Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9184–9187, October 1991
Biochemistry

Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol 4-phosphate

(chemical synthesis/cell signaling)

JAMES P. WALSH, KEVIN K. CALDWELL, AND PHILIP W. MAJERUS*

Washington University School of Medicine, Division of Hematology-Oncology, 660 South Euclid Avenue, Box 8125, St. Louis, MO 63110-1093

Contributed by Philip W. Majerus, July 29, 1991

ABSTRACT **We have synthesized phosphatidylinositol 3-phosphate from phosphatidylinositol 4-phosphate by using diisopropylcarbodiimide to promote migration of the 4-phosphate via a cyclic phosphodiester intermediate. The product was isolated by a thin-layer chromatographic method that depends on the ability of phosphatidylinositol 4-phosphate, but not phosphatidylinositol 3-phosphate, to form complexes with boric acid. The final yield of the procedure was 8% phosphatidylinositol 3-phosphate, which was ≈80% pure. The product was shown to be phosphatidylinositol 3-phosphate by the following criteria: (*i*) cochromatography with an authentic standard on borate thin-layer chromatography, (*ii*) cochromatography of the deacylated product with glycerophosphoinositol 3-phosphate on high-performance liquid chromatography, (*iii*) conversion of the product to phosphatidylinositol by homogeneous phosphatidylinositol 3-phosphate 3-phosphatase, and (*iv*) deacylation and deglyceration of the product to a compound that comigrates with inositol 1,3-bisphosphate on high-performance liquid chromatography. The availability of mass amounts of phosphatidylinositol 3-phosphate will allow further elaboration of reactions in this recently discovered pathway of phosphatidylinositol metabolism.**

Phosphatidylinositols containing phosphate esters in the 3-position of inositol represent a recently discovered pathway of phosphatidylinositol metabolism (1). These include phosphatidylinositol 3-phosphate (PtdIns3*P*), phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. The latter two compounds are formed transiently in cells in response to agonists and growth factors (2). PtdIns3*P* is present in cells under all conditions and serves as a precursor for the polyphosphorylated 3-phosphate-containing phosphatidylinositols (3). The metabolism of these lipids has been difficult to study since only trace radiolabeled compounds are available. In cells labeled with [^{3}H]inositol, PtdIns3*P* is labeled to <1% the extent of phosphatidylinositol 4-phosphate (PtdIns4*P*) (4). Whether this label incorporation reflects the level of this compound remains to be determined. Phosphatidylinositol 3-kinases (5, 6) and PtdIns3*P* 3-phosphatases (7) have been identified and isolated, but full characterization of these enzymes and definition of the further metabolism of PtdIns3*P* await the availability of PtdIns3*P* as a reagent. We now report a method for synthesis of PtdIns3*P* by chemical isomerization of PtdIns4*P*. The method employs a carbodiimide to promote migration of phosphate from the 4-position of inositol via a cyclic phosphodiester intermediate. In addition, we report a method for separation of PtdIns3*P* from PtdIns4*P*.

EXPERIMENTAL PROCEDURES

Materials. Dioleoylphosphatidylglycerol, dioleoylphosphatidylcholine, dioleoylphosphatidylserine, and soybean phosphatidylinositol were from Avanti Polar Lipids. Bovine PtdIns4*P* and all other lipids used were from Sigma. [2-^{3}H]PtdIns4*P* was from New England Nuclear. [γ-^{32}P]ATP was from Amersham. Diisopropylcarbodiimide, diisopropylethylamine, and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were from Aldrich. Technical grade ethoxyquin used for TLC was from Sigma, whereas purified ethoxyquin used in the PtdIns3*P* synthesis was from Crescent Chemical (Hauppauge, NY). Silica gel 60 plates (0.25 mm thick, without fluorescent indicator) were from Merck. Solvents were from Fisher. Fatty acid and globulin-free bovine albumin and *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) were from Sigma. Silicic acid (Bio-Sil A, 100–200 mesh) was from Bio-Rad. PtdIns3*P* 3-phosphatase was purified from rat brains as described (7). PtdIns[^{32}P]3*P* was prepared by phosphorylation of soybean phosphatidylinositol with anti-platelet-derived growth factor receptor immunoprecipitates with [γ-^{32}P]ATP as described (7). PtdIns-[^{32}P]4*P* was a gift of Linda Pike (Department of Biochemistry and Molecular Biophysics, Washington University, St. Louis). All other materials used in this work were obtained as described (7).

TLC of Phosphatidylinositol Phosphates. Phosphatidylinositol phosphates were separated in the presence of boric acid as follows. Silica gel 60 plates (5 × 20 cm) were immersed face up for 10 sec with gentle swirling in CDTA solution. This solution was prepared by stirring a mixture of 4.55 g of disodium CDTA·H_2O, 165 ml of H_2O, 330 ml of ethanol, and 3.0 ml of 10 M NaOH until the CDTA was dissolved. The plates were allowed to air-dry by standing up for 1 hr and then baked at 100°C for 10 min. Lipids were spotted 1.7 cm from the bottom of the plate. The immersion depth in the developing solution was 0.7 cm. The TLC developing solution was prepared by stirring together methanol (75 ml), $CHCl_3$ (60 ml), pyridine (45 ml), and boric acid (12 g) until the boric acid was dissolved. Water (7.5 ml), 88% (vol/vol) formic acid (3.0 ml), BHT (0.375 g), and technical grade ethoxyquin (75 μl) were then added. The plates were developed in a saturated tank and were run to the top (≈3 hr). PtdIns4*P* migrated with an R_f of 0.46, whereas the R_f of PtdIns3*P* was 0.51 (Fig. 1). Two secondary fronts were observed with R_f values of 0.66 (boric acid) and 0.22 (formate). The relative migrations of several other lipids in this system are given in Table 1.

Synthesis of PtdIns3*P*. PtdIns4*P* (5 mg, 5.0 μmol) was dissolved in 2.5 ml of $CHCl_3$/methanol, 3:2 (vol/vol). Addition of 5 μl of 1.0 M HCl was required to achieve solution. Five microcuries (1 Ci = 37 GBq) of [^{3}H]PtdIns4*P* and 5 μl of ethoxyquin (purified grade) were then added. This mixture was passed over a 0.5-ml column of pyridinium Dowex 50W-X8 that was equilibrated with CH_2Cl_2/methanol/

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PtdIns3*P*, phosphatidylinositol 3-phosphate; PtdIns4*P*, phosphatidylinositol 4-phosphate; BHT, 2,6-di-*tert*-butyl-4-methylphenol; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid.

*To whom reprint requests should be addressed.

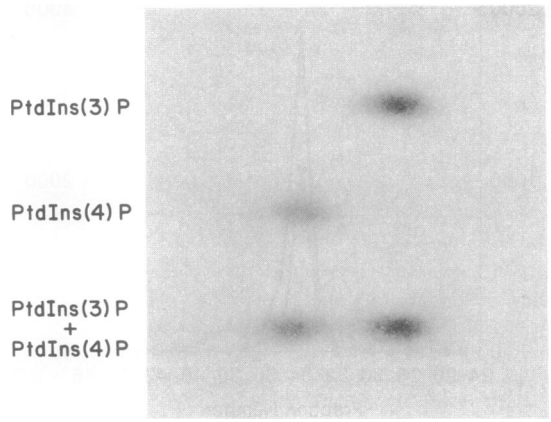

FIG. 1. Separation of phosphatidylinositol monophosphates by borate TLC. PtdIns[^{32}P]3*P* and PtdIns[^{32}P]4*P* (500 dpm of each) were spotted onto CDTA-treated TLC plates as indicated and developed in borate solution. Detection was by autoradiography at −70°C for 18 hr. The direction of chromatography is from left to right.

pyridine, 5:4:1 (vol/vol) and eluted with 4 ml of the same solvent. The solvents were removed on a rotary evaporator. Traces of solvents were removed by evaporation of an additional 5 ml of pyridine. The syrupy residue was then dissolved in 2.5 ml of pyridine containing 5.0 mM diisopropylethylamine. The reaction was initiated by addition of 12.5 μmol of *N,N'*-diisopropylcarbodiimide (62.5 μl of a freshly prepared 200 mM solution in tetrahydrofuran) (9). The reaction vessel was capped with a rubber septum, purged with argon, and stirred magnetically in the dark at room temperature for 2 days. At the end of this period, the pyridine was removed by rotary evaporation, and the residue was dissolved in 5 ml of 0.1 M HCl in tetrahydrofuran/water, 9:1 (vol/vol). This solution was allowed to stand at room temperature for 1 hr to hydrolyze the cyclic phosphates and any phosphorylureas (10, 11). The solution was then concentrated to ≈50 μl by rotary evaporation and dissolved in 1.0 ml of $CHCl_3$. The $CHCl_3$ solution was extracted twice with 1.0 ml of 1.0 M HCl in methanol/water, 1:1 (vol/vol). The volume of the final $CHCl_3$ solution was 1.1 ml. Recovery of tritium was 93%. A sample of this material was saved for analysis, and the remainder was used to purify PtdIns3*P* as described below.

Table 1. TLC migration of several lipids in the presence of boric acid

Lipid	R_f
Phosphatidylinositol	0.82
PtdIns4*P*	0.46
PtdIns3*P*	0.51
Phosphatidylinositol 4,5-bisphosphate	0.22*
Lysophosphatidylinositol	0.66*
Phosphatidylserine	0.75
Lysophosphatidylserine	0.65
Lysophosphatidic acid	0.69
Phosphatidylglycerol	0.95
Lysophosphatidylglycerol	0.79
Phosphatidylcholine	0.66*
Lysophosphatidylcholine	0.48
Lysophosphatidylethanolamine	0.66*

Twenty micrograms of each lipid was spotted onto a CDTA-treated silica gel 60 plate and developed in borate solution as described in *Experimental Procedures*. Detection was with phosphate stain (8). PtdIns3*P* was detected separately by autoradiography of a ^{32}P-labeled standard.

*These lipids comigrated with one of the secondary solvent fronts (borate, R_f 0.66; or formate, R_f 0.22) and were poorly resolved.

Purification of PtdIns3*P*. Samples (60 μl) of the product phosphatidylinositol phosphates were streaked onto 5 × 20-cm CDTA-treated silica plates. Standards of ^{32}P-labeled PtdIns3*P* and PtdIns4*P* were alternately spotted at 1-cm intervals. The plates were developed in borate solution as described above and dried under N_2. The migration of the PtdIns[^{32}P]3*P* and PtdIns[^{32}P]4*P* was determined by autoradiography at −70°C. The silica gel containing the [^{3}H]-PtdIns3*P* was scraped into a glass centrifuge tube and pulverized with a glass rod. The lower third of the [^{3}H]PtdIns3*P* band was not scraped inasmuch as a less pure product was obtained when this was done. The product was eluted from the silica from three 5 × 20-cm TLC plates by extraction twice with 2 ml of $CHCl_3$/methanol/pyridine/acetic acid/H_2O, 1:2:1:1:1 (vol/vol). The extracts were combined, and 1 ml of $CHCl_3$ and 3 ml of H_2O were added. This mixture was vortexed, and the phases were separated by a brief centrifugation. The lower, chloroform phase was transferred to a new tube and the upper phase was extracted with an additional 1 ml of $CHCl_3$. The two $CHCl_3$ phases were combined and extracted twice with 2 ml of 1 M HCl in methanol/H_2O, 1:1 (vol/vol). Phosphatidylinositol phosphates were quantatively extracted from the silica by this procedure. Traces of antioxidants still present in the [^{3}H]PtdIns3*P* were removed by passing this product over a 0.5-ml column of silicic acid in $CHCl_3$, washing with 4 ml of $CHCl_3$, and then eluting the PtdIns3*P* with 4 ml of $CHCl_3$/methanol/pyridine/acetic acid/H_2O, 1:2:1:1:1 (vol/vol). Alternatively, the TLC scrapings were washed with $CHCl_3$ to remove the antioxidants prior to elution of the [^{3}H]PtdIns3*P*.

Assay of PtdIns3*P* 3-Phosphatase. The assay was a minor modification of previously described methods (7, 12). The final assay solution contained, in a volume of 10 μl, 100 mM KCl, 20 mM Mes (pH 6.5), 2.5 mM EDTA, 1 mM dithiothreitol, albumin at 1 mg/ml, 0.5 mM dioleoylphosphatidylglycerol, 0.3–100 μM PtdIns3*P*, and 10 mM octyl β-D-glucopyranoside (octyl glucoside). Chloroform solutions of the phosphatidylglycerol and [^{3}H]PtdIns3*P*, to which PtdIns[^{32}P]3*P* had been added, were transferred into 1.5-ml polypropylene centrifuge tubes, evaporated under a stream of N_2, and placed briefly *in vacuo*. A solution containing the other assay ingredients was then added, and the lipids were suspended by two 15-sec immersions in a water bath sonifier. The reactions were initiated by adding 20 pg of enzyme and incubating at 37°C for 10 min. Dilutions of enzyme were performed in assay mixtures in which the [^{3}H]PtdIns3*P* was omitted and the dithiothreitol was increased to 5 mM. Reactions were terminated by addition of 0.5 ml of 10% trichloroacetic acid and 50 μl of 20% (vol/vol) Triton X-100 (13). The mixture was centrifuged, and the radioactivity in the aqueous and organic phases was determined by liquid scintillation counting.

Analysis of Phosphatidylinositol Phosphates. Deacylations of radiolabeled phosphatidylinositol phosphates were performed as described (4, 14) except that 20 μl of carrier lipid solution was added to each reaction. The carrier lipid solution was prepared by dissolving 100 mg of crude phosphatidylinositol (Sigma) in 1 ml of $CHCl_3$ and extracting three times with 1 ml of 1 M HCl in methanol/H_2O, 1:1 (vol/vol). A small amount of interfacial precipitate formed during these extractions and was removed. Partisil SAX HPLC of glycerophosphoinositol phosphates was performed as described (4, 15). A 30-min linear gradient of 40–425 mM ammonium formate (pH 3.5), followed by a 20-min linear gradient of 0.81–1.11 M ammonium formate (pH 3.5), was employed. The flow rate was 1 ml/min. Fractions were collected at 1-min intervals and assayed in 10 ml of Scintiverse I (Fisher). The column was washed for 10 min with 3.0 M ammonium formate (pH 3.5) and for 20 min with 40 mM ammonium formate (pH 3.5) prior to injection of the next sample.

Deglyceration of glycerophosphoinositols was performed as described (15).

RESULTS AND DISCUSSION

The preparation of PtdIns3*P* required a method to separate it from other phosphatidylinositol phosphates present in the carbodiimide reaction products. Other workers have shown that inositol isomers can be separated by methods based on the formation of borate complexes with cis-diols present in the inositol ring (16, 17). In PtdIns3*P*, the cis-diol is blocked by the phosphomonoester, and it is therefore unable to form a borate complex, whereas PtdIns4*P* has the 2,3-cis-diol available to form a borate complex. In initial experiments, TLC plates were impregnated with boric acid as described by other workers (18) and developed in several solvent systems that have been used previously to separate phosphatidylinositol phosphates (12, 19). In an acidic solvent system (19), no separation of phosphatidylinositol phosphate isomers was observed. However, in an alkaline, NH_4OH-based system (12), PtdIns3*P* migrated slightly faster than PtdIns4*P*. These results are consistent with the alkaline dependence of borate complex formation (20). Several organic bases were then tried, and good separation of PtdIns3*P* from PtdIns4*P* was obtained with pyridine. Much less separation was obtained with 2,6-lutidine. Variable separations were encountered because of migration of the boric acid on the TLC plates. This was prevented by dissolving boric acid in the developing solution rather than impregnating it into the plates. Problems were also encountered with oxidation of lipids during the drying of the plates and extraction from the silica. The presence of BHT in the solvent greatly inhibited this oxidation, and ethoxyquin abolished it. Inclusion of 0.2% BHT in the developing solution slightly improved the chromatographic separation. Both BHT and ethoxyquin were thus included in the TLC solvent. PtdIns3*P* eluted from plates developed in this manner migrated as a single spot on repeat TLC, showing no evidence of oxidation. Poor separation of PtdIns3*P* from PtdIns4*P* was obtained if other solvents (CH_2Cl_2 or isooctane/ethylacetate) were substituted for $CHCl_3$. Similar separation was obtained if formic acid was omitted and H_2O was increased from 7.5 to 15 ml, but increased tailing of the phosphatidylinositol phosphate spots occurred.

The [^{3}H]PtdIns3*P* synthetic strategy was based on observations of earlier workers that carbodiimides can promote the formation of cyclic phosphate esters across trans equatorial hydroxyls (21, 22). It was reasoned that treatment of

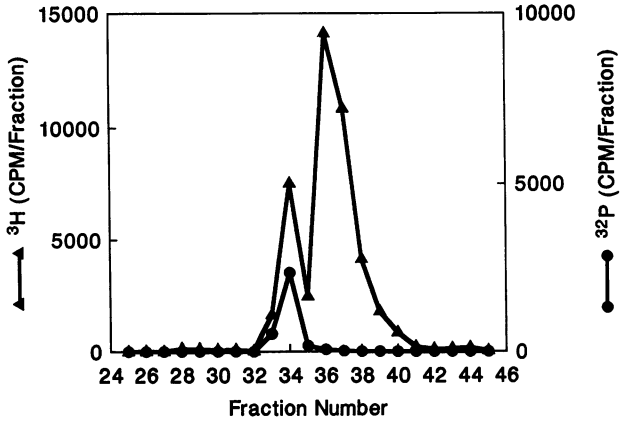

FIG. 2. Analysis of products of carbodiimide treatment of PtdIns4*P*. The carbodiimide reaction products were deacylated and analyzed by HPLC. PtdIns[^{32}P]3*P* was included as an internal standard. The HPLC fractions were assayed for ^{3}H and ^{32}P.

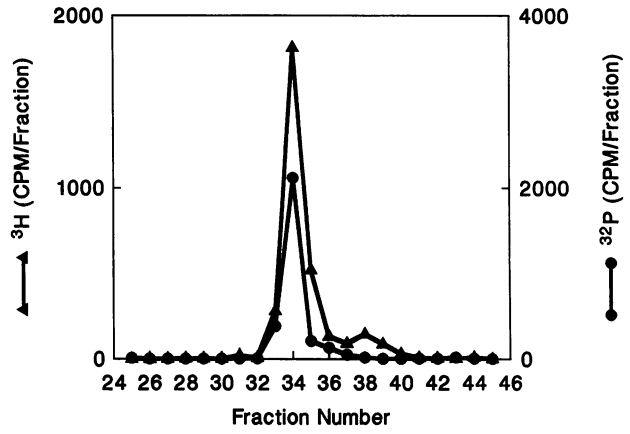

FIG. 3. Analysis of borate TLC-purified PtdIns3*P*. The final [^{3}H]PtdIns3*P* product was deacylated and analyzed by HPLC. A PtdIns[^{32}P]3*P* internal standard was included. The HPLC fractions were assayed for ^{3}H and ^{32}P.

[^{3}H]PtdIns4*P* with carbodiimide would lead to formation of a mixture of 3,4 and 4,5 cyclic phosphates, which could be hydrolyzed to a mixture of phosphatidylinositol 3-, 4-, and 5-phosphates. Assuming random formation and hydrolysis of the cyclic phosphates, the expected yield of [^{3}H]PtdIns3*P* would be 25%. Initial experiments were performed under essentially the conditions of Pizer and Ballou (21) with dicyclohexylcarbodiimide and aqueous pyridine. The deacylated products of this reaction showed the presence of an HPLC peak that comigrated with authentic glycerophosphoinositol 3-[^{32}P]phosphate. Furthermore, treatment of these reaction products with PtdIns3*P* 3-phosphatase, followed by deacylation, yielded a product that eluted from HPLC in the position of [^{3}H]glycerophosphoinositol. The initial [^{3}H]-PtdIns3*P* preparations were contaminated with significant amounts of side products that comigrated with [^{3}H]PtdIns3*P* on borate TLC. These side products were minimized by inclusion of 5 mM diisopropylethylamine in the reaction mixture to inhibit any reaction of the carbodiimide with the cyclic phosphates or phosphodiesters (23). Inclusion of a tertiary base beyond this amount only inhibited PtdIns3*P* formation and led to the appearance of new side products. Exclusion of water and reduction of the carbodiimide concentration to the minimum required for complete reaction were also helpful. When reactions were performed with

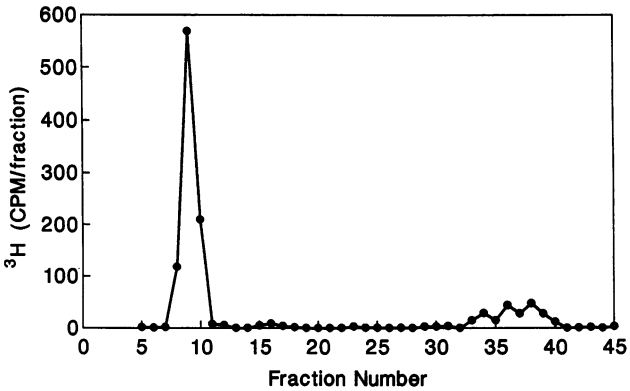

FIG. 4. Dephosphorylation of [^{3}H]PtdIns3*P*. Borate TLC-purified [^{3}H]PtdIns3*P* (2.6 nmol) was treated with 18 ng of purified type II 3-phosphatase for 20 min as described in *Experimental Procedures*, except that the reaction volume was 1.0 ml and the phosphatidylglycerol concentration was increased to 1.0 mM. The reaction products were extracted into $CHCl_3$, deacylated, and analyzed by partisil SAX HPLC. The HPLC fractions were assayed for ^{3}H as described.

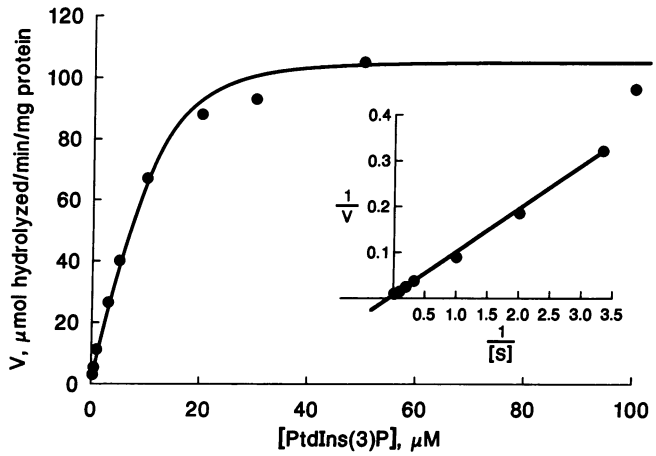

FIG. 5. Reaction velocities for the hydrolysis of PtdIns3*P* by type I 3-phosphatase at various concentrations of PtdIns3*P*. [^{3}H]PtdIns3*P* and PtdIns[^{32}P]3*P* were mixed to prepare the substrate at a specific activity of 200,000 cpm of ^{32}P per nmol. Various concentrations of the substrate were incubated with 20 pg of type I 3-phosphatase for 10 min at 37°C in a reaction volume of 10 μl. The reaction was terminated, and the release of ^{32}P was quantitated as described under *Experimental Procedures*.

dicyclohexylcarbodiimide, the dicyclohexylurea formed interfered with the TLC separation of products. Diisopropylcarbodiimide, which has a similar reactivity (9), was therefore substituted. The diisopropylurea formed was apparently removed by the methanol/H_2O washing. The HPLC profile of the deacylated products of the final procedure is shown in Fig. 2. The yield of ^{3}H comigrating with the [^{32}P]glycerophosphoinositol 3-phosphate standard was 24%, which is consistent with the theoretical yield of 25% noted above.

[^{3}H]PtdIns3*P* was purified from the carbodiimide reaction products by borate TLC. An HPLC profile of the final [^{3}H]PtdIns3*P* product that had undergone deacylation is shown in Fig. 3. Greater than 80% of the ^{3}H comigrated with the ^{32}P standard. Improved purity was not obtained by repeat TLC. The [^{3}H]glycerophosphoinositol 3-phosphate (HPLC fractions 33–35, see Fig. 3) from the deacylated [^{3}H]PtdIns3*P* was deglycerated, and the inositol phosphate products were analyzed by partisil SAX HPLC (15). Eighty-six percent of the ^{3}H comigrated with the [^{32}P]inositol 1,3-bisphosphate standard (data not shown). As an additional proof of the product, the [^{3}H]PtdIns3*P* was treated with an excess of type II 3-phosphatase. This resulted in conversion of 80% of the ^{3}H to a product that comigrated with glycerophosphoinositol after deacylation (Fig. 4). We conclude that the TLC-purified product is at least 80% PtdIns3*P*. The overall yield of PtdIns3*P* from PtdIns4*P*, based on ^{3}H recovery and correcting for the estimated purity of the final product, was 8.2%. This procedure has been repeated three times with similar yields.

The TLC-purified [^{3}H]PtdIns3*P* was used to determine the kinetic parameters for hydrolysis of PtdIns3*P* by type I 3-phosphatase (7). In this experiment, the ^{3}H-labeled substrate provided mass, and the enzyme activity was determined by measuring the release of ^{32}P from PtdIns[^{32}P]3*P*. Enzyme activity obeyed Michaelis–Menten kinetics; it displayed substrate saturation (Fig. 5). The K_m and V_{max} values determined from weighted linear regression analysis (24) of the 1/[S] vs. 1/*v* plot of the data were 7.7 μM and 116 μmol of PtdIns3*P* hydrolyzed per min per mg of protein, respectively. This result demonstrates that the [^{3}H]PtdIns3*P* that we have synthesized is a useful tool to characterize PtdIns3*P* 3-phosphatase. It follows that the molecule will be of similar utility in the study of other reactions involved in the metabolism of PtdIns3*P*.

We thank Linda Pike, Heidi Roth, and Chris Ebert for graciously providing PtdIns[^{32}P]4*P* and Tom Cunningham for assisting with development of the borate TLC method. This research was supported by Grants HL 14147 (Specialized Center for Research in Thrombosis), HL 16634, and Training Grant HL 07088 from the National Institutes of Health.

1. Carpenter, C. L. & Cantley, L. C. (1990) *Biochemistry* **29**, 11147–11156.
2. Serunian, L. A., Auger, K. R., Roberts, T. & Cantley, L. C. (1990) *J. Virol.* **64**, 281–302.
3. Cunningham, T. W. & Majerus, P. W. (1991) *Biochem. Biophys. Res. Commun.* **175**, 568–576.
4. Lips, D. L., Majerus, P. W., Gorga, F. R., Young, A. T. & Benjamin, T. L. (1989) *J. Biol. Chem.* **264**, 8759–8763.
5. Shibasaki, F., Homma, Y. & Takenawa, T. (1991) *J. Biol. Chem.* **266**, 8108–8114.
6. Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S. & Cantley, L. C. (1990) *J. Biol. Chem.* **265**, 19704–19711.
7. Caldwell, K. K., Lips, D. L., Bansal, V. S. & Majerus, P. W. (1991) *J. Biol. Chem.* **266**, in press.
8. Kates, M. (1986) *Techniques of Lipidology* (Elsevier, Amsterdam), 2nd Ed., p. 241.
9. Tartar, A. & Gesquiere, J.-C. (1979) *J. Org. Chem.* **44**, 5000–5003.
10. Brown, D. M. & Higson, H. M. (1957) *J. Chem. Soc.*, 2034–2041.
11. Dekker, C. A. & Khorana, H. G. (1954) *J. Am. Chem. Soc.* **76**, 3522–3527.
12. Lips, D. L. & Majerus, P. W. (1989) *J. Biol. Chem.* **264**, 19911–19915.
13. Rebecchi, M. J. & Rosen, O. M. (1987) *J. Biol. Chem.* **264**, 12526–12532.
14. Clarke, N. G. & Dawson, R. M. C. (1981) *Biochem. J.* **195**, 301–306.
15. Cunningham, T. C., Lips, D. L., Bansal, V. S., Caldwell, K. K., Mitchell, C. A. & Majerus, P. W. (1990) *J. Biol. Chem.* **265**, 21676–21683.
16. Angyal, S. J. & McHugh, D. J. (1957) *J. Chem. Soc.*, 1423–1431.
17. Sasaki, K., Balza, F. & Taylor, I. E. P. (1987) *Carbohydr. Res.* **166**, 171–180.
18. Fine, J. B. & Sprecher, H. (1982) *J. Lipid Res.* **23**, 660–663.
19. Traynor-Kaplan, A. E., Harris, A., Thompson, B., Taylor, P. & Sklar, L. A. (1988) *Nature (London)* **334**, 353–356.
20. Mazzeo, J. R. & Krull, I. S. (1989) *BioChromatography* **4**, 124–130.
21. Pizer, F. L. & Ballou, C. E. (1959) *J. Am. Chem. Soc.* **81**, 915–921.
22. Khorana, H. G., Tener, G. M., Wright, R. S. & Moffatt, J. G. (1957) *J. Am. Chem. Soc.* **79**, 430–436.
23. Smith, M., Moffatt, J. G. & Khorana, H. G. (1958) *J. Am. Chem. Soc.* **80**, 6204–6212.
24. Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332.