Biochem. J. (1984) **218**, 785–793
Printed in Great Britain

Analysis of the metabolic turnover of the individual phosphate groups of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate

Validation of novel analytical techniques by using ^{32}P-labelled lipids from erythrocytes

Phillip T. HAWKINS, Robert H. MICHELL and Christopher J. KIRK
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

(*Received 24 August 1983/Accepted 18 November 1983*)

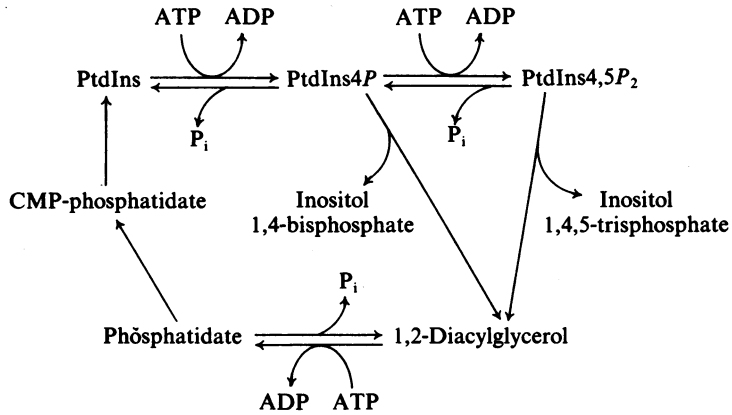
1. We have developed methods that yield estimates of the ^{32}P content of each of the individual phosphate groups of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, thus extending the information available from studies of the labelling of these lipids in intact cells or membrane preparations. The analyses are undertaken with the deacylated lipids. 2. Assay of the 5-phosphate of phosphatidylinositol 4,5-bisphosphate is achieved by the use, under conditions of first-order kinetics, of a 5-phosphate-specific phosphomonoesterase present in isolated erythrocyte membranes [Downes, Mussat & Michell (1982) *Biochem. J.* **203**, 169–177]. 3. Assay of the 4-phosphate of phosphatidylinositol 4-phosphate and of the total monoester phosphate content (4-phosphate plus 5-phosphate) of phosphatidylinositol 4,5-bisphosphate employs alkaline phosphatase from bovine intestine. The radioactivity of the 1-phosphate is that remaining as organic phosphate after exhaustive alkaline phosphatase treatment. 4. The methodology has been validated by using lipids from human erythrocytes: these contain no ^{32}P in their 1-phosphate. 5. These methods should be of substantial value in studies of the many cells that show rapid hormonal perturbations of phosphatidylinositol 4,5-bisphosphate metabolism.

Polyphosphoinositides (PtdIns4*P* and PtdIns4,5P_2) are present in tissues at low concentrations but rapidly incorporate ^{32}P, particularly into their monoester phosphate groups. Their metabolism can be dramatically perturbed by hormones and neurotransmitters, but neither the mechanism nor the function of these changes is yet understood (see Downes & Michell, 1982, for a review).

Metabolic turnover of the entire inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate headgroups of PtdIns4*P* and PtdIns4,5P_2 involves a minimum of ten enzymic steps (see Scheme 1) and yet ^{32}P-labelling studies do not usually distinguish between the individual phosphate groups in each lipid molecule. It is usually assumed that the monoesterified 4-phosphate and 5-phosphate groups of these lipids are much more metabolically active than the diesterified 1-phosphate, and that substantial changes in ^{32}P-labelling reflect changes only in the monoesterified phosphate groups. Occasionally this has been verified by the use of an unspecific phosphomonoesterase to distinguish between monoesterified and diesterified phosphate groups (Brockerhoff & Ballou, 1962; Hokin & Hokin, 1964; Galliard *et al.*, 1965).

Ideally, studies of these lipids would employ simple, routine techniques that would allow a separate analysis of each of the phosphate groups in the PtdIns4*P* and PtdIns4,5P_2 molecules. Some years ago, Prottey *et al.* (1968) demonstrated that intestinal alkaline phosphatase and wheat bran phytase showed slight specificity for the 4-phosphate and 5-phosphate, respectively, of deacylated PtdIns4,5P_2. However, the discrimination achieved in this study was not sufficient for the adoption of these enzymes as routine analytical tools. Recently we have described an inositol 1,4,5-trisphosphate 5-phosphomonoesterase that will attack deacylated PtdIns4,5P_2, leading to selective release of its 5-phosphate (Downes *et al.*, 1982). In this paper we describe the use of this enzyme,

Abbreviations used, PtdIns4*P* and PtdIns4,5P_2, phosphatidylinositol 4-phosphate and 4,5-bisphosphate respectively; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethane sulphuric acid.



Scheme 1. *Metabolic turnover of polyphosphoinositides*

together with an alkaline phosphatase that removes the 4-phosphate from deacylated PtdIns4*P* and both the 4-phosphate and 5-phosphate from deacylated PtdIns4,5P_2, to generate simple procedures for distinguishing between the various phosphate groups of the polyphosphoinositides. Validation of these techniques has been largely based on studies of deacylated [^{32}P]PtdIns4*P* and [^{32}P]PtdIns4,5P_2 from erythrocytes since these lipids have the convenient characteristic of being radioactively labelled only in their monoester phosphate groups.

Materials and methods

Materials were, unless otherwise noted, of the types and from the sources specified previously (Downes & Michell, 1981; Downes *et al.*, 1982). Bovine intestinal alkaline phosphatase was from Sigma (type P 5521). Folch fraction I/II (a polyphosphoinositide-rich lipid fraction from brain) was prepared as described by Folch (1949).

Protein was estimated according to Lowry *et al.* (1951) using bovine serum albumin as standard. ATP concentrations were estimated by using an assay linked to the production of NADPH (Lamprecht & Trautschold, 1974). Total phosphorus was assayed after wet digestion with $HClO_4$ as described by Galliard *et al.* (1965). The P_i released during enzymic degradation of samples was measured by the method of Baginski *et al.* (1967).

Isolation of deacylated PtdIns4P and PtdIns4,5P_2 and of inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate

Unlabelled glycerophosphoinositol 4-phosphate (deacylated PtdIns4*P*) and glycerophosphoinositol 4,5-bisphosphate (deacylated PtdIns4,5P_2) were isolated from a Folch I/II brain lipid fraction as described by Creba *et al.* (1983). To ensure complete resolution of the two esters on the small resin columns, each column was loaded with samples containing no more than 2–3 μmol of each ester.

^{32}P-labelled glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate were derived from ^{32}P-labelled PtdIns4*P* and PtdIns4,5P_2 of erythrocytes. Packed human erythrocytes (3–4ml) were incubated at 60% haematocrit with 1mCi of [^{32}P]P_i for 120min, as described by Downes *et al.* (1982). ^{32}P-labelled membranes were isolated and used both for the preparation of glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate (Downes *et al.*, 1982; Creba *et al.*, 1983) and, via activation of the endogenous polyphosphoinositide phosphodiesterase, for the generation of inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate (Downes *et al.*, 1982). The ammonium formate eluates in which these esters were obtained from the resin columns were neutralized with ammonium hydroxide and they were stored at −20°C. When more concentrated, salt-free samples of the esters were required, the ammonium formate was removed by freeze-drying and the esters were redissolved in a small volume of water (pH adjusted to 7.0 with cyclohexylamine).

^{32}P-labelled inositol 1,4,5-trisphosphate was also prepared by chemical degradation of isolated ^{32}P-labelled glycerophosphoinositol 4,5-bisphosphate by the method of Brown & Stewart (1966). The cyclohexylamine salt of glycerophosphoinositol 4,5-bisphosphate (12.5nmol, 50μl) was incubated with 1ml of 1mM-$NaIO_4$ for 75min, at room temperature and in the dark. A 0.1% aqueous solution of ethylene glycol (30μl) was then added

and the mixture left to stand for 10min. A 1% solution of 1,1-dimethylhydrazine (400μl; pH adjusted to 4.0 with formic acid) was added, and the sample was mixed vigorously and left at room temperature for 60min. It was then treated with 1ml of Dowex-50 (H^+ form) and the supernatant was taken and neutralized with cyclohexylamine in a final volume of 10ml. Inositol 1,4,5-trisphosphate was recovered from this mixture by anion-exchange chromatography on a small Dowex resin column (Downes & Michell, 1981).

Isolation of human erythrocyte membranes for use as inositol 1,4,5-trisphosphatase 5-phosphomonoesterase

The membranes were isolated as described by Downes *et al.* (1982). They were divided into small samples and stored at −20°C until required. A volume of 250ml of packed erythrocytes yields approx. 80ml of membranes at a protein concentration of 5–6mg/ml. This is sufficient for about 100 determinations of the 5-phosphate of glycerophosphoinositol 4,5-bisphosphate.

Selective release of the 5-phosphate from glycerophosphoinositol 4,5-bisphosphate

Samples (0.5ml) of ^{32}P-labelled glycerophosphoinositol 4,5-bisphosphate in 0.85M-ammonium formate (from neutralized column eluates, see above) were diluted to 5ml with Hepes buffer (30mM, pH7.2) and 2mM-$MgCl_2$ and erythrocyte membranes were added to a final concentration of approx. 1.0mg of protein/ml. Unless otherwise specified, incubations were at 37°C for 75min, during which time the kinetics of breakdown were first-order, leading to hydrolysis of 40–60% of the 5-phosphate. Reactions were terminated and the proportion of total ^{32}P released as [^{32}P]P_i was determined as described below. The precise extent of breakdown was calibrated by using a sample of erythrocyte glycerophosphoinositol 4,5-bisphosphate in which the proportion of ^{32}P in the 5-phosphate had been previously determined (see the Results and discussion section).

Analysis of the reaction products from incubation of glycerophosphoinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate with erythrocyte ghosts

Samples of the labelled phosphate esters were incubated with erythrocyte membranes as described above, except that the incubation volume was 10ml. Reactions were terminated with 5ml of ice-cold 10mM-EDTA, pH7.0. The membranes were removed by centrifugation at 10000*g* for 15min and the supernatants were freeze-dried until most of the ammonium formate had sublimed. The freeze-dried residue was dissolved in 10ml of 5mM-disodium tetraborate (the resulting pH was approx. 7.0) and applied to a column (4cm × 0.6cm) of Dowex-1 (100–200 mesh; formate form). The reaction products were separated as described (Downes & Michell, 1981; Creba *et al.*, 1983).

Selective release of the 4-phosphate from glycerophosphoinositol 4-phosphate

Samples (0.5ml) of ^{32}P-labelled glycerophosphoinositol 4-phosphate in 0.4M-ammonium formate (from the neutralized column eluates, see above) were diluted to 1ml with ethanolamine buffer (20mM, pH9.5), 5mM-$MgCl_2$ and approx. 0.02 units of bovine intestinal alkaline phosphatase. Incubations were at 37°C for 2h, during which time glycerophosphoinositol 4-phosphate was quantitatively converted to glycerophosphoinositol and P_i (see the Results and discussion section). Incubations were terminated and the proportion of total ^{32}P released as [^{32}P]P_i was assayed as described below.

Selective release of the 4- and 5-phosphates from glycerophosphoinositol 4,5-bisphosphate

This was achieved in a very similar manner to that described for the release of the 4-phosphate from glycerophosphoinositol 4-phosphate, except that more alkaline phosphatase was required. Samples (0.235ml) of ^{32}P-labelled glycerophosphoinositol 4,5-bisphosphate were assayed in place of 0.5ml samples of glycerophosphoinositol 4-phosphate (so that the final concentration of ammonium formate was approx. 0.2M in each case) and approx. 0.6 units/ml of alkaline phosphatase were present so as to achieve quantitative conversion of glycerophosphoinositol 4,5-bisphosphate to glycerophosphoinositol and P_i within 2h (see the Results and discussion section).

Measurement of ^{32}P-labelled P_i released during enzymic degradation of phosphate esters

The method is based on that of Martin & Doty (1949). Incubations were terminated by the addition of ice-cold $HClO_4$ to a final concentration of 1M. After centrifugation to remove protein, a sample of the supernatant was taken and ammonium molybdate was added to a final concentration of 1% (w/v). Two volumes of isobutyl alcohol/toluene (1:1, v/v) were added and the samples were mixed vigorously. The two phases were separated by centrifugation and a sample of the upper phase was dispensed into 3vol. of Triton/toluene scintillation fluid and its radioactivity determined. When alkaline phosphatase was used, the protein concentration was so low that the first centrifugation step could be safely omitted.

Results and discussion

Selective chemical or enzymic methods for the analysis of the individual phosphate groups of PtdIns4*P* and PtdIns4,5P_2 might in principle be applied either to the intact lipids or to some isolated portion of these molecules. Both approaches are practical possibilities, since recent reports have described both a PtdIns4,5P_2 5-phosphomonoesterase (Roach & Palmer, 1981) and an inositol 1,4,5-trisphosphate 5-phosphomonoesterase that also degrades glycerophosphoinositol 4,5-bisphosphate (Downes & Michell, 1981; Downes *et al.*, 1982).

We chose to develop selective degradation procedures applicable to the water-soluble phosphate esters produced by alkaline deacylation of the lipids, rather than to the intact lipids, for two reasons. First, degradation of these water-soluble phosphate esters by added enzymes would occur in an homogenous aqueous phase, and would not involve the uncertainties of enzyme access that are implicit in enzyme attack upon dispersed lipid substrates. Secondly, we have recently made extensive use of Dowex-1 anion-exchange columns for separating the deacylation products of PtdIns4*P* and PtdIns4,5P_2 derived from intact cells (Downes & Michell, 1981, 1982; Creba *et al.*, 1983), so these esters were readily available in a form suitable for analysis. They are normally present in the eluates from Dowex 1 columns at very low concentrations and in a medium of quite high ionic strength. For example, 20ml of an approx. 0.1 μM solution of glycerophosphoinositol 4,5-bisphosphate in 0.75M-ammonium formate/0.1M-formic acid might be derived from a 50μl sample of erythrocytes. The objective of this study was to produce simple routine methods of analysis that would be directly applicable to samples of this type.

Validation of the specific removal of the 5-phosphate from glycerophosphoinositol 4,5-bisphosphate

Downes *et al.* (1982) concluded that the inositol 1,4,5-trisphosphate phosphomonoesterase of erythrocyte membranes selectively removed the 5-phosphate from both inositol 1,4,5-trisphosphate and glycerophosphoinositol 4,5-bisphosphate. For the former compound this conclusion was based on a rigorous demonstration of the liberation of a *para* isomer of inositol bisphosphate (i.e. of inositol 1,4-bisphosphate rather than the 1,5- or 4,5-isomers), but no equally simple approach was available for glycerophosphoinositol 4,5-bisphosphate. In that case, therefore, we made use of the fact that ^{32}P is unequally distributed between the 4- and 5-phosphate of PtdIns4,5P_2 extracted from erythrocytes that have been incubated with [^{32}P]P_i for 2h. We

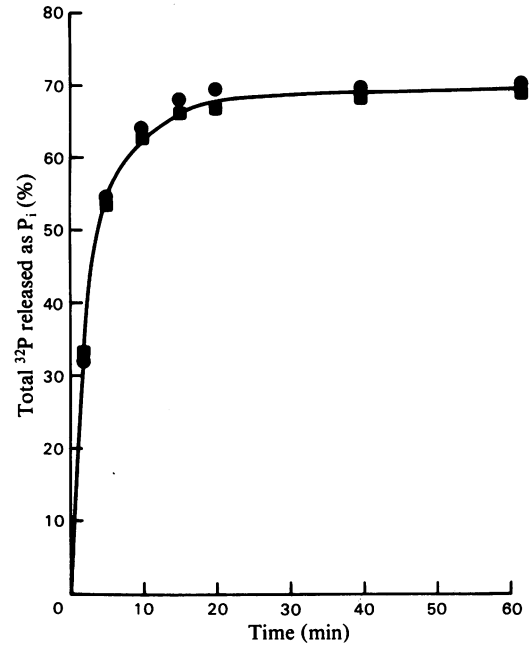


Fig. 1. *Determination of the ^{32}P-labelling of the 4- and 5-phosphates of inositol 1,4,5-trisphosphate*

Samples of inositol 1,4,5-trisphosphate, derived either by phosphodiesterase attack on PtdIns4,5P_2 (■) or by chemical degradation of glycerophosphoinositol 4,5-bisphosphate (●), were incubated with erythrocyte membranes as described in the Materials and methods section. At various times aliquots of the incubations were taken and the proportion of total ^{32}P released as inorganic phosphate was estimated. Results are means of duplicate determinations from a single experiment.

demonstrated that the distribution of radioactivity between organic and inorganic phosphate amongst the products of phosphomonoesterase attack upon inositol 1,4,5-trisphosphate and upon glycerophosphoinositol 4,5-bisphosphate were the same (provided that both esters were isolated from the same erythrocyte ghosts, and hence from the same PtdIns4,5P_2).

Since the key step in the procedure to be described below is the use of the erythrocyte membrane inositol trisphosphate phosphomonoesterase to remove the 5-phosphate from glycerophosphoinositol 4,5-bisphosphate, we initially sought to confirm more rigorously the specificity of its attack on the 5-phosphate of this ester. In order to do this we isolated from the same batch of erythrocyte ghosts: (*a*) glycerophosphoinositol 4,5-bisphosphate derived from PtdIns4,5P_2 by alkaline methanolysis; (*b*) inositol 1,4,5-trisphosphate derived from this glycerophosphoinositol 4,5-bisphosphate by chemical degradation (Brown &

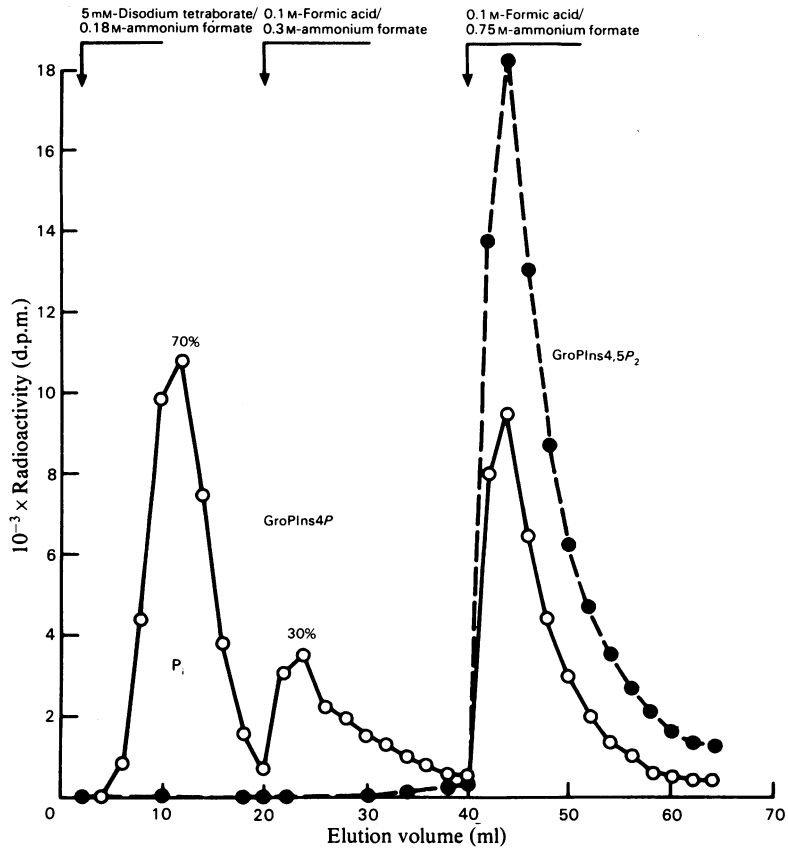


Fig. 2. *Analysis of the reaction products derived from erythrocyte membrane-catalysed hydrolysis of glycerophosphoinositol 4,5-phosphate*

Samples of ^{32}P-labelled glycerophosphoinositol 4,5-bisphosphate (Gro*P*Ins4,5P_2) were incubated at 37°C with erythrocyte membranes for 75min. The reaction products were isolated as described in the Materials and methods section. This treatment led to approx. 50% hydrolysis of the 5-phosphate (○, see the text). In control incubations, EDTA (final concentration 3.3mM) was added before addition of the membranes (●). Results are from a single representative experiment. Similar results were obtained in five further experiments.

Stewart, 1966); and (*c*) inositol 1,4,5-trisphosphate derived from PtdIns4,5P_2 by Ca^{2+} activation of the membrane polyphosphoinositide phosphodiesterase (see the Methods section). When the two inositol 1,4,5-trisphosphate preparations were exhaustively treated with erythrocyte ghosts both released 69% of their ^{32}P as [^{32}P]P_i (Fig. 1). Thus both preparations had 69% of their ^{32}P in the 5-phosphate and 31% in the 4-phosphate; their identity implies that the half to two-thirds of the membrane PtdIns4,5P_2 that is broken down on addition of Ca^{2+} to erythrocyte membranes is metabolically indistinguishable from the unhydrolysed PtdIns4,5P_2. We then exposed samples of glycerophosphoinositol 4,5-bisphosphate to erythrocyte ghosts for long enough to achieve degradation of about half of the ester and separated the products of this hydrolysis by anion-exchange chromatography. Of the ^{32}P in the labelled products, 70% appeared as [^{32}P]P_i and 30% as glycerophosphoinositol phosphate (Fig. 2). The fact that the distribution of radioactivity between the P_i (putative 5-phosphate) and glycerophosphoinositol phosphate (putative 4-phosphate) is identical with that observed with inositol 1,4,5-trisphosphate from the same erythrocytes confirms that the 5-phosphate of glycerophosphoinositol 4,5-bisphosphate was removed specifically, as claimed by Downes *et al.* (1982).

The rate of hydrolysis of glycerophosphoinositol 4,5-bisphosphate by erythrocyte ghosts was proportional to substrate concentration at least up to 10 μM (Fig. 3). This implies that hydrolysis of this ester should be a first-order process at the very low

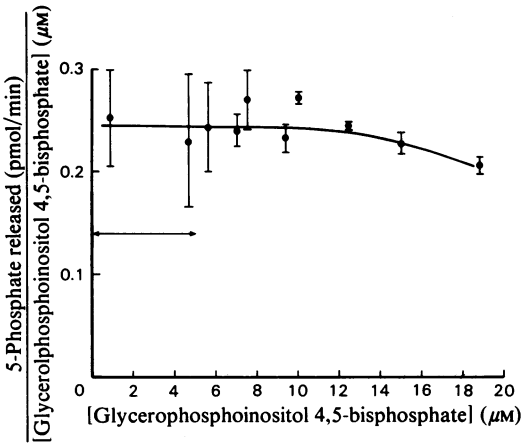


Fig. 3. *Influence of glycerophosphoinositol, 4,5-bisphosphate concentration*

Samples of ^{32}P-labelled glycerophosphoinositol 4,5-bisphosphate (cyclohexylamine salt in water) were incubated with erythrocyte membranes in a final volume of 50μl as described in the Materials and methods section, except that a combination of membrane concentration (approx. 0.03mg of protein) and time (40min) were chosen so as to allow ⩽15% hydrolysis of the 5-phosphate. The [^{32}P]P_i released was corrected for the specific radioactivity of the 5-phosphate. Results are means±S.D. of three independent incubations. ↔ represents the range of glycerophosphoinositol 4,5-bisphosphate concentrations that might be present in the column eluates obtained during routine tissue extractions.

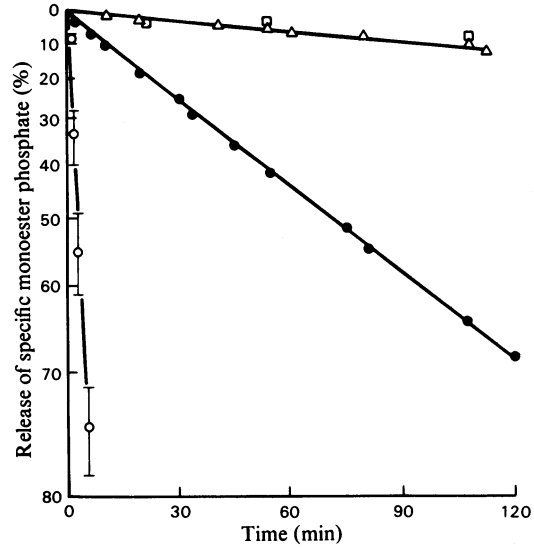


Fig. 4. *First-order kinetics of the hydrolysis of inositol 1,4,5-trisphosphate, inositol 1,4-bisphosphate, glycerophosphoinositol 4,5-bisphosphate and glycerophosphoinositol 4-phosphate by human erythrocyte membranes*

Samples of ^{32}P-labelled esters were incubated with erythrocyte membranes as described in the Materials and methods section. At various times aliquots of the reaction mixture were taken for assay of the [^{32}P]P_i released. ○, Inositol 1,4,5-trisphosphate (mean±S.D. from three separate experiments). ●, glycerophosphoinositol 4,5-bisphosphate; □, inositol 1,4-bisphosphate and △, glycerophosphoinositol 4-phosphate (each point represents an individual determination from one of three separate experiments).

glycerophosphoinositol 4,5-bisphosphate concentrations present in the eluates from Dowex columns. This was found to be so (Fig. 4).

In the study by Downes *et al.* (1982), it was noted, using a substrate concentration that was approximately $2 \times K_m$ for inositol 1,4,5-trisphosphate, that glycerophosphoinositol 4,5-bisphosphate was attacked about one-eighth as fast as inositol 1,4,5-trisphosphate. Under the conditions of first-order degradation chosen for routine analytical use of this enzyme this disparity was even greater. The half-lives of inositol 1,4,5-trisphosphate and glycerophosphoinositol 4,5-bisphosphate under these conditions were 2.5–3.0min and 60–75min, respectively (Fig. 4); i.e. the first-order rate constants differed by about a factor of 30.

The simplest way to apply the inositol trisphosphate phosphatase to the analysis of the 5-phosphate of glycerophosphoinositol 4,5-bisphosphate would be to incubate for a period long enough to achieve essentially complete breakdown. However, this would take many hours (e.g. approx. 7h for 98% hydrolysis). This was not feasible since during such prolonged incubations the erythrocyte ghost preparation released a small amount of radioactivity from inositol 1,4-bisphosphate and glycerophosphoinositol 4-phosphate (Fig. 4). This activity against the diphosphate esters is probably independent of the activity against inositol 1,4,5-trisphosphate and glycerophosphoinositol 4,5-bisphosphate, since it exhibits a different sensitivity to various inhibitors (Zn^{2+}, Cd^{2+}, Ag^+ and F^-; P. T. Hawkins & C. E. King, unpublished work). However, we have not found an inhibitor that shows sufficient discrimination to eliminate selectively this phosphatase in routine use.

The general characteristics of the inositol trisphosphate 5-phosphomonoesterase were described by Downes *et al.* (1982). We have noted two additional properties that facilitate its routine use as an analytical tool. It is stable during storage of erythrocyte membranes at −20°C for several weeks, and its activity is almost undiminished in the presence of ammonium formate at concentrations up to approx. 0.1M. This latter property

facilitates its application to the analysis of phosphate esters in diluted eluates from Dowex 1 anion exchange columns. In order to check for possible variability of the phosphatase activity of different ghost preparations, we isolated ghosts from erythrocytes of a variety of blood groups, both within a day or two of donation and after becoming outdated by three weeks of storage for transfusion. No systematic variations were observed: the first-order rate constants for attack upon glycerophosphoinositol 4,5-bisphosphate under our standard incubation conditions (approx. 1 mg of protein/ml) were $(5.98 \pm 0.4) \times 10^{-3} min^{-1}$ (mean ± S.E.M. for ten separate ghost preparations).

Calibration of the assay of 5-phosphate release from glycerophosphoinositol 4,5-bisphosphate by erythrocyte ghosts

When a series of samples are all incubated identically under conditions giving first-order breakdown then exactly the same proportion of the substrate will be degraded in every sample. We therefore chose to incubate all glycerophosphoinositol 4,5-bisphosphate samples under conditions which achieved 40–60% hydrolysis of the 5-phosphate (the exact figure was in each case dependent on the activity of the particular erythrocyte ghost preparation), with negligible attack on the 4-phosphate. The precise extent of this hydrolysis was determined by assaying in parallel the extent of hydrolysis of a 'standard' sample of glycerophosphoinositol bisphosphate.

In order to prepare and calibrate this 'standard' sample, samples of labelled inositol 1,4,5-trisphosphate and glycerophosphoinositol 4,5-bisphosphate were isolated from one batch of erythrocytes that had been incubated with [^{32}P]P_i for 2 h (see the Methods section). Such samples usually have 60–70% of their ^{32}P in the 5-phosphate (see above). A sample of inositol trisphosphate was then incubated under conditions leading to complete removal of the 5-phosphate without attack on the 4-phosphate (Fig. 1), thus defining precisely the distribution of ^{32}P between the 4- and 5-phosphates of this ester and of the glycerophosphoinositol 4,5-bisphosphate (see above).

A sample of such a 'standardized' preparation of glycerophosphoinositol 4,5-bisphosphate was included in every experimental determination of 5-phosphate release as applied to diluted column eluates. This yielded a value for the fraction of the total 5-phosphate released from the 'standard' sample; this correction factor was used to convert the value of released radioactivity for each unknown sample to a value of the total radioactivity in the 5-phosphate.

We have routinely used ^{32}P-labelled glycerophosphoinositol 4,5-bisphosphate from erythrocytes for calibrating our assays: this is easily prepared from erythrocyte ghosts at high specific radioactivity, but its radioactivity decays with a half-life of only 14 days. In principle, it would also be possible to use glycerophosphoinositol 4,5-bisphosphate prepared from PtdIns4,5P_2 that had been labelled with [^{3}H]inositol for this purpose. This would be stable during prolonged storage, so avoiding the need to prepare frequently fresh batches of 'standard' glycerophosphoinositol 4,5-bisphosphate. However, its use would also entail some disadvantages: (1) it would be expensive to prepare in substantial quantities at high specific radioactivity; and (2) the analysis of its reaction products would call for quantitative separation of glycerophosphoinositol 1,4,5-trisphosphate and glycerophosphoinositol 1,4-bisphosphate by ion-exchange chromatography, a potentially less precise procedure than the separation of organic and inorganic phosphate by solvent partition in the presence of molybdate ions.

Validation of the specific release of 4-phosphate from glycerophosphoinositol 4-phosphate

Glycerophosphoinositol 4-phosphate from erythrocyte PtdIns4*P* is labelled with ^{32}P only in its 4-phosphate. As has been demonstrated previously (Hokin & Hokin, 1964), this radioactivity can be quantitatively released as [^{32}P]P_i using alkaline phosphatase from *Escherichia coli* or bovine intestine. Under the same conditions, bovine intestinal alkaline phosphatase releases only one-half of the total phosphate of unlabelled glycerophosphoinositol 4-phosphate as P_i, demonstrating that this enzyme preparation exhibits no phosphodiesterase activity against glycerophosphoinositol 4-phosphate or glycerophosphoinositol (Fig. 5). Hydrolysis of glycerophosphoinositol 4-phosphate by alkaline phosphatase is sufficiently rapid for routine assays to measure quantitative release of the 4-phosphate. The routine conditions chosen (see the Methods section) represent a combination of enzyme concentration and incubation period that are 2-fold greater than the minimum needed for quantitative hydrolysis.

Validation of the specific release of both the 4- and 5-phosphates from glycerophosphoinositol 4,5-bisphosphate

Quantitative degradation of glycerophosphoinositol 4,5-bisphosphate derived from ^{32}P-labelled erythrocyte PtdIns4,5P_2 required much higher quantities of alkaline phosphatase than did the degradation of glycerophosphoinositol 4-phosphate (see the Methods section). Presumably the rate-limiting step is attack upon the first phosphate in the vicinal 4,5-bisphosphate grouping. Even in the presence of this much higher enzyme concen-

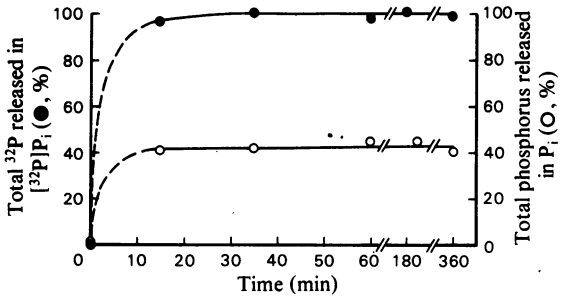


Fig. 5. *Selective release of the 4-phosphate from glycerophosphoinositol 4-phosphate by alkaline phosphatase*
Samples of ^{32}P-labelled and unlabelled glycerophosphoinositol 4-phosphate (derived from erythrocytes and Folch fraction I/II respectively) were incubated together with alkaline phosphatase (1 unit/ml) as described in the Materials and methods section. At various times, aliquots of the incubation were taken for analysis of the ^{32}P-labelled (●) and unlabelled (○) inorganic phosphate released. Each point represents the mean of duplicate incubations.

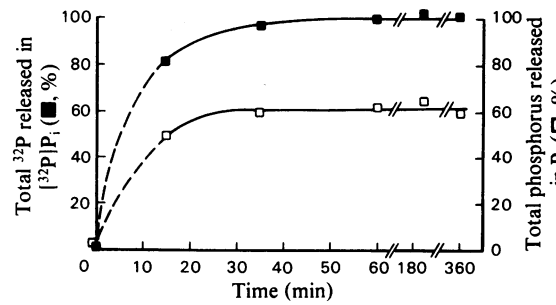
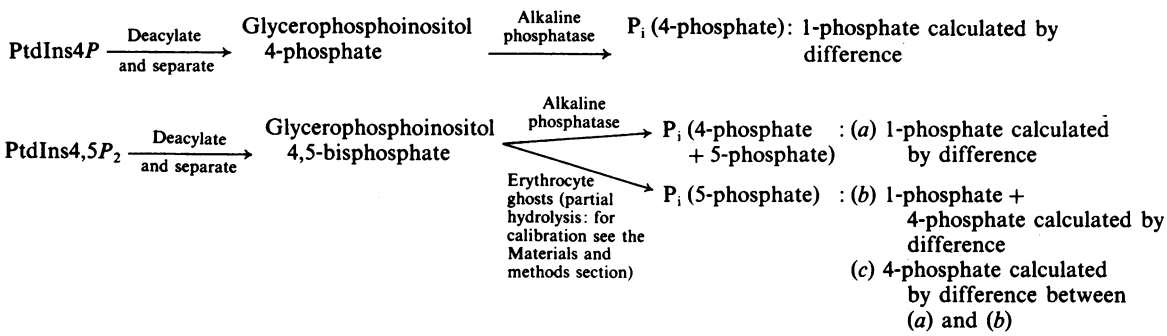


Fig. 6. *Selective release of the 4- and 5-phosphates from glycerophosphoinositol 4,5-bisphosphate by alkaline phosphatase*
Samples of ^{32}P-labelled and unlabelled glycerophosphoinositol 4,5-bisphosphate (derived from erythrocytes and Folch fraction I/II respectively) were incubated together with alkaline phosphatase (5 units/ml) as described in the Materials and methods section. At various times, aliquots of the incubation were taken for analysis of the ^{32}P-labelled (■) and unlabelled (□) inorganic phosphate released. Each point represents the mean of duplicate incubations.



Scheme 2. *Strategy for analysing the distribution of radioactivity amongst the phosphate groups of PtdIns4P and PtdIns4,5P_2*

tration, only two-thirds of the phosphate of unlabelled glycerophosphoinositol 4,5-bisphosphate was released as P$_i$, again validating the use of this intestinal preparation as a phosphodiesterase-free phosphomonoesterase (Fig. 6). As with glycerophosphoinositol 4-phosphate, the routine assay conditions chosen incorporate a large margin of safety over the minimum enzyme concentration and incubation period needed to yield quantitative degradation.

Strategy used for enzyme degradation of PtdIns4P and PtdIns4,5P_2

This is summarized in Scheme 2. A complete description of the distribution of ^{32}P between the 1-phosphate and 4-phosphate of PtdIns4*P* can be obtained simply by the application of alkaline phosphatase, whereas a complete description of the distribution of radioactivity between the three phosphates of PtdIns4,5P_2 can only be obtained by treating separate samples of the labelled glycerophosphoinositol 4,5-phosphate with alkaline phosphatase and erythrocyte ghosts and then combining the results.

We are grateful to the MRC for the award of a Research Studentship to P.T.H.

References

Baginski, E. S., Zak, B. & Foa, P. P. (1967) *Clin. Chem.* **13**, 326–330

Brockerhoff, H. & Ballou, C. E. (1962) *J. Biol. Chem.* **237**, 49–52

Brown, D. M. & Stewart, J. C. (1966) *Biochim. Biophys. Acta* **125**, 413–421

Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **212**, 733–747

Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140

Downes, C. P. & Michell, R. H. (1982) *Cell Calcium* **3**, 467–502

Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203**, 169–177

Folch, J. (1949) *J. Biol. Chem.* **177**, 505–519

Galliard, J., Michell, R. H. & Hawthorne, J. N. (1965) *Biochim. Biophys. Acta* **106**, 551–563

Hokin, L. E. & Hokin, M. R. (1964) *Biochim. Biophys. Acta* **84**, 563–575

Lamprecht, W. & Trautschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 2101–2110, Academic Press, New York

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Martin, J. B. & Doty, D. M. (1949) *Anal. Chem.* **21**, 965–967

Prottey, C., Salway, J. G. & Hawthorne, J. N. (1968) *Biochim. Biophys. Acta* **164**, 238–251

Roach, P. D. & Palmer, F. B. St. C. (1981) *Biochim. Biophys. Acta* **661**, 323–333