by NMR in all of these experiments.

The question whether the observed hydrolysis of Ins(1:2cyc)P was due to an intrinsic cyclic phosphodiesterase activity of the *B. cereus* PI-PLC or caused by a contaminating activity in the preparation was addressed by using monoclonal antibodies specific for the enzyme. One of these monoclonals, A72-24, strongly inhibited the PI-PLC activity in the PtdIns cleavage assay (A. Kuppe, K. K. Hedberg, J. J. Volwerk, and O. H. Griffith, unpublished results). Another monoclonal, L26-7, had no inhibitory effect and was used here as a control. Figure 4 shows the reaction progress curves for the hydrolysis of D-Ins(1:2cyc)P in the presence of the two monoclonal antibodies. In the presence of L26-7, the reaction proceeded with a rate similar to that observed without added antibody under otherwise similar conditions (approximately 50% hydrolysis in 3 h; see also Figure 3), while the rate of hydrolysis was much slower in the presence of A72-24. The effects of these highly specific monoclonal antibodies indicate that the PI-PLC from *B. cereus* is responsible for the hydrolysis of the cyclic inositol phosphate.

Discussion

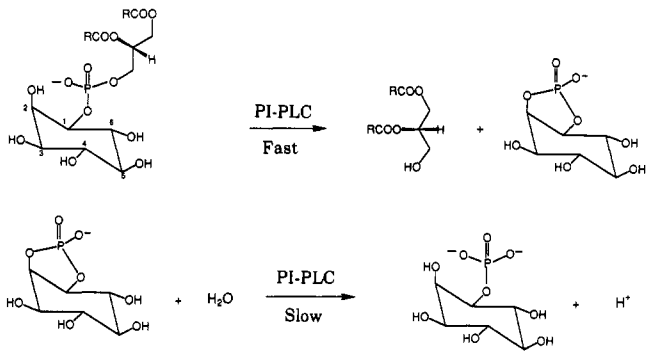
The present study examines the inositol phosphate product(s) formed upon cleavage of PtdIns by the phosphatidylinositol-specific phospholipase C (PI-PLC) from *B. cereus* using ^{31}P NMR. It is generally thought that cleavage of PtdIns by bacterial PI-PLCs produces *sn*-1,2-diacylglycerol and cyclic inositol phosphate, but there are some conflicting reports in the literature. Early observations by Ikezawa et al. (1976) have indicated that cleavage of PtdIns by the *B. cereus* PI-PLC produces 1,2-diacylglycerol and a mixture of Ins(1)P and Ins(1:2cyc)P. However, in a subsequent study performed under similar conditions with the PI-PLC from *B. thuringiensis* (Taguchi et al., 1980), only the cyclic phosphate product was identified. This difference cannot be explained by a difference in enzyme source, since it is now known that the PI-PLCs from *B. cereus* and *B. thuringiensis* are almost identical in their amino acid sequences (Henner et al., 1988; Kuppe et al., 1989) and are functionally indistinguishable (Volwerk et al., 1989a). In more recent studies Lin and Tsai (1989) and Lin et al. (1990) investigated the stereochemistry of the PI-PLCs from *B. cereus* and guinea pig uterus using synthetic 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoinositol. For both enzymes it was found that the steric course of formation of Ins(1:2cyc)P involves inversion at the phosphorus. However, under their conditions formation of noncyclic inositol phosphates by the *B. cereus* PI-PLC was not observed.

For analysis of the mode of action of the *B. cereus* PI-PLC as a function of pH and other experimental conditions, we selected NMR spectroscopy because it permits quantitative analysis of the reaction mixtures as a function of time without requiring additional sample manipulations such as extraction and product separation, which could introduce artifacts. For example, inositol cyclic phosphate is acid labile and could hydrolyze to the noncyclic form during extraction under the strongly acidic conditions usually employed during organic solvent extractions in enzyme assays of PI-PLC.

The main results obtained here can be summarized as follows: (1) ^{31}P NMR is an excellent tool for identifying inositol phosphates in reaction mixtures and time-course experiments such as those reported here; (2) at enzyme concentrations in the ng/mL range the PI-PLC from *B. cereus* cleaves PtdIns, generating *sn*-1,2-diacylglycerol and D-Ins(1:2cyc)P; (3) at 1000-fold higher enzyme concentrations the same products are formed initially, but the cyclic inositol phosphate is further hydrolyzed to D-Ins(1)P; (4) the PI-PLC-catalyzed hydrolysis of Ins(1:2cyc)P occurs in a regio- as well as stereospecific fashion and is not affected by added Ca^{2+} ions or EDTA; (5) the hydrolysis of inositol cyclic phosphate is an intrinsic activity of the PI-PLC since it is inhibited by a monoclonal antibody specific for the *B. cereus* enzyme which is known to strongly inhibit the PI-PLC-catalyzed cleavage of PtdIns (A. Kuppe, K. K. Hedberg, J. J. Volwerk, and O. H. Griffith, unpublished results).

The hydrolysis of D-Ins(1:2cyc)P occurs with an apparent rate that is about 1000-fold slower than the cleavage of PtdIns. Enzyme concentrations in the μg/mL range rather than ng/mL are required for detection by ^{31}P NMR under otherwise similar conditions. From the known specific activity of

Scheme I



the *B. cereus* PI-PLC for cleavage of PtdIns [approximately 1500 μmol/(min·mg) at pH 7.5 in the presence of deoxycholate (Volwerk et al., 1989b)] the specific activity for hydrolysis of D-Ins(1:2cyc)P can therefore be estimated to be on the order of 1–2 μmol/(min·mg). A similar specific activity can be estimated from the initial slope of the progress curve for D-Ins(1:2cyc)P in Figure 3. This activity may be significantly higher under optimal conditions, e.g., saturating concentrations of the cyclic phosphate. By comparison, the specific activities of purified mammalian PI-PLCs range from <1 to 30 μmol/(min·mg) depending on the tissue source, the phosphoinositide used as substrate, and the calcium concentration (Crooke & Bennett, 1989). The turnover of the *B. cereus* PI-PLC hydrolyzing inositol cyclic phosphate, though much lower than its turnover when cleaving PtdIns, is therefore comparable to that of the mammalian PI-PLCs cleaving their phosphoinositide substrate(s).

On the basis of the results described here we propose that hydrolysis of PtdIns to diacylglycerol and Ins(1)P by the PI-PLC from *B. cereus* proceeds via a two-step mechanism, as summarized in Scheme I. The first step produces *sn*-1,2-diacylglycerol and D-Ins(1:2cyc)P, with the enzyme acting as a phosphotransferase promoting a direct intramolecular attack of the 2-hydroxyl of the inositol ring on the phosphate group. In the second step the enzyme acts as a regio- and stereospecific cyclic phosphodiesterase promoting nucleophilic attack of a water molecule (or hydroxyl ion) on the cyclic phosphate group and producing D-Ins(1)P. The initial cleavage of PtdIns by the phospholipase thus proceeds via an *intra*molecular phosphotransfer reaction involving the *cis*-hydroxyl in the 2-position of the inositol ring, whereas the subsequent conversion of the cyclic phosphate proceeds via an *inter*molecular hydrolysis reaction. The close proximity of the 2-hydroxyl participating in the phosphotransfer reaction may help explain the substantial difference in the apparent rates of PtdIns cleavage and Ins(1:2cyc)P hydrolysis we observe. Large rate enhancements due to participation of neighboring groups (proximity effect) in intramolecular reactions of model compounds and in enzyme catalysis are well documented (Jencks, 1969; Bruice & Benkovic, 1966a), including those involving formation of cyclic phosphodiesters (Bruice & Benkovic, 1966b). However, additional factors may play a role. For example, cleavage of PtdIns takes place at the lipid–water interface while hydrolysis of the water-soluble Ins(1:2cyc)P occurs in solution. The phenomenon of interfacial activation, i.e., the enhanced rates of conversion of substrates organized at the lipid–water interface generally displayed by lipolytic enzymes (Verger & De Haas, 1976), thus may contribute to the relatively fast rate of PtdIns cleavage by the *B. cereus* phospholipase.

A well-known example of an enzyme cleaving its substrate by a two-step mechanism is the hydrolysis of the phosphodiester bonds of ribonucleic acids catalyzed by pancreatic ribonuclease, RNase A (Walsh, 1979; Hammes, 1982). In the first step, cleavage of the RNA chain by the RNase occurs via an intramolecular phosphotransfer reaction involving the 2′-hydroxyl of the ribose ring with formation of a pyrimidine 2′,3′-cyclic phosphate intermediate. In the second step, the cyclic intermediate is hydrolyzed to the 3′-phosphate final product. RNase thus catalyzes two reactions, a phosphotransfer and a hydrolysis reaction, presumably using the same catalytic residues in the active site. Interestingly, comparison of the rate constants for the RNase-catalyzed phosphotransfer reaction of cytidine or uridine dinucleotides with the rate constants for the hydrolysis of the corresponding cyclic phosphates shows differences of up to 3 orders of magnitude, depending on the nature of the leaving alcohol (Witzel, 1963). The similar pathways of phosphodiester cleavage by RNase and the *B. cereus* PI-PLC suggest that the catalytic mechanisms of these enzymes may have elements in common.

Cyclic phosphodiesterase activity has not been previously described for any of the prokaryotic or eukaryotic PI-PLCs studied so far. It is therefore of interest to compare the mode of action of the *B. cereus* PI-PLC with that of other phospholipases C with similar specificities. One related group of enzymes are the phospholipases C involved in the cleavage of glycosyl-PtdIns moieties such as those found in the membrane anchor of the variant surface glycoprotein (VSG) of *T. brucei* and other proteins (Ferguson & Williams, 1988). Structural analysis of the VSG anchor suggests that cleavage by the endogenous phospholipase C can result in the formation of a *myo*-inositol cyclic monophosphate (Ferguson et al., 1985), but it is not known whether this enzyme displays cyclic phosphodiesterase activity.

A second group of interesting PI-PLCs are the enzymes derived from mammalian cells involved in the phosphoinositide-dependent pathway of transmembrane signal transduction (Rhee et al., 1989). Early observations by Dawson et al. (1971) have been confirmed more recently (Wilson et al., 1985; Majerus et al., 1986, 1988; Kim et al., 1989; Lin et al., 1990) and indicate that cleavage of phosphoinositides by mammalian PI-PLCs produces cyclic and noncyclic inositol (poly)phosphates simultaneously in pH-dependent ratios, with the ratio of cyclic/noncyclic products decreasing at higher pH values. A straightforward interpretation of these observations is that two competing reactions occur simultaneously in these enzymes: nucleophilic attack of the 2-hydroxyl of the inositol group on the phosphorus producing cyclic inositol phosphates or nucleophilic attack of hydroxyl ions from the medium producing noncyclic products in a one-step hydrolysis (Majerus et al., 1988; Lin et al., 1990). Under physiological conditions the latter would be predominant in accordance with the role of noncyclic inositol trisphosphate as an intracellular secondary messenger (Berridge & Irvine, 1989). PI-PLC-catalyzed hydrolysis of the cyclic phosphates formed has not been observed in these systems. However, the possibility that the hydrolysis of phosphoinositides by the mammalian PI-PLCs occurs in two steps involving transient cyclic inositol phosphate intermediates presently cannot be excluded and deserves further study.

It will be interesting to see what structural differences between the bacterial and mammalian enzymes correlate with this apparent difference in mechanism of action. If the mammalian and microbial PI-PLCs are evolutionarily related, the former must have evolved into a form that allows the one-step hydrolysis of phosphoinositides to compete more effectively with the energetically more favorable (Bruice &

Benkovic, 1966a,b; Jencks, 1969) intramolecular phosphotransfer reaction or, alternatively, into a form that is capable of more efficient hydrolysis of the cyclic phosphate intermediates. This may be related to the dependence of the mammalian PI-PLCs on Ca^{2+} ions for activity. The bacterial enzymes do not require metal ions for cleavage of PtdIns (Ikezawa & Taguchi, 1981; Low, 1981), and as shown here, the cyclic phosphodiesterase activity of the *B. cereus* PI-PLC also is independent of Ca^{2+}. Finally, we note that the stereospecific hydrolysis of Ins(1:2cyc)P by the *B. cereus* PI-PLC may prove useful in the enantiospecific synthesis of inositol derivatives.

ACKNOWLEDGMENTS

We are grateful to Drs. Charles E. Klopfenstein and Sandip K. Sur for help and expert advice regarding use of the Nicolet NT-360 instrument, Dr. Karen K. Hedberg for help and expert advice regarding the production of monoclonal antibodies, John A. Koke for continued supply of pure phospholipase C, and Drs. H. Stewart Hendrickson and John F. W. Keana for stimulating discussions.

Registry No. PI-PLC, 63551-76-8; D-Ins(1:2cyc)P, 43119-57-9; D-Ins(1)P, 15421-51-9; phosphotransferase, 9031-09-8; inositol cyclic 1,2-phosphate 2-phosphodiesterase, 9076-91-9.

REFERENCES

Berridge, M. J. (1986) in *Receptor Biochemistry and Methodology* (Putney, J. W., Ed.) Vol. 7, pp 25–47, Liss, New York.

Berridge, M. J., & Irvine, R. F. (1989) *Nature 341*, 197–205.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bruice, T. C., & Benkovic, S. J. (1966a) *Bioorganic Mechanisms*, Vol. 1, pp 119–195, W. A. Benjamin, New York.

Bruice, T. C., & Benkovic, S. J. (1966b) *Bioorganic Mechanisms*, Vol. 2, pp 35–55, W. A. Benjamin, New York.

Crooke, S. T., & Bennett, C. F. (1989) *Cell Calcium 10*, 309–323.

Dawson, R. M. C., Freinkel, N., Jungalwala, F. B., & Clarke, N. (1971) *Biochem. J. 122*, 605–607.

Duthu, B., Houalla, D., & Wolf, R. (1988) *Can. J. Chem. 66*, 2965–2974.

Ferguson, M. A. J., & Williams, A. F. (1988) *Annu. Rev. Biochem. 57*, 285–320.

Ferguson, M. A. J., Low, M. G., & Cross, G. A. M. (1985) *J. Biol. Chem. 260*, 14547–14555.

Hammes, G. G. (1982) *Enzyme Catalysis and Regulation*, pp 110–121, Academic Press, New York.

Henner, D. J., Yang, M., Chen, E., Hellmiss, R., Rodriguez, H., & Low, M. G. (1988) *Nucleic Acids Res. 16*, 10383.

Hereld, D., Hart, G. W., & Englund, P. T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8914–8918.

Ikezawa, H., & Taguchi, R. (1981) *Methods Enzymol. 71*, 731–741.

Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T., & Ohyabu, T. (1976) *Biochim. Biophys. Acta 450*, 154–164.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, Chapter 1, pp 7–40, McGraw-Hill, New York.

Kim, J. W., Ryu, S. H., & Rhee, S. G. (1989) *Biochem. Biophys. Res. Commun. 163*, 177–182.

Kuppe, A., Evans, L. M., McMillen, D. A., & Griffith, O. H. (1989) *J. Bacteriol. 171*, 6077–6083.

Lin, G., & Tsai, M.-D. (1989) *J. Am. Chem. Soc. 111*, 3099–3101.

Lin, G., Bennett, C. F., & Tsai, M.-D. (1990) *Biochemistry 29*, 2747–2757.

Low, M. G. (1981) *Methods Enzymol. 71*, 741–746.

Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bansal, V. S., & Wilson, D. B. (1986) *Science 234*, 1519–1526.

Majerus, P. W., Connolly, T. M., Bansal, V. S., Inhorn, R. C., Ross, T. S., & Lips, D. L. (1988) *J. Biol. Chem. 263*, 3051–3054.

Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. A. (1981) *Biochemistry 20*, 833–840.

Molotkovsky, J. G., & Bergelson, L. D. (1971) *Tetrahedron Lett. 50*, 4791–4794.

Rhee, S. G., Suh, P.-G., Ryu, S.-H., & Lee, S. Y. (1989) *Science 244*, 546–550.

Taguchi, R., & Ikezawa, H. (1978) *Arch. Biochem. Biophys. 186*, 196–201.

Taguchi, R., Asahi, Y., & Ikezawa, H. (1980) *Biochim. Biophys. Acta 619*, 48–57.

Verger, R., & De Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.

Volwerk, J. J., Koke, J. A., Wetherwax, P. B., & Griffith, O. H. (1989a) *FEMS Microbiol. Lett. 61*, 237–242.

Volwerk, J. J., Wetherwax, P. B., Evans, L. M., Kuppe, A., & Griffith, O. H. (1989b) *J. Cell. Biochem. 39*, 315–325.

Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, pp 199–207, W. H. Freeman & Co., New York.

Watanabe, Y., Ogasawara, T., Nakahira, H., Matsuki, T., & Ozaki, S. (1988) *Tetrahedron Lett. 29*, 5259–5262.

Wilson, D. B., Connolly, T. M., Bross, T. E., Majerus, P. W., Sherman, W. R., Tyler, A. N., Rubin, L. J., & Brown, J. E. (1985) *J. Biol. Chem. 260*, 13496–13501.

Witzel, H. (1963) in *Progress in Nucleic Acid Research* (Davidson, J. N., & Cohn, W. E., Eds.) Vol. 2, pp 221–256, Academic Press, New York.