Phosphomonoesterase Hydrolysis of Polyphosphoinositides in Rat Kidney

PROPERTIES AND SUBCELLULAR LOCALIZATION OF THE ENZYME SYSTEM

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The properties of diphosphoinositide and triphosphoinositide phosphatases from rat kidney homogenate were studied in an assay system in which non-specific phosphatase activity was eliminated. The enzymes were not completely metal-ion dependent and were activated by Mg^{2+} . The detergents sodium deoxycholate, Triton X-100 and Cutscum inhibited the reaction; cetyltrimethylammonium bromide only activated when added with the substrate and in the presence of Mg^{2+} . Both enzymes had a pH optimum of 7.5. Ca^{2+} and Li^+ both activated triphosphoinositide phosphatase, but Ca^{2+} inhibited and Li^+ had little effect on diphosphoinositide phosphatase. Cyclic AMP had no effect on either enzyme. The enzymes were three times more active in kidney cortex than in the medulla. On subcellular fractionation of kidney-cortex homogenates by differential and density-gradient centrifugation, the distribution of the enzymes resembled that of thiamin pyrophosphatase (assayed in the absence of ATP), suggesting localization in the Golgi complex. However, the distribution differed from that of the liver Golgi marker galactosyl-transferase. Activities of both diphosphoinositide and triphosphoinositide phosphatases and thiamin pyrophosphatase were low in purified brush-border fragments. Further experiments indicate that at least part of the phosphatase activity is soluble.

Sloane-Stanley (1953) showed that brain phosphoinositide fractions were rapidly attacked by brain homogenates with the release of free and combined phosphate as well as free and combined inositol. Rodnight (1956) later performed experiments to suggest that two enzymes, a phosphomonoesterase and a phosphodiesterase, were present. Work with pure triphosphoinositide (phosphatidyl-*myo*-inositol 4,5-bisphosphate)‡ indicated that the lipid was broken down first to diphosphoinositide (phosphatidyl-*myo*-inositol 4-phosphate) and then to phosphatidylinositol (Dawson & Thompson, 1964; Thompson & Dawson, 1964):

Andrade & Huggins (1964) using a phosphoinositide complex from kidney showed that the mono-

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‡ The names diphosphoinositide and triphosphoinositide are retained in the text since they are widely known and there is as yet no internationally agreed systematic nomenclature.

esterified phosphate of the polyphosphoinositides is more active metabolically than the diesterified phosphate. Similarly, Santiago-Calvo *et al.* (1964) found a high turnover of the phosphoinositides in various tissues. Phosphatidylinositol bisphosphate phosphatase (EC 3.1.3.36) has been investigated in brain (Dawson & Thompson, 1964; Salway *et al.*, 1967; Sheltawy *et al.*, 1972) and kidney (Lee & Huggins, 1968*a,b*), but work on the subcellular distribution of this enzyme has led to conflicting observations. Salway *et al.* (1967) and Harwood & Hawthorne (1969) studying brain found that triphosphoinositide phosphatase was localized in the particle-free cytoplasmic fraction. Under different assay conditions, using 'pH 5 supernatant' as activator, Sheltawy *et al.* (1972) reported a distribution of the brain phosphatase similar to that of the plasma-membrane marker 5'-nucleotidase, but different from that of alkaline phosphatase. However, Lee & Huggins (1968*a,b*), using a very simple assay procedure for the enzyme, found enrichment in the microsomal fraction of kidney, the distribution being similar to that of glucose 6-phosphatase. The questions that needed an answer were as follows. (1) In an assay eliminating non-specific phosphatase activity, was the enzyme still membrane-bound? (2) If so, was it specifically associated with the plasma membrane? (3) In optimum assay conditions, worked out for both triphosphoinositide and diphosphoinositide, are the two activities similarly distributed? This paper reports

the development of assays, the characterization and the subcellular distribution of the two phosphatases.

Materials and Methods

Materials

N-Acetylglucosamine, UDP-galactose, Dowex 2 (X8), sodium pyruvate, rotenone, ouabain, 5-methylresorcinol and thiamin pyrophosphate were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Silica CC7 was obtained from Canlab Ltd. (Edmonton, Alberta, Canada) and UDP-[U-¹⁴C]galactose from Atomic Energy of Canada Ltd., Ottawa, Canada. Cutscum (iso-octylphenoxy-polyethoxyethanol) was obtained from Kodak Ltd. (Kirkby, Liverpool, U.K.) and Triton X-100 from Lennig Chemicals Ltd. (Jarrow, Co. Durham, U.K.).

Methods

Preparation of homogenate. In all experiments 3-month-old Sprague-Dawley rats of either sex maintained on a diet of rat-cake and water *ad libitum* were used. The rats were killed by a blow to the neck and the kidneys quickly removed and homogenized in 0.32M-sucrose adjusted to pH 7.2 with Tris-HCl buffer (5 ml/g of tissue). A Teflon-glass Potter homogenizer was used with a radial clearance of 0.2 mm and a pestle rotating at 600 rev./min. Six complete strokes were made over a period of 1 min at 4°C. Further sucrose was added to give a 10% (w/v) homogenate and the suspension was filtered through muslin cloth.

Preparation of subcellular fractions. The kidneys were homogenized as described above using three up-and-down strokes of a Potter or Dounce homogenizer, the pestle (radial clearance 0.2 mm) rotating at 150 rev./min. The homogenate was then fractionated by the method of Sedgwick & Hübscher (1965).

Subfractionation of microsomal membranes. The microsomal fraction was prepared from the kidney cortices from three rats. The homogenate was centrifuged at 10000g for 20 min and the resulting pellet was washed three times with the 0.32M-sucrose solution. The microsomal fraction was then recovered by centrifuging the supernatant at 150000g for 1 h. The pellets were resuspended in a small volume of 0.25M-sucrose and subfractionated by the procedure of Clarke & Dawson (1972).

Brush-border preparation. Kidney cortex was fractionated by the method of Kinne & Kinne-Saffran (1969) to yield a preparation of brush-border fragments.

Preparation of substrates. The polyphosphoinositides were prepared by column chromatography on either DEAE-cellulose (Hendrickson & Ballou, 1964) or oxalate-impregnated silica gel (Cooper &

Hawthorne, 1973). Purity of the samples was shown by thin-layer and paper chromatography. Emulsions of the lipids (sodium salts) in water were prepared for use in the enzyme assays and could be stored at 4°C for 6 months without detectable breakdown.

Chemical methods. Protein was estimated by the method of Weichselbaum (1946) with bovine serum albumin (fraction F) as standard. P_i was determined either by the method of King (1932) (for 5–60 µg of phosphorus) or that of Baginski *et al.* (1967) (for 0.3–8 µg of phosphorus). DNA was estimated by the method of Burton (1956). The initial extraction was with hot trichloroacetic acid by the method of Schneider (1957). RNA was determined by the method of Hatcher & Goldstein (1969) with CdCl₂ as precipitant.

Triphosphoinositide phosphatase (EC 3.1.3.36). The final assay system was developed as described in the Results section and its composition was as follows. The mixture had a total volume of 0.5 ml and the following components were preincubated at 0°C for 10 min: 45 mM-Tris-HCl buffer, pH 7.5; 1 mM-MgCl₂; 1 mM-NaF; 3 mM-cysteine (freshly prepared), pH 7.5; 4 mg of bovine serum albumin (type F; fatty acid-free) and about 0.5 mg of homogenate protein. After a further preincubation for 2 min in a shaking water bath at 37°C, the reaction was started by the addition of 100 µl of a mixture of triphosphoinositide (sodium salt) and cetyltrimethylammonium bromide (Cetavlon) in a molar ratio of 1:2 (made by mixing equal volumes of 10 mM-triphosphoinositide emulsion in water and 20 mM-Cetavlon). After incubation at 37°C for 30 min with continuous shaking, the reaction was stopped by the addition of 0.1 ml of 5% (w/v) bovine serum albumin (fraction V) followed, after shaking, by 0.6 ml of chilled 10% (w/v) trichloroacetic acid. After 10 min at 0°C the precipitate was removed by centrifugation at 1000g for 10 min and P_i release was determined in the clear supernatant. When the method of Baginski *et al.* (1967) is used, it is important to ensure that the concentration of trichloroacetic acid in the sample is 10–15% (w/v). Generally 0.5 ml of the supernatant was taken and made up to 1 ml with 0.5 ml of 20% trichloroacetic acid. In each experiment, controls were also incubated, one without substrate and one without tissue preparation, to correct for endogenous release of P_i and for traces of P_i in the substrate.

Diphosphoinositide phosphatase (EC 3.1.3.36). This was assayed under the same conditions, except that the MgCl₂ concentration was 1.5 mM and the reaction was started by the addition of 150 µl of a mixture of diphosphoinositide (sodium salt) and Cetavlon in an equimolar ratio (made by suitably mixing 10 mM-diphosphoinositide emulsion in water and 20 mM-Cetavlon).

Enzyme assays. Cytochrome oxidase (EC 1.9.3.1) was assayed basically by the method of Wharton &

Tzagoloff (1967) except that 0.1% Triton X-100 and 5mg of bovine serum albumin (fatty acid-free) were included.

For catalase (EC 1.11.1.6), the composition of the assay mixture was as follows: 10mM-imidazole-HCl buffer, pH7.2; 1% (v/v) Triton X-100; 0.1% bovine serum albumin (fatty acid-free); enzyme sample, in a total volume of 0.4ml. After preincubation of the assay mixture for 10min at 0°C, the reaction was started by the addition of 0.1ml of 100mM-H₂O₂ (also precooled to 0°C). Incubation was for 5min and the reaction was terminated by the addition of 2.4ml of titanium sulphate reagent (Snell & Snell, 1949). Water (3ml) was added and the tubes were well mixed. The yellow complex was then assayed spectrophotometrically at 400nm. Enzyme-free blanks were used to determine the original concentration of H₂O₂ and standards of H₂O₂ were assayed concurrently. The enzyme does not exhibit zero-order kinetics, but the reaction rate was proportional to enzyme concentration under the conditions used. Only 0.02% of the substrate was used up during the incubation.

Acid phosphatase (EC 3.1.3.2) was assayed in the presence of 4mM-EDTA and 0.1% Triton X-100 (Hübscher & West, 1965). For β -galactosidase (EC 3.2.1.23) the method of Sellinger *et al.* (1960) was used. Glucose 6-phosphatase (EC 3.1.3.9) was assayed in the presence of 4mM-EDTA and 2mM-KF as described by Hübscher & West (1965). Stetten & Ghosh (1971) found that the liver enzyme was stimulated by preincubation with NH₄⁺ ions. No increase in activity was found with the kidney enzyme and this preliminary operation was omitted.

For arylesterase (EC 3.1.1.2), the assay of Shephard & Hübscher (1969) was used except that 5mg of bovine serum albumin (fatty acid-free) was added to ensure linearity between protein concentration and product formed. (Na⁺+K⁺)-stimulated adenosine triphosphatase (EC 3.6.1.3) was assayed by the method of Kai *et al.* (1968) except that the blank without Na⁺ and K⁺ ions included 1mM-ouabain. Jørgensen & Skou (1969) reported stimulation of rabbit kidney (Na⁺+K⁺)-adenosine triphosphatase

by sodium deoxycholate; however, this was not observed in rat kidney homogenate. Ca²⁺-activated adenosine triphosphatase (EC 3.6.1.3) was assayed by the method of Parkinson & Radde (1971). K⁺-stimulated nitrophenylphosphatase was assayed by the method of Kinne & Kinne-Safran (1969).

Thiamin pyrophosphatase was assayed by the method of Yamazaki & Hayaishi (1968), except that 3mM-cysteine and 1mM-NaF were included to eliminate non-specific alkaline and acid phosphatases respectively. The enzyme was assayed in the presence and absence of 0.2mM-ATP as activator. ATP was included in the no-substrate blank to allow for ATPase (adenosine triphosphatase) activity. For galactosyltransferase the assay of Fleischer *et al.* (1969) was used and for alkaline phosphatase (EC 3.1.3.1) the methods of Kinne & Kinne-Safran (1969) or Hübscher & West (1965) were used. Lactate dehydrogenase (EC 1.1.1.27) was assayed as described by Salway *et al.* (1967) except that 20 μ l of a saturated ethanolic solution of rotenone was included to prevent mitochondrial NADH dehydrogenase activity (Dr. P. G. Shakespeare, personal communication) and the reaction started by the addition of pyruvate.

Results and Discussion

Preliminary subcellular fractionation of kidney cortex

Two rat kidneys were homogenized in 0.32M-sucrose, pH7.5, filtered through muslin and made up to 10% (w/v) with more sucrose solution. The homogenate was centrifuged at 4300g for 10min to pellet nuclei, mitochondria and cell debris. After three washings with sucrose solution, the combined supernatants were centrifuged at 100000g for 60min to sediment membranes and leave the particle-free supernatant. Triphosphoinositide phosphatase was then assayed in a system containing 45mM-Tris-HCl buffer, pH7.2, kidney homogenate (2mg of protein) and water to a final volume of 1ml. After 30min the reaction was stopped by the addition of ice-cold 10% (w/v) trichloroacetic acid and P_i in the supernatant determined. The results in Table 1 show

Table 1. *Preliminary subcellular fractionation of kidney cortex*

For details see the text. The relative specific activity is relative to the homogenate.

Sample	Protein		Triphosphoinositide phosphatase		
	Total (mg)	Total (%)	Specific activity	Total (%)	Relative sp. activity
Homogenate	223.5	100.0	2.37	100.0	1.00
Nuclear-mitochondrial fraction	83.0	37.2	1.44	22.6	0.61
Membrane fraction	60.6	27.2	3.69	42.1	1.55
Supernatant	77.6	34.8	5.74	84.3	2.42
Recovery (%)		99.2		149.0

Fig. 1. Effect of NaF, EDTA and cysteine on (a) alkaline phosphatase, (b) acid phosphatase, (c) triphosphoinositide phosphatase and (d) diphosphoinositide phosphatase

For the polyphosphoinositide phosphatases the reaction mixture had a volume of 1 ml and the following composition: 45 mM-Tris-HCl buffer, pH 7.2; kidney homogenate (2 mg of protein); EDTA (○), cysteine (▲) or NaF (●) in various amounts. These compounds were incubated at 0°C for 10 min. After warming to 37°C, the reaction was started by the addition of 0.1 ml of 10 mM-phosphoinositide and stopped after 10 min by the addition of 0.2 ml of 5% bovine serum albumin, followed by 1.2 ml of ice-cold 10% (w/v) trichloroacetic acid. Alkaline and acid phosphatase assays are described in the text.

most of the activity in the supernatant fraction, which would tend to confirm the results found for brain (Salway *et al.*, 1967; Harwood & Hawthorne, 1969), but contradict those of Lee & Huggins (1968a,b) who claimed the kidney enzyme to be microsomal. The recovery of the enzyme was high, suggesting latency, but freezing and thawing did not increase activity.

Because of these results it was decided to characterize the polyphosphoinositide phosphatase in the homogenate. If the enzymes were characterized in the supernatant that showed highest activity, subsequent subcellular fractionation experiments could be misleading, especially in view of the apparent latency.

Elimination of acid and alkaline phosphatase activities

Since non-specific phosphatase activity is extremely high in kidney, and since alkaline phosphatase, at least, can hydrolyse the polyphosphoinositides (Prottey *et al.*, 1968), it was necessary to eliminate any contributions by these enzymes in the polyphosphoinositide phosphatase assays. Hübscher & West (1965) have shown that EDTA inhibits alkaline phosphatase and NaF inhibits acid phosphatase in cat intestine. To show whether these inhibitors are also specific for the rat kidney enzymes, alkaline and acid phosphatase were assayed in their presence with 2-glycerophosphate as substrate. A 10 min preincubation was used to allow the inhibitors to act. The results

Table 2. Metal-ion requirements of polyphosphoinositide phosphatases

The assay conditions are given in the legend to Fig. 2.

Metal ion (2mM)	Triphosphoinositide phosphatase (nmol of P _i /min per mg of protein) Undialysed	Diphosphoinositide phosphatase (nmol of P _i /min per mg of protein)	
		Before dialysis	After dialysis
—	6.02	10.48	10.40
Mg ²⁺	10.08	9.53	15.12
Mn ²⁺	0.76	3.95	7.80
Ca ²⁺	1.70	7.96	11.13
Zn ²⁺	0	0	10.64
Hg ²⁺	0	1.36	9.45
Ba ²⁺	0.19	6.60	9.92
Co ²⁺	5.36	2.93	—
Cd ²⁺	0	0.61	—
Fe ²⁺	0	0	—
Ni ²⁺	4.96	6.46	—
Cu ²⁺	0.29	1.56	—
Sr ²⁺	1.87	8.71	—

Fig. 2. Effect of Mg²⁺ on polyphosphoinositide phosphatases

Assay conditions follow those of Fig. 1 except that EDTA was omitted and 3 mM-cysteine included. Triphosphoinositide phosphatase: ○, undialysed. Diphosphoinositide phosphatase: ■, dialysed; ●, undialysed.

in Fig. 1 show that EDTA is a potent inhibitor of alkaline phosphatase, but slightly stimulates acid phosphatase; NaF, however, completely inhibits acid phosphatase but is without effect on alkaline phosphatase. Polyphosphoinositide phosphatases were then assayed in a simple system in the presence of these inhibitors. Fig. 1 shows that although NaF had no effect up to a concentration of 20 mM, EDTA gave a constant inhibition of about 50%. This agrees with the results of Dawson & Thompson (1964), who also found that the inhibition of brain triphosphoinositide phosphatase by EDTA was not progressive. Since this would seem to indicate metal dependency, it was necessary to find another inhibitor for alkaline phosphatase. Cysteine was found to be suitable for this purpose, complete inhibition of the enzyme being obtained at 3 mM (Fig. 1). Cysteine was completely without effect on acid phosphatase, triphosphoinositide phosphatase or diphosphoinositide phosphatase at concentrations up to 20 mM. Under the final assay conditions for polyphosphoinositide phosphatases, with cysteine and NaF as inhibitors of non-specific phosphatase activity, no P_i was released from 2-glycerophosphate by kidney homogenate. Though the homogenate showed little non-specific phosphatase activity towards the polyphosphoinositides (Fig. 1) the assay is designed for subcellular fractions (e.g. brush-border membranes) which may be high in phosphatase.

Effect of detergents

Three different types of detergent were used: (i) non-ionic, Cutscum and Triton X-100 (0.1–1.0%);

(ii) cationic, Cetavlon (0.5–2.0 mM); (iii) anionic, sodium deoxycholate (0.1–0.5%).

Kidney homogenate was preincubated for 10 min at 0°C with one of the above detergents in the assay mixture and the reaction started by the addition of substrate (or substrate–Cetavlon premixed). After 30 min incubation the reaction was stopped and P_i determined. Inhibition was obtained with all four detergents, the strongest (80%) being with 0.5% sodium deoxycholate. Diphosphoinositide phosphatase, however, was unaffected by Cetavlon when the reaction was started with substrate–Cetavlon premixed.

Metal-ion activation (Table 2)

Triphosphoinositide phosphatase was activated only by Mg²⁺ ions. Diphosphoinositide phosphatase was not activated by any of the ions tested. However, when the homogenate was dialysed against double-de-ionized water overnight, Mg²⁺ activated the enzyme. The Mg²⁺ ion-requirement curves are shown in Fig. 2. The optimum for triphosphoinositide phosphatase was 1 mM-Mg²⁺, above which concentration there was inhibition. With dialysed homogenate,

Fig. 3. Effect of Cetavlon on polyphosphoinositide phosphatases

The conditions are those of Fig. 2 with the following exceptions. The reaction was started by the addition of the substrate-Cetavlon mixture. Triphosphoinositide phosphatase: ○, 1 mM-MgCl₂ throughout; diphosphoinositide phosphatase: ●, 1.5 mM-MgCl₂; ■, dialysed homogenate, 1.5 mM-MgCl₂; ▲, no MgCl₂.

diphosphoinositide phosphatase required 1.5 mM-Mg²⁺ for optimum activity, but Mg²⁺ had no effect on the undialysed enzyme. The reason for this difference is not known and solution of the problem would require investigation of the purified enzymes.

Effect of Cetavlon in the presence of Mg²⁺

Triphosphoinositide phosphatase. When the reaction was started by the addition of substrate-Cetavlon premixed in various amounts (0.1 ml of 10 mM-triphosphoinositide with 0.1–0.5 ml of 5 mM-Cetavlon), with Mg²⁺ present, activity of the enzyme was almost doubled when the detergent concentration reached 2 mM (Fig. 3).

Diphosphoinositide phosphatase. Since the metal-ion-requirement experiments had shown that this phosphatase was only activated by Mg²⁺ when the enzyme preparation was dialysed, the effect of Cetavlon on both dialysed and non-dialysed homogenate was studied (Fig. 3). The reaction was started by a substrate-Cetavlon mixture as above. In the absence of Mg²⁺, Cetavlon inhibited the phosphatase at concentrations above 1.5 mM. With dialysed homogenate in the presence of 1.5 mM-Mg²⁺, the detergent inhibited activity at all concentrations. However, with non-dialysed homogenate activity rose by over 50%, reaching a peak at 1.0–1.5 mM, above which there was marked inhibition.

The fact that the cationic detergent Cetavlon has to be added with the substrate before activation occurs would indicate that the enzyme is less able to dephosphorylate the substrate until the negative charges on the monoesterified phosphate groups have been decreased or neutralized. These conclusions were reached by Dawson & Thompson (1964) in their studies on brain triphosphoinositide phosphatase. A long-chain base, such as Cetavlon, would tend to lie alongside a phosphoinositide molecule with the polar heads adjacent and the long hydrocarbon chains attracted together by Van der Waals forces. Since no Cetavlon activation of diphosphoinositide phosphatase was observed with dialysed homogenate and Mg²⁺, it is possible that a third factor present in the non-dialysed preparation can also activate this enzyme. The pH 5-supernatant factor of Sheltawy *et al.* (1972) appears to be different, since it resists dialysis (Dawson & Thompson, 1964).

Substrate requirements (Fig. 4)

In the absence of Cetavlon, the substrate concentration curve for triphosphoinositide phosphatase showed a peak at 2 mM substrate, above which concentration there was loss of activity. When Cetavlon was added with the substrate, the optimum concentration was lowered to 1 mM, marked inhibition occurring at higher concentrations. For diphosphoinositide phosphatase essentially the same picture was obtained. However, in the absence of Cetavlon, the optimum was reached at 3 mM substrate and activity remained constant up to 9 mM, beyond which inhibition occurred. In the presence of Cetavlon there was marked inhibition at concentrations above the optimum 1.5–2 mM.

The very sharp peaks obtained when Cetavlon was used seemed to indicate that the molar ratio of Cetavlon/substrate was important. To test this the phosphatases were assayed by starting the reaction with substrate-detergent mixtures in different ratios. The final concentrations of triphosphoinositide and diphosphoinositide were kept constant at 1 and 2 mM respectively. Fig. 5 shows that for triphosphoinositide phosphatase the critical lipid/Cetavlon molar ratio is 1:2, whereas for the diphosphoinositide enzyme it is 1:1. When the substrate curve was re-determined, keeping the molar ratio of Cetavlon/diphosphoinositide constant at 1:1, a plateau was reached at 2 mM as in the more usual enzyme-substrate curve (Fig. 4c). A similar result was obtained for triphosphoinositide phosphatase when the Cetavlon/lipid ratio was kept constant (Fig. 4a).

Reaction rates

The mixture (total vol. 25 ml) contained 45 mM-Tris-HCl buffer, pH 7.2, 1 mM-NaF, 3 mM-cysteine and 1.5 mM-MgCl₂. After 5 min preincubation at 37°C

Fig. 4. Effect of substrate concentration

Conditions are given in the legend to Fig. 3 except that the reaction volume was decreased to 0.5 ml and the reaction was started with a substrate-Cetavlon mixture. (a) Triphosphoinositide phosphatase, (b) and (c) diphosphoinositide phosphatase. ■, Constant lipid/Cetavlon molar ratio (a, triphosphoinositide/Cetavlon 1:2; b, diphosphoinositide/Cetavlon 1:1); ▲, 2 mM-Cetavlon (triphosphoinositide) or 1 mM-Cetavlon (diphosphoinositide) in all assays; ●, without Cetavlon.

Fig. 5. Effect of substrate-Cetavlon molar ratio on polyphosphoinositide phosphatases

Conditions are given with Fig. 3 except that the concentration of diphosphoinositide was 2 mM and triphosphoinositide was 1 mM. Cetavlon was added with the substrate in the ratios shown. ●, Triphosphoinositide; ○, diphosphoinositide.

the reaction was started by the addition of equimolar diphosphoinositide-Cetavlon mixture to give a final concentration of 2 mM-diphosphoinositide. With triphosphoinositide as substrate the lipid-Cetavlon mixture added gave final concentrations of 2 mM-Cetavlon and 1 mM-triphosphoinositide. Duplicate 1 ml samples were removed at different time-intervals and pipetted into 0.2 ml of 5% (w/v) bovine serum albumin. After shaking, 1.2 ml of ice-cold 10% trichloroacetic acid was added. P_1 was determined after centrifugation.

For triphosphoinositide phosphatase the reaction rate was constant for 30 min, activity falling off after this time. The reaction rate of diphosphoinositide phosphatase, on the other hand, fell off almost immediately. One explanation is that an inhibitor (possibly fatty acid from endogenous phospholipase A) was being produced. Since albumin has an affinity for fatty acids, its effect on the phosphatases was studied. Triphosphoinositide phosphatase was slightly stimulated, but diphosphoinositide phosphatase was considerably activated, the optimum bovine serum albumin concentration being 8 mg/ml. In the presence of albumin at this concentration, the reaction rate was constant for 30 min.

Enzyme concentration

The reaction rate for diphosphoinositide phosphatase was proportional to enzyme concentration over the range 0–1.6 mg of homogenate protein. However,

Fig. 6. Effect of pH on polyphosphoinositide phosphatases

For assay conditions see Fig. 5; 4mg of albumin was included. ■, Diphosphoinositide phosphatase; ●, triphosphoinositide phosphatase. A Tris-HCl buffer was used at each pH value.

the curve for triphosphoinositide phosphatase only became linear when 8 mg of bovine serum albumin/ml was included.

Effect of pH

Measurement of the pH before and after the reaction showed that no variation had taken place. Both enzymes showed a peak at pH 7.5 (Fig. 6). This is lower than the optimum found for triphosphoinositide phosphatase by Lee & Huggins (1968a), but the difference may be due to the fact that alkaline phosphatase activity was not eliminated in their system.

Effect of Ca^{2+}

In a partially purified preparation of triphosphoinositide phosphatase Dawson & Thompson (1964) found that the enzyme was activated by Ca^{2+} only if the Mg^{2+} concentration was sub-optimal. In the present work, Ca^{2+} activated kidney triphosphoinositide phosphatase both in the presence and the absence of 0.5 mM- MgCl_2 . Diphosphoinositide phosphatase, however, was inhibited at all concentrations of Ca^{2+} between 0.5 and 2 mM (Fig. 7). The lower activity of triphosphoinositide phosphatase with 2 mM- Ca^{2+} in Table 2 is due to the absence of Cetavlon.

Fig. 7. Effect of Ca^{2+} on polyphosphoinositide phosphatases

Assay conditions are given with Fig. 5, only the inclusion of 4 mg of albumin and MgCl_2 concentration being different. Triphosphoinositide phosphatase: ■, 0.5 mM- MgCl_2 ; ●, without MgCl_2 . Diphosphoinositide phosphatase: □, 0.5 mM- MgCl_2 ; ○, without MgCl_2 .

Effect of Li^+

Li^+ ions are known to decrease inositol transport in kidney (Pearson & Jenner, 1971) and brain (Allison & Stewart, 1971). Effects are also observed on the permeability of the epithelial membrane of toad urinary bladder (Bentley & Wasserman, 1972) and the action of antidiuretic hormone (Harris & Jenner, 1972). Triphosphoinositide phosphatase activity was increased by 40% in the presence of 3 mM- Li^+ ions. Diphosphoinositide phosphatase activity was only slightly increased.

Effect of cyclic AMP

Polyphosphoinositide phosphatases were assayed under optimum conditions in the presence of different concentrations of cyclic AMP (0.01–5 mM). An apparent stimulation of phosphate release occurred (up to 100% increase at 5 mM-cyclic AMP). However, in the presence of 10 or 20 mM-caffeine (which alone had no effect on the phosphatases) the effect was abolished. Caffeine itself is a potent inhibitor of 3':5'-cyclic AMP phosphodiesterase (EC 3.1.4.17;

Fig. 8. Mixed-substrate experiment with polyphosphoinositide phosphatase

The assay mixture had the following composition: 45 mM-Tris-HCl buffer, pH 7.2, 3 mM-cysteine, 1 mM-NaF, 1 mM-MgCl₂, 4 mg of bovine serum albumin, kidney homogenate (0.39 mg of protein), total volume 0.5 ml. The reaction was started by the addition of diphosphoinositide and stopped after 30 min at 37°C as described in the legend to Fig. 1. ○, Diphosphoinositide only; △, enzyme pre-incubated with 2 mM-triphosphoinositide for 2 min at 37°C.

Robison *et al.*, 1968), hence it was probable that the apparent stimulation of the phosphatases was really due to AMP hydrolysis. When AMP was included in the assay mixtures (without polyphosphoinositide) at the above concentrations, release of P_i was more than enough to account for any increase observed in the presence of cyclic AMP. Hence it was concluded that cyclic AMP had no effect on the polyphosphoinositide phosphatases.

Are there two separate polyphosphoinositide phosphatases?

The differing responses of triphosphoinositide and diphosphoinositide phosphatases to dialysis, metal-ion activation and detergent might suggest that each enzyme is a separate entity. The pH optimum was the same for each reaction, however, and other properties were similar, so a mixed substrate experiment was performed (Fig. 8). Addition of diphosphoinositide to enzyme already saturated with triphosphoinositide gave very little increase in reaction rate. This is the behaviour expected of one enzyme; with two separate enzymes an additive effect would be seen. It will be necessary to repeat this study with purified enzyme preparations, but for the present it can be concluded that there is only one phosphatase, capable of hydrolysing both diphosphoinositide and triphos-

Table 3. Activities of diphosphoinositide and triphosphoinositide phosphatase in kidney-cortex and medulla homogenates

Specific activity values are means $\pm$ S.D. for the number of preparations in parentheses.

Sample	Specific activity (nmol/min per mg of protein)	
	Diphosphoinositide phosphatase	Triphosphoinositide phosphatase
Cortex (6)	20.7 $\pm$ 2.5	14.8 $\pm$ 0.8
Medulla (3)	7.6 $\pm$ 0.4	5.1 $\pm$ 0.4

phoinositide. In the following sections therefore triphosphoinositide phosphatase refers to the enzyme assayed with triphosphoinositide as substrate and diphosphoinositide phosphatase indicates assays with diphosphoinositide as substrate.

Activities of triphosphoinositide and diphosphoinositide phosphatases in cortex and medulla

Kidney cortex and medulla were separated with the aid of a dissecting microscope. Each sample (two kidneys) was homogenized in 0.32 M-sucrose, pH 7.5, to a final concentration of 10% (w/v). Polyphosphoinositide phosphatases were then assayed under optimum conditions. Table 3 shows that both diphosphoinositide and triphosphoinositide phosphatase are about three times more active in the cortex than in the medulla. Further investigation of the subcellular distribution of these enzymes was performed with the cortex only.

Fractionation in Potter-Elvehjem or Dounce homogenizers

Kidney cortex was homogenized by using either a Potter-Elvehjem or a Dounce (spherical) pestle and fractionated as described in the Materials and Methods section. The inositide enzymes and markers were then assayed (Table 4 and Fig. 9). All enzymes investigated gave recoveries in the 90–110% region. The markers used and their presumed localization in the cell are shown in Table 5. The reason for assaying so many enzymes is twofold. (i) The kidney contains many cell types and therefore a particular enzyme may not be exclusively localized in one organelle in all the cells. (ii) The behaviour of organelles not normally investigated in this type of experiment (peroxisomes, Golgi apparatus etc.) was also studied. It is necessary to investigate as many cell components as possible so as to minimize the risk of confusing the investigated activity with the wrong organelle.

Assay of triphosphoinositide and diphosphoinositide phosphatases under optimum conditions showed much of the activity to be particulate. In the experiments with the Potter-Elvehjem homogenizer, the

Table 4. Subcellular distribution of markers and polyphosphoinositide phosphatase in kidney cortex

Specific activities are given as μg of DNA-P/mg of protein for DNA and nmol/min per mg of protein for the enzymes. (1) Fractionations using the Potter-Elvehjem homogenizer. The means $\pm$ s.d. of three experiments are shown except where indicated (* = mean of two experiments). (2) Fractionations using the Dounce homogenizer.

Percentage distribution in fractions									
Assay		Nuclear	Mitochondrial	Light		Microsomal	Supernatant	Recovery (%)	Homogenate sp. activity
				mitochondrial					
Protein	(1)	20.7 ± 2.2	14.7 ± 2.9	10.1 ± 1.0		14.8 ± 0.9	39.7 ± 1.9	98.6 ± 1.9	—
	(2)	31.5	15.0	6.5		10.7	36.3	99.5	—
DNA	(1)	90.7 ± 1.7	0.7 ± 0.0	2.3 ± 0.0		6.3 ± 1.8	0.0 ± 0.0	99.0 ± 1.8	1.68 ± 0.15
	(2)	89.9	3.8	1.5		4.9	0.0	99.1	1.57
Cytochrome c oxidase	(1)	16.9 ± 6.9	59.8 ± 11.3	15.7 ± 2.3		7.9 ± 1.7	0.0 ± 0.0	99.2 ± 1.4	111 ± 15
	(2)	55.0	34.7	7.7		2.7	0.0	99.2	33.6
β-Galactosidase	(1)	14.8 ± 0.3	5.9 ± 0.0	6.2 ± 0.6		3.5 ± 0.8	69.7 ± 4.6	104.4 ± 0.3	6.1 ± 2.4
	(2)	18.9	4.9	4.0		3.6	68.6	95.6	4.6
Acid phosphatase	(1)	21.7 ± 4.3	13.5 ± 3.0	13.6 ± 3.0		24.4 ± 3.4	26.8 ± 7.7	96.5 ± 2.2	55.6 ± 8.0
	(2)	40.0	16.4	12.2		20.2	11.2	93.3	44.3
Catalase	(1)*	7.3 ± 2.5	2.8 ± 0.8	10.0 ± 3.4		4.4 ± 1.9	75.5 ± 8.5	101.9 ± 2.0	102 ± 10
	(2)	12.2	1.9	28.5		15.2	42.3	92.0	50.0
Lactate dehydrogenase	(1)	3.2 ± 0.4	0.9 ± 0.8	1.5 ± 0.4		4.8 ± 6.2	89.8 ± 6.6	97.1 ± 3.1	650 ± 25
	(2)	9.8	1.6	1.8		8.9	77.9	95.4	258
Alkaline phosphatase	(1)	12.3 ± 6.2	8.6 ± 2.0	25.9 ± 5.1		46.0 ± 4.8	7.2 ± 4.0	94.7 ± 3.7	217 ± 49
	(2)	28.8	17.7	17.3		36.3	0.0	103.5	132
K ⁺ -stimulated nitrophenyl-phosphatase	(1)*	21.1 ± 2.9	14.3 ± 0.0	26.8 ± 4.1		28.6 ± 3.1	9.2 ± 4.3	97.1 ± 4.8	5.82 ± 0.70
	(2)	46.8	14.5	20.1		18.6	0.0	98.5	7.59
Ca ²⁺ -stimulated ATPase	(1)*	21.7 ± 0.6	11.6 ± 1.5	20.0 ± 2.7		46.7 ± 0.6	0.0 ± 0.0	108.6 ± 5.9	67.3 ± 21.6
	(2)	34.6	21.9	10.9		30.9	1.7	93.4	177
(Na ⁺ + K ⁺)-stimulated ATPase	(1)*	17.5 ± 4.3	12.6 ± 10.0	39.4 ± 5.4		30.5 ± 8.8	0.0 ± 0.0	101.0 ± 3.9	79 ± 9.9
	(2)	—	—	—		—	—	—	—
Arylesterase	(1)	10.0 ± 5.2	4.0 ± 1.4	12.8 ± 8.1		60.7 ± 9.0	12.5 ± 5.6	95.8 ± 5.1	127 ± 17
	(2)	14.1	6.6	10.6		57.0	11.7	96.2	63.5
Glucose 6-phosphatase	(1)	8.6 ± 4.7	5.5 ± 4.1	8.1 ± 1.9		55.0 ± 8.4	22.8 ± 4.2	92.9 ± 0.4	125 ± 10
	(2)	17.2	15.7	12.2		33.1	21.8	104.1	59.1
Galactosyltransferase	(1)*	14.0 ± 0.2	3.8 ± 0.1	20.9 ± 1.1		52.2 ± 0.8	9.1 ± 0.8	107.6 ± 1.1	3.05 ± 0.04
	(2)	37.8	7.1	13.8		39.1	2.1	99.1	1.22
Thiamin pyrophosphatase (+ATP)	(1)	10.5 ± 0.4	8.0 ± 0.8	17.0 ± 1.3		40.9 ± 0.8	23.6 ± 1.9	98.8 ± 4.6	57.0 ± 12.2
	(2)	14.3	11.2	10.8		35.4	28.2	99.4	42.9
Thiamin pyrophosphatase (no ATP)	(1)	10.3 ± 0.9	6.8 ± 2.7	22.0 ± 1.1		30.1 ± 2.1	30.8 ± 2.6	95.9 ± 0.7	29.3 ± 5.7
	(2)	21.0	14.2	12.0		21.5	31.3	101.4	33.9
Triphosphoinositide phosphatase	(1)	13.4 ± 4.4	8.4 ± 2.3	15.3 ± 2.9		26.2 ± 2.6	36.7 ± 5.2	98.8 ± 2.0	16.1 ± 0.2
	(2)	22.9	15.5	10.5		19.8	31.4	100.0	12.6
Diphosphoinositide phosphatase	(1)	15.6 ± 1.3	9.1 ± 3.6	14.9 ± 1.4		23.1 ± 3.7	37.3 ± 2.4	100.6 ± 7.5	18.9 ± 1.7
	(2)	20.0	11.2	9.7		21.2	38.0	103.3	14.4

Table 5. Marker localization

Most of the work has been done with liver and the precise localization of certain enzymes (*) is less well defined for kidney. Alkaline phosphatase is established as a marker for kidney brush border. References: de Duve (1971), de Duve *et al.* (1962), Roodyn (1965, 1967) and Goldfischer *et al.* (1964).

Marker	Location in cell
DNA	Nucleus
Cytochrome <i>c</i> oxidase (EC 1.9.3.1)	Mitochondrion
Acid phosphatase (EC 3.1.3.2)	Lysosome
β -Galactosidase (EC 3.2.1.23)	Lysosome+supernatant
Catalase (EC 1.11.1.6)	Peroxisome
Glucose 6-phosphatase (EC 3.1.3.9)	Endoplasmic reticulum
Arylesterase* (EC 3.1.1.2)	Endoplasmic reticulum?
Alkaline phosphatase (EC 3.1.3.1)	Brush border
Ca ²⁺ -ATPase* (EC 3.6.1.3)	Plasma membrane
(Na ⁺ +K ⁺)-ATPase (EC 3.6.1.3)	Plasma membrane (basolateral)
K ⁺ -nitrophenylphosphatase	Plasma membrane (basolateral)
Thiamin pyrophosphatase	Golgi apparatus
Galactosyltransferase*	Golgi apparatus
Lactate dehydrogenase (EC 1.1.1.27)	Cell sap

Fig. 9. Subcellular distribution of polyphosphoinositide phosphatases with marker enzymes in kidney cortex

Relative specific activity represents the percentage of total enzyme activity recovered in a fraction/percentage of total protein recovered in that fraction. N, Nuclear fraction; Mt, mitochondrial fraction; LMt, light mitochondrial fraction; Mic, microsomal fraction; S, supernatant. (a)–(e) Potter–Elvehjem homogenizer (vertical bars represent s.d.); (f)–(j) Dounce homogenizer.

subcellular distribution of the inositide enzymes (Tables 4 and 6) resembled those of acid phosphatase and thiamine pyrophosphatase (assayed without ATP). Acid phosphatase is thought to be mainly

present in the lysosome, but it is unlikely that the inositide phosphatases are present here, since they did not show any of the latency properties normally associated with lysosomal enzymes. Further, most

Table 6. *Correlation of marker histograms with that of triphosphoinositide phosphatase*

The results are based on Table 4. Histograms of the percentage distribution of the different enzymes in the sub-fractions are each compared with the triphosphoinositide phosphatase histogram. Areas which overlap are expressed as the percentage of the overlapping plus non-overlapping areas.

Homogenizer ...	Correlation of area (%)	
	Potter-Elvehjem	Dounce
Triphosphoinositide phosphatase	100.0	100.0
Diphosphoinositide phosphatase	96.5	92.1
Thiamin pyrophosphatase (-ATP)	89.4	96.8
Acid phosphatase	86.6	79.9
Thiamin pyrophosphatase (+ATP)	83.6	84.0
K ⁺ -stimulated nitrophenyl-phosphatase	72.5	66.4
Glucose 6-phosphatase	71.2	84.8
Alkaline phosphatase	69.6	68.7
Galactosyltransferase	67.8	62.4
Arylesterase	65.5	62.6
β -Galactosidase	65.7	62.8
(Na ⁺ +K ⁺)-stimulated ATPase	63.3	—
Ca ²⁺ -stimulated ATPase	63.3	70.4
Catalase	61.2	46.6
Lactate dehydrogenase	47.1	53.5
Cytochrome c oxidase	45.0	49.2
DNA	22.7	33.5

lysosomal enzymes have highest activity at acid pH, but the inositide phosphatases had a pH optimum of 7.5. When fractionation was performed with the Dounce homogenizer, lysosomal damage was decreased considerably as shown by the much lower supernatant acid phosphatase activity (Table 4). However, as Table 6 shows, the distribution of triphosphoinositide and diphosphoinositide phosphatases remained unchanged and still resembled thiamin pyrophosphatase (assayed without ATP). Assay of polyphosphoinositide phosphatases in the presence of 0.1–5 mM-ATP showed that activities were unaffected.

Microsomal and brush-border fractions

When the microsomal fraction was centrifuged on a discontinuous sucrose gradient, the distribution of polyphosphoinositide phosphatases strongly resembled that of thiamin pyrophosphatase and was clearly distinguishable from the pattern of the other enzymes. Thiamin pyrophosphatase assayed in the presence of ATP showed a pattern that resembled glucose 6-phosphatase more closely (Fig. 10).

Preparation of the brush-border fraction of kidney cortex by the method of Kinne & Kinne-Saffran (1969) yielded a preparation in which alkaline phosphatase, the brush-border marker, was enriched 8.4-fold, whereas (Na⁺+K⁺)-ATPase, the plasma-membrane marker, which is present mainly in the basolateral membrane, was increased 4.4-fold (Table 7). This is in agreement with the results of Kinne & Kinne-Saffran (1969). Glucose 6-phosphatase and acid phosphatase were absent. Polyphosphoinositide phosphatases, however, showed only low activity in this preparation, as did thiamin pyrophosphatase assayed without ATP.

Thiamin pyrophosphatase is interesting in that histochemical techniques show it to be present in the Golgi apparatus of many tissues (Goldfischer *et al.*, 1964). Staining of the endoplasmic reticulum is also observed and is stimulated by ATP (Goldfischer *et al.*, 1971). Hence assay of thiamin pyrophosphatase in the absence of ATP should show more of the Golgi component and less of the endoplasmic reticulum component. Fig. 10 bears this out for kidney. However, assay of galactosyltransferase, which has been shown to be a very specific marker for the Golgi apparatus in liver, but not as yet in kidney (Morré, 1971; Morré *et al.*, 1971), showed a very different distribution in the primary fractions, most of the activity being present in the microsomal fraction (Table 4).

All the experiments performed would seem to indicate therefore that the distributions of diphosphoinositide and triphosphoinositide phosphatases resemble that of thiamin pyrophosphatase (without ATP).

Supernatant-distribution experiments

Since appreciable activity of triphosphoinositide and diphosphoinositide phosphatase had been found in the supernatant fraction, it was desirable to know whether the enzymes are freely soluble or had been released during fractionation. Studies performed with homogenates of different tissue concentrations can give clues to adsorption or desorption (Anderson & Green, 1967).

Results of an experiment of this type are shown in Fig. 11. Only lactate dehydrogenase, RNA and possibly β -galactosidase showed any effect. Lactate dehydrogenase and β -galactosidase followed the pattern expected for a soluble enzyme adsorbed to a new surface produced at homogenization. RNA in the kidney is present in the soluble phase in appreciable amounts but is also attached to the endoplasmic reticulum in the form of ribosomes (Reid, 1956). This marker followed the pattern for a particulate component released into the supernatant, which presumably represents release of ribosomal RNA. The polyphosphoinositide phosphatases, however, behaved in the

Fig. 10. Distribution of polyphosphoinositide phosphatases and marker enzymes after centrifuging kidney-cortex microsomal fraction on a sucrose gradient

Fractions from the discontinuous gradient are numbered as follows: 1, material in 0.25M-sucrose and at interface; 2, 0.4–0.6M-sucrose interface; 3, 0.6–0.85M-sucrose interface; 4, 0.85–1.1M-sucrose interface; 5, 1.1–1.3M-sucrose interface; 6, pellet in 1.3M-sucrose. Relative specific activity represents percentage of total enzyme activity of fraction/percentage of total microsomal protein recovered in that fraction. Microsomal specific activities (nmol/min per mg of protein) were as follows (recoveries in parentheses): (a) alkaline phosphatase 459 (95); (b) (Na⁺ + K⁺)-ATPase 143 (104); (c) acid phosphatase 83 (106); (d) glucose 6-phosphatase 279 (100); (e) thiamin pyrophosphatase + ATP 103 (97); (f) thiamin pyrophosphatase 79 (98); (g) diphosphoinositide phosphatase 22 (97); (h) triphosphoinositide phosphatase 17 (96); protein (97). The percentages of protein recovered in each fraction were: 1, 5.4%; 2, 14.0%; 3, 9.9%; 4, 16.7%; 5, 31.8%; 6, 22.2%.

Table 7. Kidney brush-border preparation

Specific activities are given as nmol/min per mg of protein. Relative specific activity represents specific activity of fraction/specific activity of homogenate. N.D., No activity detectable.

	Homogenate		Brush border	
	Specific activity	Relative sp. activity	Specific activity	Relative sp. activity
Alkaline phosphatase	244	1.0	2057	8.4
(Na ⁺ + K ⁺)-ATPase	106	1.0	471	4.4
Glucose 6-phosphatase	74	1.0	N.D.	0.0
Acid phosphatase	49	1.0	N.D.	0.0
Thiamin pyrophosphatase (–ATP)	27	1.0	10	0.4
Thiamin pyrophosphatase (+ATP)	62	1.0	81	1.3
Diphosphoinositide phosphatase	18	1.0	6	0.3
Triphosphoinositide phosphatase	13	1.0	4	0.3

same way as arylesterase and acid phosphatase, suggesting that the portion of activity found in the supernatant fraction is freely soluble. This is confirmed by the observation that no further activity was released

into the supernatant after six washings of the particulate fraction.

A further possibility is that soluble activity in the supernatant is really concealed in vesicles in the intact

Fig. 11. Enzyme activities in rat kidney-cortex supernatant from homogenates of different concentrations

Kidney cortex from male rats was homogenized under standard conditions in 0.32M-sucrose, pH 7.4, using three strokes of a Potter-Elvehjem homogenizer (radial clearance 0.25 mm) rotating at 150 rev./min. Homogenates were filtered through muslin and centrifuged for 60 min at 105 000g. Curve (A), lactate dehydrogenase; (B), RNA; (C), protein; (D), triphosphoinositide phosphatase; (E), arylesterase; (F), β -galactosidase; (G), diphosphoinositide phosphatase; (H), acid phosphatase.

cell and is released into the supernatant mechanically on homogenization. This seems unlikely since use of the more gentle Dounce homogenizer minimized lysosomal acid phosphatase release into the supernatant, but the percentage of triphosphoinositide and diphosphoinositide phosphatases present in this fraction remained unchanged (Table 4 and Fig. 9).

Subcellular localization of polyphosphoinositide phosphatases

These experiments illustrate the dangers of studying the distribution of an enzyme before optimum assay conditions have been worked out. When the distribution of triphosphoinositide phosphatase was determined with a simple assay system (Table 1), most of the activity was found in the supernatant although a high recovery seemed to indicate that a latent enzyme was present. After characterization of the homogenate, further subcellular distribution studies revealed that the greater part of the activity was particulate.

Lee & Huggins (1968a,b) postulated that triphosphoinositide phosphatase was present in endoplasmic reticulum, since its distribution in primary fractions resembled that of glucose 6-phosphatase. The present more detailed study does not support this conclusion. Both diphosphoinositide and triphosphoinositide phosphatase seem to be associated with the Golgi membranes of kidney. In addition there is activity in the supernatant fraction, but the same applies to the Golgi marker, thiamin pyrophosphatase.

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