Biochem. J. (1986) 235, 775–783 (Printed in Great Britain)

Turnover of phosphomonoester groups and compartmentation of polyphosphoinositides in human erythrocytes

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The turnover of phosphomonoester groups of phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] was investigated in human erythrocytes by short-term labelling with [^{32}P]P_i. The procedure applied ensured a quantitative extraction of erythrocyte polyphosphoinositides as well as their reliable separation for the determinations of pool sizes and specific radioactivities. The pool sizes of phosphatidylinositol (PtdIns), PtdIns4*P* and PtdIns(4,5)P_2 are 25, 11 and 44 nmol/ml of cells respectively. Under steady-state conditions, the phosphorylation fluxes from [γ-^{32}P]ATP into PtdIns4*P* and PtdIns(4,5)P_2 are in the ranges 14–22 and 46–94 nmol·h^{-1}·ml of $cells^{-1}$ respectively. Only 25–60% of total PtdIns4*P* and 6–10% of total PtdIns(4,5)P_2 take part in the rapid tracer exchange, i.e. are compartmentalized. In isolated erythrocyte ghosts, the turnover of PtdIns4*P* approximately corresponds to that in intact erythrocytes, although any compartmentation can be excluded in this preparation. Under the conditions of incubation employed, the turnover of PtdIns(4,5)P_2 is more than one order of magnitude smaller in isolated ghosts than that obtained for intact erythrocytes.

INTRODUCTION

Polyphosphoinositides undergo a rapid turnover of their phosphomonoester groups in erythrocytes, but the role of this process has not been established definitely (Girand *et al.*, 1984). Most previous studies have reported changes only in total radioactivity, which may be difficult to interpret. More recently, changes in radioactivity as well as in lipid content were studied in liver in order to define better the dynamics of polyphosphoinositides (Litosch *et al.*, 1983).

Much of the information regarding the inositide content of erythrocyte membranes is subject to uncertainty (Allan, 1982), because of the risk of degradation of polyphosphoinositides in the course of membrane isolation and lipid extraction. Careful determinations of the inositide levels revealed that PtdIns4*P* and PtdIns(4,5)P_2 predominated over PtdIns, although there is a wide variety in the values reported ((Allan & Michell, 1978; Minenko *et al.*, 1981; Maretzki *et al.*, 1983; Girand *et al.*, 1984).

With regard to the turnover of phosphomonoester groups of polyphosphoinositides in intact erythrocytes, mainly qualitative data are available. They are derived from long-term labelling experiments with [^{32}P]P_i, i.e. they are performed during the pre-stationary phase (Reich, 1968), where the specific radioactivities of intrasystemic compounds equilibrate slowly with that of the precursor pool, a condition hampering quantitative data evaluation.

The present paper examines the dynamic phase (Reich, 1968) of the ^{32}P flux into the polyphosphoinositides with wide and changing differences in the specific radioactivities of precursor and products by an appropriate short-term sampling. Improved experimental procedures are described in order to obtain valid pool sizes of inositides and specific radioactivities of [γ-^{32}P]ATP, PtdIns4*P* and PtdIns(4,5)P_2, because incomplete removal of P-containing impurities results in wrong calculation of flux rates. Generally, the specific-radioactivity time courses of the 4-*P* of PtdIns4*P* and 4- plus 5-*P* of PtdIns(4,5)P_2 were determined. The only way in which the flux model applied for data evaluation could be made to match the measured radiolabelling time course was to invoke compartmentation of polyphosphoinositides.

MATERIALS AND METHODS

Materials

Carrier-free neutral [^{32}P]P_i solution (10 mCi = 370 MBq/ml) and neutral [γ-^{32}P]ATP solution (2 mCi = 74 MBq/ml) were obtained from Zentralinstitut für Kernforschung (Rossendorf/Dresden, German Democratic Republic). PtdIns, PtdIns4*P*, PtdIns(4,5)P_2 and other phospholipid standards were obtained from Sigma, ADP and ATP from Boehringer, Alcian Blue 8 GS from Fluka, phenylmethanesulphonyl fluoride from Serva and silica-gel HR 'reinst' from Merck. Other chemicals were of the highest purity available from commercial sources. Organic solvents were glass-distilled before use.

Heparinized blood was withdrawn from healthy donors and centrifuged for 10 min at 4 °C and 1400 *g*. Plasma and buffy coat were removed and the packed erythrocytes washed three times with cold wash solution containing 50 mM-Tris/maleate/100 mM-NaCl/5 mM-KCl/1 mM-Na_2HPO_4/5.5 mM-glucose, pH 7.4 (adjusted at 37 °C). The erythrocytes were used immediately after the washing procedure.

Abbreviations used: PtdIns, PtdIns4*P* and PtdIns(4,5)P_2, phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate respectively.

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Radiolabelling time course

Intact erythrocytes. A 10 ml portion of washed and pelleted erythrocytes (15 min at 1400 *g*; haematocrit about 90%) were incubated for 5 min at 37 °C. The labelling was started by pouring the erythrocytes into an equal volume of wash solution at 37 °C containing, in addition, 1.4 mM-$CaCl_2$, 0.8 mM-$MgCl_2$ and about 80 μCi (3 MBq) of [^{32}P]P_i/ml. In a second series of experiments the erythrocytes were washed and preincubated for 15–20 min at 37 °C in wash solution containing 1.4 mM-$CaCl_2$ and 0.8 mM-$MgCl_2$ (haematocrit about 45%). The labelling was then started by adding 1 ml of incubation solution with about 800 μCi (30 MBq) of [^{32}P]P_i to the incubation mixture. The results of both series of experiments showed no noticeable differences in the measured radiolabelling time courses. Thus they were combined for data evaluation (see Fig. 1 below). After 1–15 min of incubation with shaking, 4 ml of incubation mixture was frozen down to −65 °C within about 20 s in a bath of solid CO_2/methanol and stored on solid CO_2.

In order to assess by comparison the absence of secondary tracer fluxes during the 'freeze stop', the following experiment was carried out. Cells were lysed by pouring 4 ml of incubation mixture into 70 ml of ice-cold lysis buffer containing 2 mM-P_i, 2 mM-EDTA and 1 mM-EGTA, pH 7.2 (adjusted at room temperature). The membranes were pelleted by 15 min centrifugation at 14000 *g* and 0–1 °C, washed thrice with 16 ml of ice-cold lysis buffer and submitted to lipid extraction as described below. Simultaneously, 4 ml of the same labelled incubation mixture was submitted to freeze stop and our standard procedure of membrane isolation (see below). Both the methods applied for termination of labelling and preparation of ghosts resulted in identical specific radioactivities of PtdIns4*P* and PtdIns(4,5)P_2. Much higher specific radioactivities were obtained, however, after a preceding separation of cells from the labelled incubation medium by pouring the incubation mixture into a large volume of ice-cold unlabelled iso-osmotic buffer and pelleting the cells in the cold.

For convenience we preferred our standard procedure with freeze stop and membrane isolation in the presence of Alcian Blue (see below).

Isolated membranes. Saponin ghosts (about 50 mg of ghost protein) obtained as described below were preincubated for 10–20 min at 37 °C in 25 ml of a solution containing 50 mM-Tris/HCl/100 mM-KCl/1 mM-EGTA/3.5 mM/$MgCl_2$/2 mM-ATP, pH adjusted at 37 °C to 7.2, in order to wait for the main phase of net synthesis of polyphosphoinositides which would have obscured the examination of the steady-state turnover of polyphosphoinositides. The labelling was started by the addition of 67 μCi (2.5 MBq) of [γ-^{32}P]ATP dissolved in 1 ml of incubation solution. After 5–60 min of incubation with shaking, 5 ml of the incubation mixture was mixed with 12 ml of ice-cold solution containing 6% (w/v) trichloroacetic acid and 2 mM-unlabelled ATP. The membranes were pelleted by 3 min centrifugation at 14000 *g* and submitted to lipid extraction.

An advantage of iso-osmotic saponin membranes is that they do not reseal during incubation at 37 °C. They are permeable to dextran, pass to the bottom of dextran cushions and show identical activities of Ca^{2+}-transporting ATPase in the absence and in the presence of 0.02% Triton X-100 (D. Brox, R. Klinger & H. Petermann, unpublished work). Hypo-osmotic ghosts, however, often reseal at temperatures above 0 °C after return to iso-osmoticity as well as under conditions of decreased ionic strength (Lee *et al.*, 1985; Nash & Meiselman, 1985).

Membrane isolation

A 14 ml portion of an ice-cold filter solution containing 154 mM-NaCl, 2 mM-EDTA, 1 mM-EGTA and 0.005% of the haemagglutinating polycationic dye Alcian Blue, pH 7.6 (adjusted at room temperature) (Halbhuber *et al.*, 1977) was added to the frozen incubation mixture. The mixture was thawed carefully, with stirring, so that the temperature did not rise above 0–1 °C, and then centrifuged for 5 min at 14000 *g* and 0 °C. The pelleted membranes were washed twice or thrice with 16 ml of ice-cold filter solution containing 154 mM-NaCl, 0.5 mM-EDTA, 0.1 mM-EGTA and 0.001% Alcian Blue. The pH was adjusted to 8.0 at room temperature. The membranes were pelleted each time by 5 min centrifugation at 14000 *g* and 0 °C. Nearly haemoglobin-free ghosts were obtained by this procedure in about 45 min.

Control experiments revealed that only traces of radioactivity could be detected in PtdIns4*P* and PtdIns(4,5)P_2 when unlabelled erythrocytes were subjected to membrane isolation and lipid extraction after addition of 19 μCi (0.7 MBq) of [γ-^{32}P]ATP to the first wash solution applied for thawing the frozen incubation mixture (see above).

In order to assess by comparison (*a*) possible disturbances of lipid extraction and/or separation by Alcian Blue and (*b*) the risk of degradation of PtdIns4*P* and PtdIns(4,5)P_2 in the process of membrane isolation, two other membrane preparations were performed. One preparation was made in the presence of Alcian Blue exactly as described above, but with the addition of 1 mM-neomycin to all solutions employed [because this substance is thought to inhibit a breakdown of PtdIns(4,5)P_2 (Roach & Palmer, 1981)]. The other preparation, carried out as described by Klinger *et al.* (1980), was a conventional iso-osmotic procedure with saponin haemolysis in the absence of Alcian Blue and took about 120 min in the cold.

Membranes produced on haemolysis of erythrocytes with saponin were also used in labelling experiments with isolated ghosts (see above). The preparation of ghosts for these experiments was, however, performed with two modifications in order to reduce proteolysis of membrane proteins, especially of Ca^{2+}-ATPase, and the breakdown of polyphosphoinositides that was observed after longer incubations of ghosts at 37 °C. Leucocytes of the heparinized blood sample were removed by passage through a cotton column as described by Busch & Pelz (1966). All solutions subjected to haemolysis and membrane isolation contained 0.5 mM-phenylmethanesulphonyl fluoride [added from a stock solution (100 mM) in propan-2-ol].

Lipid extraction

The following ice-cold additions were made to membranes obtained from 4 ml of incubation mixture: 1 ml of 2 mM-EDTA, 6 ml of methanol (dropwise addition) and 3 ml of chloroform. This mixture was stirred for 30 min under N_2 at room temperature and centrifuged for 5 min at 6000 *g*. The supernatant was removed and the extraction continued by stirring the

sediment for 5 min in 9 ml of chloroform/methanol (1:2, v/v) at room temperature. A 2.5 ml portion of 0.75 M-HCl was then added and, after stirring for 15 min at room temperature under N_2, 6 ml of chloroform and 5 ml of 2 M-KCl were added to this mixture together with the first supernatant. After vigorous mixing, phase separation was performed by 10 min centrifugation at 4000 *g*. The lower organic phase was removed and the upper phase was washed once with chloroform. The combined organic phases were then neutralized with NH_3 vapour and evaporated to dryness in a stream of N_2 at 35 °C. The lipids were finally dissolved in 0.3 ml of benzene/ethanol (4:1, v/v) and stored in a small stoppered plastic vessel on solid CO_2.

Control experiments with addition of labelled and purified PtdIns4*P* or PtdIns(4,5)P_2 to unlabelled isolated membranes revealed that about 90% of the total radioactivity added was extracted by this procedure and could be recovered in the corresponding spots after t.l.c.

Lipid chromatography

Aliquots of the lipid extract (about 2.5 μmol of lipid P) were applied to silica-gel HR thin-layer plates (20 cm × 20 cm; 6 g of dry gel suspended in 20 ml of 0.07% potassium oxalate) previously heated at 110 °C for 90 min, chromatographed in the first direction in chloroform/methanol/25% NH_3/water (90:74:13:8, by vol.) and in the second direction by the method of Schick *et al.* (1984) (modified) in chloroform/methanol/acetic acid/water (98:13:50:10, v/v). Between the two runs, plates were dried for 15 min at room temperature and for another 15 min at 70 °C. After development of the chromatograms, the spots were coloured with iodine vapour, or strips of X-ray film (ORWO XR 1; 16 h) were applied for autoradiography. Unlabelled phospholipid standards were used in parallel runs for identification of the spots. The method described shows a good separation of all three inositides and phosphatidic acid. In corresponding autoradiograms (not shown), only PtdIns4*P*, PtdIns(4,5)P_2 and phosphatidic acid were labelled. Traces of radioactivity clearly separated from PtdIns(4,5)P_2 could be detected at the start and between start and PtdIns(4,5)P_2.

Deacylation and chromatography of deacylation products

In order to check the purity of the PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 spots, a transformation of these compounds and a separation of the transformation products in an independent separation system were performed in one additional experiment. The identity of specific radioactivities of the non-transformed and the transformed compounds was considered to be a good indicator of a reliable purification by the t.l.c. system described above. Since PtdIns does not become labelled with [^{32}P]P_i in erythrocytes, a mixed lipid extract from ^{32}P-labelled thrombocytes and erythrocytes was used in this experiment.

The areas of four identical chromatograms that autoradiographically corresponded to PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 were scraped off, combined and eluted with 10 ml of chloroform/methanol/water (1:2.2:1, v/v). The three eluates were dried *in vacuo*, deacylated separately by alkaline hydrolysis, and the single deacylated phospholipids separated on Dowex 1 (formate form columns as described by Downes & Michell (1981). Peak-radioactivity fractions of eluates containing glycerophosphoinositol, glycerophosphoinositol 4-phosphate and 4,5-bisphosphate were freeze-dried, dissolved in 0.1 ml of water and submitted to one-dimensional t.l.c. in order to remove P_i impurities derived from the eluents of the Dowex 1 separation. The t.l.c. system used for this purpose was a minor modification of the system used for the separation of ATP described by Randerath (1962) and provided efficient and rapid separation of P_i ($R_F = 0.56$), glycerophosphoinositol ($R_F = 0.40$) and its 4-phosphate ($R_F = 0.32$) and 4,5-bisphosphate ($R_F = 0.23$) in distinct spots. Briefly, thin-layer plates of silica-gel H (20 cm × 20 cm; 7 g of dry gel suspended in 14 ml of 0.045% EDTA) were used and chromatographed in butanol-1-ol/acetone/acetic acid/5 M-NH_3/water (19:14:8:7:7, by vol.). Then strips of X-ray film (ORWO XR 1, 16 h) were used for autoradiography.

The results shown in Table 1 indicate that the specific radioactivities of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 approximately correspond to those of their deacylation products. Thus the t.l.c. system applied for lipid separation yields sufficiently pure inositides.

Selective release of 5-phosphate from deacylated PtdIns(4,5)P_2

In most experiments the mean specific radioactivity of the 4-*P* plus the 5-*P* of PtdIns(4,5)P_2 was determined (see below) and used together with that of the 4-*P* of PtdIns4*P* for parameter estimation. Only in two additional experiments was the specific radioactivity of 4-*P* of PtdIns(4,5)P_2 measured separately (see Table 3 below) in order to ensure that labelled PtdIns4*P* acted as precursor of PtdIns(4,5)P_2, and hence that the model applied for data evaluation (see below) was valid.

For this purpose, lipid extracts obtained after 20 min incubation of erythrocytes with [^{32}P]P_i were treated by the procedures of Downes & Michell (1981), Downes *et al.*

Table 1. Specific radioactivities of inositides before and after deacylation

PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 were subjected to determination of specific radioactivities after purification by t.l.c. and after conversion of the purified compounds into their deacylation products and a further separation of these products by column chromatography.

	Sp. radioactivity (c.p.m./nmol of labelled *P*)		
	PtdIns	4-*P* of PtdIns4*P*	4-*P* + 5-*P* of PtdIns(4,5)P_2
Before deacylation	74	585	141
After deacylation	66	563	114

(1982) and Hawkins *et al.* (1984) with minor modifications. Briefly, 8 μg (total mass) of labelled glycerophosphoinositol 4,5-bisphosphate derived from the column chromatography of deacylated PtdIns(4,5)P_2 was incubated with shaking for 4 h at 37 °C in 4 ml of an incubation solution containing 30 mM-Hepes, pH 7.0, 2 mM-$MgCl_2$, 0.1 mM-EGTA and 1.5 mg of protein/ml of erythrocyte membranes prepared by the method of Klinger *et al.* (1980). After this time, about 50% of the 5-*P* of glycerophosphoinositol 4,5-bisphosphate was hydrolysed. Iso-osmotic saponin ghosts used by us as a source of inositol-trisphosphate phosphatase have a lower enzyme activity than hypo-osmotic ghosts (see Downes & Michell, 1981), which results in a longer half-time of hydrolysis than that reported by Hawkins *et al.* (1984). The incubation was terminated by the addition of an equal volume of 20% (w/v) trichloroacetic acid. The mixture was kept on ice for 10 min and the precipitated proteins were sedimented by centrifugation. The supernatant was neutralized after removal of trichloroacetic acid and then applied to a Dowex 1 (formate form) column. The peak-radioactivity fractions of the eluates were freeze-dried, dissolved in 0.1 ml of water and submitted to the one-dimensional t.l.c. described in the above subsection.

The P_i fraction obtained in the separation of deacylation products by column chromatography was (*a*) heavily contaminated with non-labelled P_i derived from the ghosts applied as a source of inositol-trisphosphate phosphatase and (*b*) also contaminated with labelled P_i derived from 4-*P* of glycerophosphoinositol 4-phosphate and 4,5-bisphosphate owing to the presence of a low phosphomonoesterase activity in ghosts specific for the 4-position (Hawkins *et al.*, 1984). Thus only the specific radioactivities of 4-*P* and 4-*P* plus 5-*P* of PtdIns(4,5)P_2 could be determined, whereas that of 5-*P* had to be calculated from these data.

Purification of ATP

After incubation of erythrocytes with [^{32}P]P_i or of ghosts with [γ-^{32}P]ATP, 0.2 ml of the incubation mixture was mixed with 0.6 ml of 0.8 M-$HClO_4$ and centrifuged. The supernatant was neutralized with 5 M-K_2CO_3, and the $KClO_4$ precipitate was removed by centrifugation. The supernatant was adjusted to pH 1–3 with HCl and then placed on a charcoal column (0.5 cm × 3.0 cm; Aktivkohle Merck, 30–50 mesh) and the column was washed with 30 ml of 0.1 M-HCl and with water until neutral. The absorbed nucleotides were eluted with 5 ml of ethanol/2 M-NH_3 (1:1, v/v), dried *in vacuo* at 37 °C, dissolved in 0.1 ml of water and taken for t.l.c. by the method of Randerath (1962). The areas on the chromatogram corresponding to ATP and, in a few cases, also those of ADP and AMP, were scraped off and analysed for phosphate content and radioactivity. Specific radioactivity of total phosphorus of ATP stemming from incubations with intact erythrocytes was multiplied by 1.5 in order to obtain the specific radioactivities of the β-*P* and the γ-*P* of ATP, which were in radioactive equilibrium at all incubation times examined. This conclusion is based on the finding that the specific radioactivity of total phosphorus of ADP multiplied by 2 (α-*P* of ADP and ATP was unlabelled) was identical with that of total phosphorus of ATP multiplied by 1.5. The rapid radioactive equilibrium between β-*P* of ADP and β-*P* and γ-*P* of ATP is due to the high concentration of adenylate kinase in erythrocytes (see Jakobasch *et al.*, 1974*a*). The labelling of AMP derived from labelled ATP by acid hydrolysis at 100 °C could be neglected. [γ-^{32}P]ATP used for radiolabelling of PtdIns4*P* and PtdIns(4,5)P_2 of ghosts contained traces of radioactivity in the β-position, which slowly increased during 60 min incubation. This was due to traces of adenylate kinase activity in ghosts, which caused a dilution of the specific radioactivity of γ-*P* by partly randomizing the label between β-*P* and γ-*P*. After 60 min incubation, the specific radioactivity of β-*P* reached about 10% of that of γ-*P*. This was taken into account for calculating specific radioactivities of the γ-*P* of ATP.

Determination of pool sizes and of specific radioactivities of inositides

The areas on the thin-layer chromatograms corresponding to the inositides as well as those corresponding to phosphatidylcholine and phosphatidylethanolamine were removed by scraping, submitted to wet ashing with 1 ml of Lowry solution [75 ml of 70% (w/v) $HClO_4$, 278 ml of conc. H_2SO_4 and water to 2 litres] and then analysed for phosphate content by using the micro method of Bartlett (1959). Corresponding blanks were run in parallel.

In order to calculate the content of inositides per ml of erythrocytes from the amount found in the chromatogram, the following method was adopted. The contents of phosphatidylcholine and phosphatidylethanolamine were first determined to be 885 ± 75 (10) and 782 ± 77 (10) nmol/ml of packed erythrocytes (10^{10} cells) respectively. For each chromatogram, the ratios phosphatidylcholine (1 ml of cells)/phosphatidylcholine (chromatogram) and phosphatidylethanolamine (1 ml of cells)/phosphatidylethanolamine (chromatogram) yielded two independent values of the calibration factor. The amounts of inositides found in the chromatogram were multiplied by the mean calibration factor in order to obtain the amounts per ml of cells.

In an aliquot of the final coloured solution in the P_i determination the radioactivity was measured by scintillation counting. The same procedure was applied to the eluates of the t.l.c. of deacylated polyphosphoinositides. Since the phosphodiester groups of PtdIns4*P* and PtdIns(4,5)P_2 were unlabelled, specific radioactivities of 4-*P* of PtdIns4*P* and of 4-*P* plus 5-*P* of PtdIns(4,5)P_2 were calculated by multiplying specific radioactivities of total phosphorus by 2 and 1.5 respectively.

Computer analysis of data

The purpose of the computer analysis was to extract the flux constants from the measured specific radioactivity time course, which give the flux rates when multiplied by the size of the corresponding receptor pools. Since the pool sizes did not change during the incubation time, the condition of stationary pools could be applied. A flux model was derived from the metabolic map in accordance with earlier proposals (Brockerhoff & Ballou, 1962*a*,*b*; Peterson & Kirschner, 1970). The following differential equations were formulated for the description of the ^{32}P exchange:

$$\dot{x}_{4\text{-}P \text{ of PtdIns4}P} = k_1(x_{\text{ATP}\,\gamma\text{-}P} - x_{4\text{-}P \text{ of PtdIns4}P}) + k_2(x_{4\text{-}P \text{ of PtdIns(4,5)}P_2} - x_{4\text{-}P \text{ of PtdIns4}P})$$

$$\dot{x}_{4\text{-}P \text{ of PtdIns(4,5)}P_2} = k_3(x_{4\text{-}P \text{ of PtdIns4}P} - x_{4\text{-}P \text{ of PtdIns(4,5)}P_2})$$

$$\dot{x}_{5\text{-}P \text{ of PtdIns(4,5)}P_2} = k_3(x_{\text{ATP}\,\gamma\text{-}P} - x_{5\text{-}P \text{ of PtdIns(4,5)}P_2})$$

where x = specific radioactivity in c.p.m./nmol of P in the indicated position, $\dot{x} = dx/dt$, k_1, k_2, k_3 = flux constants in min^{-1},

$$k_1 = \rho_1/(\phi_{PtdIns4P} \cdot C_{PtdIns4P}),$$
$$k_2 = \rho_2/(\phi_{PtdIns4P} \cdot C_{PtdIns4P}),$$
$$k_3 = \rho_2/(\phi_{PtdIns(4,5)P_2} \cdot C_{PtdIns(4,5)P_2}),$$

ρ_1 = phosphorylation flux (nmol·min^{-1} × ml of $cells^{-1}$) from PtdIns into PtdIns4P = dephosphorylation flux from PtdIns4P into PtdIns, ρ_2 = phosphorylation flux (nmol·min^{-1}·ml of $cells^{-1}$) from PtdIns4P into PtdIns(4,5)P_2 = dephosphorylation flux from PtdIns(4,5)P_2 into PtdIns4P, ϕ = compartmentation factor representing the fraction of the total pool taking part in the rapid tracer exchange ($\phi \leqslant 1$), and C = pool size in nmol·ml of $cells^{-1}$. Since metabolic stationarity was assumed C and ρ are constant. Because the measured pool size comprised the metabolically active plus the inactive compartment, the measured specific radioactivity $(x_{meas.}) = x \cdot \phi$.

$x_{ATP\,\gamma\text{-}P} = \alpha \cdot t$ was a known function of time (see Fig. 1). Depending on the actual dosage of [^{32}P]P_i in different experiments, α was of the order of 310 c.p.m./min per nmol of P. Thus all measured specific radioactivities were converted into a unit value for α of 310, i.e. to a unit dosage of [^{32}P]P_i.

The system of linear differential equations was solved analytically. The parameters were estimated by a non-linear least-squares method (Marquardt, 1963) on a desk calculator (Hewlett–Packard 9820 A) using the pool sizes stated in the first line of Table 2 and the specific radioactivities stated in Fig. 1.

The time courses of the specific radioactivities $x_{4\text{-}P \text{ of PtdIns4}P}$, $x_{4\text{-}P \text{ of PtdIns(4,5)}P_2}$ and $x_{5\text{-}P \text{ of PtdIns(4,5)}P_2}$ may be predicted by the analytical solutions of the linear differential equations if k_1, k_2 and k_3 are given as well as possible compartmentation factors $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$. These parameters are not independent, because the ratio of the pool sizes

$$C_{PtdIns(4,5)P_2}/C_{PtdIns4P} = 4 = k_2 \cdot \phi_{PtdIns4P}/(k_3 \cdot \phi_{PtdIns(4,5)P_2})$$

is known from the experiments depicted in Table 2. Therefore, for fitting the radioactivity time courses as predicted by the model to the observed radioactivities, only k_1, k_3, $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ had to be found, and k_2 could be calculated from them. For fitting,

$$x_{4\text{-}P \text{ plus } 5\text{-}P \text{ of PtdIns(4,5)}P_2}$$

was calculated from the solutions of the differential equations as

$$(x_{4\text{-}P \text{ of PtdIns(4,5)}P_2} + x_{5\text{-}P \text{ of PtdIns(4,5)}P_2})/2$$

(cf. the subsection 'Determination of pool sizes and...' above).

The parameters k_1, k_3, $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ show a certain interdependence, because increasing compartmentation factors may be compensated for, but only over a very limited range, by decreasing k_1 and k_3, and vice versa. Thus, for k_1, k_3, $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$, possible ranges rather than definite values may be extracted from the data. To explore these ranges, different pairs of values were assigned to $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ and the appropriate values of k_1 and k_3 were fitted. One should expect the fitted curves to deviate significantly from the experimental points as long as the values assigned to $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ are wrong, but not such deviations if these parameters are located in the neighbourhood of their correct values.

For detecting significant deviations, the residuals

$$x_{4\text{-}P \text{ of PtdIns4}P,\ fit} - x_{4\text{-}P \text{ of PtdIns4}P,\ exp.}$$

and

$$x_{4\text{-}P \text{ plus } 5\text{-}P \text{ of PtdIns(4,5)}P_2,\ fit} - x_{4\text{-}P \text{ plus } 5\text{-}P \text{ of PtdIns(4,5)}P_2,\ exp.},$$

i.e. the differences between fitted and measured radioactivities, were used. The numbers of signs and the numbers of runs were tested as described by Cornish-Bowden & Eisenthal (1974). Briefly, the rationale of these tests is as follows. The residuals are assumed to be distributed independently from each other and to be positive and negative with equal probability. Thus the numbers of positive and negative residuals should not differ too much for an acceptable fit. Furthermore, if the residuals are sorted in the order of increasing time, their signs should change randomly, i.e. there should be not too few runs of equal signs (e.g. the sequence + + + + + + − − − − contains only two runs, which is very improbably produced by chance).

For each fitted $x_{4\text{-}P \text{ of PtdIns4}P}$ and $x_{4\text{-}P \text{ plus } 5\text{-}P \text{ of PtdIns(4, 5)}P_2}$ curve, both the numbers of equal signs and the numbers of runs were compared with their test statistics. For counting the numbers of runs, mean specific radioactivities were used for multiple measurements at the same time. If at least one of these four numbers was out of its 95% confidence interval, the corresponding combination of k and ϕ values was rejected; otherwise it was considered acceptable.

Table 2. Pool sizes of inositides in erythrocyte membranes after different preparation procedures

Results are means ± S.D. for the number of experiments given in parentheses. Saponin ghosts were examined before and after 20 min incubation at 37 °C in the presence of 3.5 mM-$MgCl_2$ and 2 mM-ATP, pH 7.2.

Type of preparation	Pool size (nmol/ml of erythrocytes)		
	PtdIns	PtdIns4P	PtdIns(4,5)P_2
Alcian Blue*	24.8 ± 5.3 (27)	11.1 ± 1.1 (27)	43.7 ± 3.0 (24)
Saponin			
Before incubation	23.8 ± 8.0 (3)	10.2 ± 2.6 (3)	45.8 ± 1.8 (3)
After incubation	17.4 ± 2.9 (8)	15.9 ± 3.4 (8)	39.7 ± 3.0 (8)

* Parallel experiments with Alcian Blue plus neomycin gave identical results.

RESULTS

Inositide pool sizes

Acid lipid extraction giving satisfactory yields of PtdIns4*P* and PtdIns(4,5)P_2 cannot be applied to erythrocytes because of the concomitant extraction of haem pigments, which greatly disturb chromatographic lipid separations. The other approach consists of preparing haemoglobin-free ghosts before applying acid lipid extraction. This, however, involves the risk of partial degradation of polyphosphoinositides (Allan & Michell, 1978; Mack & Palmer, 1984). In order to assess this risk, we compared inositide pool sizes in three different membrane preparations.

The haemagglutinating dye Alcian Blue was used for the first preparation in order to speed it up. The same preparation was carried out in parallel in the presence of neomycin. The third preparation was a conventional procedure with saponin haemolysis, but under controlled temperature conditions (no rise above 5 °C during the preparation). All three preparations gave nearly identical inositide pool sizes (Table 2), which makes improbable a gross distortion of pool sizes during the preparation, which varied in length between 45 and 120 min. Further evidence for this conclusion may be the observation (last line of Table 2) that there is only a small net synthesis of PtdIns4*P* after 20 min incubation of ghosts in the presence of ATP and Mg^{2+}, conditions which, according to the results of Schneider & Kirschner (1970), may lead to a net synthesis of PtdIns4*P* and PtdIns(4,5)P_2 when these pools are initially small.

Turnover of polyphosphoinositides in intact erythrocytes

The turnover of PtdIns4*P* and PtdIns(4,5)P_2 was studied by ^{32}P tracer experiments. The experiments were designed such as to avoid the main difficulties which often hamper the tracer approach (Reich, 1968), namely incomplete purification of the metabolites in question, secondary tracer fluxes and an unsuitable sampling time. Our sampling time was restricted mainly to the short dynamic phase, with widely changing differences in the specific radioactivities of precursor and products. The results are shown in Fig. 1.

The specific radioactivity of the universal precursor ATP γ-*P* increased linearly from 1 to 15 min of incubation. Later on, the increase became somewhat smaller (not shown) and was no longer linear with time. PtdIns4*P* and PtdIns(4,5)P_2 were at first slowly labelled, as might be expected, and then approached an approximately linear increase in the specific radioactivities of their phosphomonoester groups, although on a much lower level than the precursor ATP.

The experimental values were submitted to data evaluation as described in the Materials and methods section. With compartmentation factors $\phi_{PtdIns4P} = \phi_{PtdIns(4,5)P_2} = 1$, i.e. assuming homogeneous pools for both inositides without any compartmentation, the best fit resulted in values for $k_1 = 0.017\ min^{-1}$ and $k_3 = 0.0105\ min^{-1}$. The phosphorylation fluxes into PtdIns4*P* and PtdIns(4,5)P_2 calculated with these data and the pool sizes stated in Table 2 (first line) are then 11 and 28 $nmol \cdot h^{-1} \cdot ml$ of $cells^{-1}$ respectively. There were, however, systematic differences between calculated (see broken lines in Fig. 1) and observed specific radioactivities, i.e. both the numbers of runs and the numbers of equal signs were out of their 95% confidence intervals. Therefore the hypothesis of no compartmentation was not acceptable for fitting the radiolabelling time courses of both inositides.

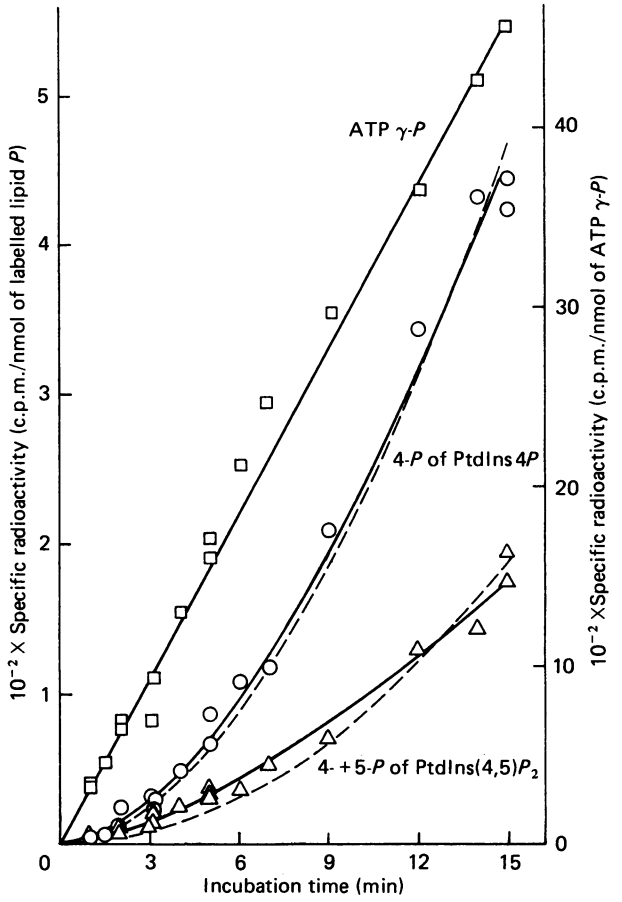

Fig. 1. Radiolabelling time courses in intact erythrocytes per unit dosage of $[^{32}P]P_i$

□, ATP γ-*P*; ○, 4-*P* of PtdIns4*P*; △, 4-*P* plus 5-*P* of PtdIns(4,5)P_2. Continuous lines show computer-generated best fits of the data, assuming compartmentation of PtdIns4*P* and PtdIns(4,5)P_2 ($\phi_{PtdIns4P} = 0.35$ and $\phi_{PtdIns(4,5)P_2} = 0.075$). Broken lines are computer-generated best fits of the data assuming homogeneous pools of polyphosphoinositides ($\phi_{PtdIns4P} = \phi_{PtdIns(4,5)P_2} = 1$). The data were derived from five experiments with five different erythrocyte samples. Two samples (1, 3, 7 and 15 min and 2, 5 and 12 min sampling time) were labelled without preincubation. Three samples (2, 3.11 and 5 min, 1.5, 3.16, 5 and 15 min and 4, 6, 9 and 14 min sampling time) were labelled after 15–20 min preincubation in the incubation medium (see the Materials and methods section). Both series were combined for data evaluation. Each point is the mean for duplicate t.l.c. runs of the same lipid extract and the same ATP preparation respectively.

Thus different pairs of values were assigned to $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$, and the appropriate values of k_1 and k_3 were fitted. A series of such calculations (not shown) revealed that acceptable fits (the numbers of equal signs and the numbers of runs for each fitted $x_{4\text{-}P\ of\ PtdIns4P}$ and $x_{4\text{-}P\ plus\ 5\text{-}P\ of\ PtdIns(4,5)P_2}$ curve within their 95% confidence intervals) were possible only with $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ in the ranges 0.25–0.60 and 0.06–0.10 respectively. The corresponding ranges for k_1 and k_3 were then 0.13–0.03 and 0.60–0.18 respectively.

Table 3. Specific radioactivities of 4-*P* and 5-*P* of PtdIns(4,5)P_2

The mean specific radioactivity of 4-*P* plus 5-*P* of deacylated PtdIns(4,5)P_2 was determined before and after incubation with ghosts as a source of inositol-trisphosphate phosphatase, that of 4-*P* after incubation with ghosts and separation of glycerophosphoinositol 4-phosphate derived from glycerophosphoinositol 4,5-bisphosphate. The specific radioactivity of 5-*P* was calculated with these data. In addition, the ratio 5-*P*/4-*P* is given. All values shown are the percentages of the specific radioactivities of the different *P*-positions of PtdIns(4,5)P_2 relative to that of ATP γ-*P*. The labelled compounds were obtained after 20 min incubation of erythrocytes with [^{32}P]P_i.

	Relative specific radioactivity (%)				
	4-*P* plus 5-*P*		4-*P*	5-*P*	5-*P*/4-*P*
Expt.	Before	After incubation			
1	6.4	6.7	4.7	8.7	1.9
2	8.5	8.5	4.6	12.5	2.7

The phosphorylation fluxes into PtdIns4*P* and into PtdIns(4,5)P_2 calculated with these data were then in the ranges 22–14 and 94–46 nmol·h^{-1}·ml of cells^{-1} respectively.

From these results we conclude that most probably only a minor part of the total PtdIns(4,5)P_2 pool takes part in the rapid turnover of its phosphomonoester groups, whereas the major part shows a much slower turnover. The existence of two metabolically different compartments may also be derived from the data for the PtdIns4*P* pool. The metabolically active fraction of the total pool, however, is not as small as that of PtdIns(4,5)P_2.

These conclusions are supported by data on the ratio of specific radioactivities (5-*P*/4-*P*) of PtdIns(4,5)P_2. We found a ratio of 2–3 after 20 min labelling time (Table 3). The ratio reported by Hawkins *et al.* (1984) was about 2 after 120 min labelling time. A computer run with the program described in the Materials and methods section predicted ratios of 9 and 1.1 respectively in the absence of any compartmentation of polyphosphoinositides after 20 and 120 min. This is not in agreement with the experimental values. The ratio was, however, predicted to be about 3 after 20 min labelling time, when compartmentation factors of 0.35 and 0.075 were assumed for PtdIns4*P* and PtdIns(4,5)P_2 respectively (cf. legend to Fig. 1).

Turnover of polyphosphoinositides in isolated erythrocyte membranes

Turnover rates of phosphomonoester groups of polyphosphoinositides were determined in isolated erythrocyte membranes for comparison with those in intact erythrocytes. Ghosts were preincubated with unlabelled ATP so that newly synthesized polyphosphoinositides contained no radioisotope. Under our conditions of membrane preparation, the main phase of net synthesis was completed after 20 min preincubation. This made it possible to study turnover rates under steady-state conditions with fairly constant pool sizes of all inositides close to those in intact cells during a further 60 min incubation with [γ-^{32}P]ATP. Longer incubation periods were avoided because a constant decrease in polyphosphoinositide pool sizes was then observed.

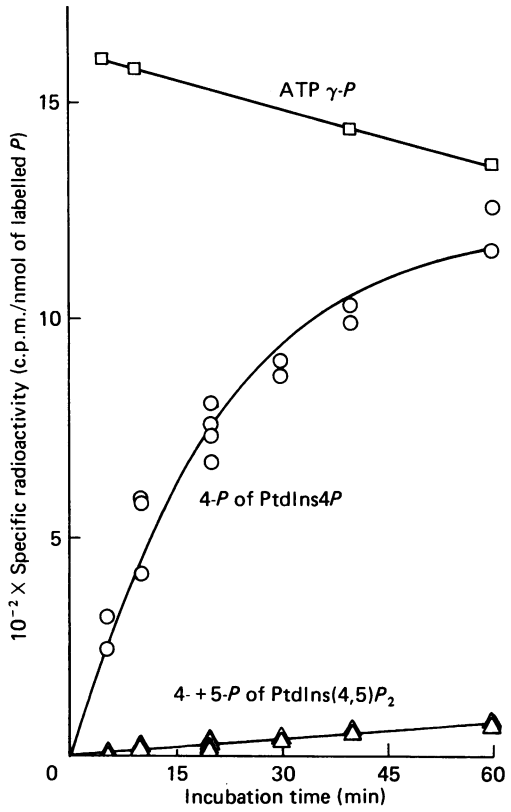

Fig. 2. Radiolabelling time courses in isolated erythrocyte membranes per unit dosage of [γ-^{32}P]ATP

□, ATP γ-*P*; ○, 4-*P* of PtdIns4*P*; △, 4-*P* plus 5-*P* of PtdIns(4,5)P_2. Continuous lines are computer-generated best fits of the data, assuming homogeneous pools of PtdIns4*P* and PtdIns(4,5)P_2 without compartmentation. Ghosts were incubated in an iso-osmotic medium simulating conditions *in vivo* at 37 °C and pH 7.2 in the presence of 3.5 mM-$MgCl_2$ and 2 mM-ATP under steady-state conditions. After 20 min preincubation with unlabelled ATP, the labelling was started by the addition of [γ-^{32}P]ATP. The data were derived from two experiments with two different samples of erythrocyte membranes.

After 60 min of incubation the specific radioactivity of PtdIns4*P* (Fig. 2) was clearly approaching radioactive equilibrium with that of the precursor [γ-^{32}P]ATP, the specific radioactivity of which somewhat decreased during this time owing to traces of adenylate kinase. The isotope incorporation into PtdIns(4,5)P_2 was much slower than that into PtdIns4*P*. Computer evaluation of the experimental data with a program analogous to that applied for data evaluation of intact erythrocytes revealed the following. Assuming homogeneous pools for both inositides without any compartmentation, i.e. $\phi_{PtdIns4P} = \phi_{PtdIns(4,5)P_2} = 1$, the best fit gave the continuous lines in Fig. 2 with $k_1 = 0.0345$ min^{-1} and $k_3 = 0.00114$ min^{-1}. The flux rates from the γ-*P* of ATP into the 4-*P* of PtdIns4*P* and 5-*P* of PtdIns(4,5)P_2 calculated with these data and those of Table 2 (last line) are then 35 and 3 nmol·h^{-1}·ml of cells^{-1} respectively.

When these data are compared with those of the foregoing section, one can conclude that the flux into 4-*P*

of PtdIns4*P* is somewhat higher in isolated erythrocyte membranes and that into 5-*P* of PtdIns(4,5)P_2 more than one order of magnitude lower than those obtained for intact erythrocytes. Taking into account the scatter of the data, one may be safe in concluding that the flux rate into 4-*P* of PtdIns4*P* in isolated erythrocyte membranes is in the same range as that observed in intact erythrocytes. In addition, in this membrane preparation there is no hint of a compartmentation of PtdIns4*P*.

Concerning PtdIns(4,5)P_2, the flux rate into 5-*P* is clearly very much lower in isolated membranes than in intact erythrocytes. Because of its slow turnover, the data presented do not allow conclusions concerning a compartmentation of PtdIns(4,5)P_2 in isolated membranes, a situation that is shown to be highly probable in intact cells.

DISCUSSION

The results of the present labelling studies were obtained by using an improved chromatographic procedure for isolating the three inositides in a pure state and in quantities sufficient for simultaneous determinations of pool sizes and specific radioactivities of their labelled phosphomonoester groups. The short-term sampling applied was expected to define the dynamics of the polyphosphoinositides in more detail than previous long-term experiments.

With regard to PtdIns4*P* and PtdIns(4,5)P_2 pool sizes, our results agree with those reported previously (Minenko *et al.*, 1981; Maretzki *et al.*, 1983; Girand *et al.*, 1984) for human erythrocytes and with those reported for pig erythrocytes and pig erythrocyte membranes (Peterson & Kirschner, 1970; Schneider & Kirschner, 1970).

After incubation of our ghosts with concentrations of ATP, Mg^{2+} and K^+ close to those in intact erythrocytes, we found a small, but distinct, increase of the PtdIns4*P* pool size. From experiments with isolated erythrocyte membranes (Schneider & Kirschner, 1970; Buckley & Hawthorne, 1972; Quist, 1982), it is known that a remarkable net synthesis of polyphosphoinositides occurs after their pool sizes have decreased during storage of blood and/or during the process of membrane isolation. Since we observed only small changes after incubation in a medium allowing net synthesis of polyphosphoinositides under simulated conditions *in vivo*, we conclude that the pool sizes shown in Table 2 are close to those in intact erythrocytes.

It has been claimed (Allan, 1982) that significant amounts of PtdIns may be 'artefactual' in origin, since it is possible to transform nearly all PtdIns of human and pig erythrocyte membranes into polyphosphoinositides under extreme incubation conditions (Schneider & Kirschner, 1970; Buckley & Hawthorne, 1972). This conclusion does not take into account, however, the dependence of the enzyme-catalysed cycles of phosphorylation and dephosphorylation of the inositol ring on the available intracellular effectors.

There are only a few quantitative data on the extent of tracer fluxes from [γ-^{32}P]ATP into the phosphomonoester groups of polyphosphoinositides in human erythrocytes. Recently, Maretzki *et al.* (1983) calculated with their long-term labelling data a share of about 20% ATP consumption for the polyphosphoinositide phosphorylation cycle. Irrespective of the extent of compartmentation of polyphosphoinositides, our turnover rates are much lower. Assuming an ATP production of 2–3 μmol·h^{-1}·ml of cells^{-1} (Jakobasch *et al.*, 1974*b*; Till *et al.*, 1977), an analogous calculation with our data reveals that the turnover of polyphosphoinositides utilizes no more than 2–5% of the total energy output of erythrocytes under steady-state conditions. We suppose that differences in the experimental techniques might be responsible for this discrepancy.

A further important result of the present study is the tracer kinetic evidence that only a limited fraction of the total polyphosphoinositides of erythrocytes seems to take part in the rapid tracer exchange, whereas the main part is metabolically more inert. Further experiments with different other methods are, however, needed to confirm this result and to elucidate the nature of this apparent compartmentation.

With regard to isolated membranes, the radiolabelling time course of 4-*P* of PtdIns4*P* and the steady-state flux rates calculated with these data clearly indicate that (*a*) the enzymes catalysing the phosphorylation–dephosphorylation cycle of PtdIns4*P* are present in our membrane preparation and (*b*) that the whole PtdIns4*P* pool is available to these enzymes. Quantitatively, this flux is in the same range in isolated membranes incubated under simulated conditions *in vivo* and in intact erythrocytes. The calculated steady-state flux rate is comparable with that reported by Quist (1982) for rabbit erythrocyte membranes, but lower than that reported by Schneider & Kirschner (1970) for pig erythrocyte membranes. A few other data in the literature are not comparable because they do not differentiate between net synthesis and steady-state fluxes.

With regard to the very low flux rate into 5-*P* of PtdIns(4,5)P_2, our results indicate that either the enzymes involved are partly lost in the membrane preparation or PtdIns(4,5)P_2 has become available to these enzymes only to a very limited extent. It is known (Roach & Palmer, 1981) that a highly active PtdIns(4,5)P_2-specific phosphomonoesterase is located in the cytosol of human erythrocytes which is lost in the process of membrane preparation. On the other hand, the results reported by Quist (1982) and Marche *et al.* (1982) for rabbit and pig erythrocyte membranes suggest that changing amounts of PtdIns(4,5)P_2 are available to the enzymes of its turnover under different incubation conditions. Further experiments are needed to elucidate the reasons for the very low turnover of PtdIns(4,5)P_2 in isolated human erythrocyte membranes.

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Received 5 June 1985/28 October 1985; accepted 2 January 1986