***sn*-Glycero(3)phosphoinositol Glycerophosphohydrolase**

A NEW PHOSPHODIESTERASE IN RAT TISSUES

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1. A phosphodiesterase that cleaves glycerophosphoinositol into glycerophosphate and inositol has been detected in rat tissues. 2. The enzyme requires Mg^{2+} (Mn^{2+}) and has a pH optimum of 7.7. 3. The richest sources of the enzyme are kidney and intestinal mucosa. In pancreas subcellular fractions it occurs largely in the microsomal fraction. 4. The enzyme is inhibited by excess substrate and by the reaction product glycerophosphate. 5. Temperature-stability studies and other observations distinguish the enzyme from other membrane-bound phosphodiesterases active at an alkaline pH e.g. glycerophosphoinositol inositolphosphohydrolase, glycerophosphocholine diesterase, inositol cyclic phosphate phosphodiesterase and phosphodiesterase I.

Glycerophosphoinositol is present in the water-soluble fraction of many tissues (Seamark *et al.*, 1968; Koch & Diringer, 1974; R. M. C. Dawson & N. Clarke, unpublished work) and it has been presumed that this arises by the total enzymic deacylation of phosphatidylinositol. There have been a number of reports of the formation of glycerophosphoinositol from this phospholipid by subcellular preparations from various tissues (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968; Quinn, 1973) and by seminal plasma (Scott & Dawson, 1968). We have described an enzyme system in rat liver lysosomes that brings about the complete deacylation of phosphatidylinositol (Irvine *et al.*, 1978).

A phosphodiesterase is present in rat tissues that hydrolyses glycerophosphoinositol into inositol monophosphate and glycerol (glycerophosphoinositol inositolphosphohydrolase; Dawson & Hemington, 1977). The present paper reports the presence of an additional independent phosphodiesterase in the same tissues, which hydrolyses the same substrate into glycerophosphate and inositol (glycerophosphoinositol glycerophosphohydrolase). Although the richest sources of this phosphodiesterase in rat are the kidney and intestinal mucosa, most of the observations in the present paper have been obtained by using particulate fraction and subfractions from pancreas. This is because with this latter tissue the glycerophosphate formed is not significantly converted into P_i by phosphomonoesterase, whereas with the former tissues, P_i is the main product. In addition, pancreas is known to contain an active deacylation system for forming glycerophosphoinositol from phosphatidylinositol (White *et al.*, 1971).

Experimental

Preparation of tissue particulate fractions and subfractions

After dissection from the recently killed rat, the tissue was homogenized in a Potter-type blender [0.008 in (206 μ m) clearance; Aldridge *et al.*, 1960] with 9 vol. of water at 0°C. The homogenate was centrifuged at 100000 g_{av} . for 1 h, the pellet collected and resuspended in the same volume of water and re-collected by centrifuging (100000 g_{av} . for 1 h). It was then resuspended in the same volume of water and dialysed against water at 4°C for 18–24 h. Subcellular fractions of rat pancreas were prepared by the method of Tartakoff & Jamieson (1974).

*Preparation of 1-(*sn*-glycero(3)[^{32}P]phospho)-D-myoinositol*

[^{32}P]Phosphatidylinositol was prepared by using baker's yeast as described by Irvine *et al.* (1978). The impure phosphatidylinositol fraction eluted from the alumina column was converted into glycero[^{32}P]phosphoinositol and purified as described by Dawson & Hemington (1977).

Glycerophosphoinositol glycerophosphohydrolase assay

Glycero[^{32}P]phosphoinositol (1 μ mol, about 20000 d.p.m.) in 15 mM-collidine/HCl buffer, pH 7.6, containing 7.7 mM- $MgCl_2$, was incubated with the tissue particulate fraction (equivalent to 20–40 mg tissue wet wt.) for 1 h at 37°C in a total incubation volume of 0.65 ml. The reaction was stopped by adding in rapid succession 0.05 ml of 5% (w/v) serum albumin solution and 0.5 ml of 20% (w/v) trichloroacetic acid

(at 0°C) and cooling in ice. After centrifuging at 0°C (3000g_{av.} for 5 min) the supernatant was washed 3 times with equal volumes of diethyl ether, neutralized with NH₃ vapour, and a portion (about half) subjected to paper ionophoresis at pH 3.6 with internal glycerophosphate (5 µg of P) and P_i (5 µg of P) as markers (Dawson & Hemington, 1977). The glycerophosphate produced by the enzyme, together with any P_i formed, were located and their radioactivity determined by the methods described previously by Irvine *et al.* (1977). Control incubations carried out in the absence of enzyme showed virtually no radioactivity in either glycerophosphate or P_i.

In certain experiments designed to identify the product of the reaction containing no phosphorus the activity was terminated with an excess of chloroform/methanol (2:1, v/v). The polyols were investigated after separation by ionophoresis and paper chromatography (Dawson & Hemington, 1977). In this and in the other assays (below) zero-order conditions were always maintained throughout the incubation.

Other phosphodiesterase assays

These were assayed by the methods described in the references listed as follows: phosphodiesterase I, EC 3.1.4.1 (Razzell, 1961a); glycerophosphocholine diesterase, EC 3.1.4.2 (Dawson, 1956); inositol 1:2-cyclic monophosphate 2-phosphohydrolase (Dawson & Clarke, 1972); glycerophosphoinositol inositolphosphohydrolase (Dawson & Hemington, 1977).

Reproducibility

Unless otherwise stated, the results are typical of at least three independent experiments of a similar type.

Results

Identification of products of enzymic reaction

The possible ³²P-labelled products that could arise by the decomposition of glycerophosphoinositol were separated and characterized by paper ionophoresis at pH 3.6. These ran with the following mobilities relative to P_i: inositol 1-phosphate, 0.69; inositol 1:2-cyclic monophosphate, 0.76; glycerol 3-phosphate, 0.84; glycerol 1:2-cyclic monophosphate, 1.04. The substrate itself had a mobility relative to P_i of 0.64. Under the standard incubation conditions with spleen, pancreas, brain and liver particulate fractions, virtually all the radioactivity liberated from glycerophosphoinositol, when assayed as described in the Experimental section, was found in glycerophosphate (Table 1), although traces of P_i and inositol 1-phosphate could be detected. With particulate preparations from kidney and intestinal mucosa, although glycerophosphoinositol could be detected, the predominant product was [³²P]P_i. This had presumably been formed by secondary action of alkaline phosphatase, an enzyme abundant in these tissues. Paper chromatography of the incubation products obtained by using the pancreas enzyme showed the production of free inositol with the same mobility and colour-shade response as inositol markers. No free glycerol could be detected. The intensity of the enzymically produced inositol spot, compared visually with a graded series of inositol marker concentrations, indicated that the inositol product was approximately stoichiometric to the glycerophosphate produced.

pH optimum

The pH optimum of the pancreatic phosphodiesterase measured in a series of Tris/HCl or collidine/

Table 1. *Distribution of glycerophosphoinositol glycerophosphohydrolase in rat tissues*

Homogenates of the tissues were incubated with glycerophosphoinositol for 1 h, under the conditions described in the Experimental section. Radioactive products were analysed by ionophoresis. Values in parentheses are the total products for each tissue.

Tissue	Products		Total (μg of P/100mg of protein per h)
	(μg of P/g wet wt. of tissue per h)		
Liver	Glycerophosphate	51.7	39.6
	P _i	3.0 (54.7)	
Pancreas	Glycerophosphate	132.7	100.1
	P _i	5.4 (138.1)	
Brain	Glycerophosphate	34.4	40.3
	P _i	7.2 (41.6)	
Spleen	Glycerophosphate	62.9	78.6
	P _i	4.7 (67.6)	
Kidney	Glycerophosphate	40.3	498.2
	P _i	532.6 (572.9)	
Intestinal mucosa	Glycerophosphate	24.7	353.0
	P _i	137.8 (162.5)

Fig. 1. The pH optima of glycerophosphoinositol glycerophosphohydrolase, glycerophosphocholine diesterase and phosphodiesterase I in particulate preparations from rat pancreas

The enzymes were assayed in a series of buffers by the methods given in the Experimental section. Symbols: ○, glycerophosphoinositol glycerophosphohydrolase activity in collidine/HCl buffer; ●, glycerophosphoinositol glycerophosphohydrolase activity in Tris/HCl buffer; □, glycerophosphocholine diesterase activity in glycine/NaOH buffer; △, phosphodiesterase I activity in glycine/NaOH buffer; ▲, phosphodiesterase I activity in 2-amino-2-methylpropane-1-3-diol/HCl buffer.

Fig. 2. Effect of bivalent metal ions on glycerophosphoinositol glycerophosphohydrolase in particulate fractions from rat pancreas

The enzyme was assayed as stated in the Experimental section with the ^{32}P -labelled products being determined after paper ionophoresis. The results are typical of three independent experiments. Symbols: ●, glycerophosphate released with Mg^{2+} added; ○, glycerophosphate released with Ca^{2+} added; ▲, P_i released with Mg^{2+} added; △, P_i released with Ca^{2+} added. The addition of EDTA (1.2mm) decreased the endogenous activity to zero.

HCl buffers was 7.7 (Fig. 1). The pH optimum of phosphodiesterase I in the same preparation was 9.3 (Fig. 1), which can be compared with a value of 9.1 obtained for this enzyme in pig kidney by Razzell (1961b). Glycerophosphocholine diesterase in the pancreas preparation had a pH optimum of 9.0 (Fig. 1); Baldwin & Cornatzer (1968) have recorded an optimum activity at pH 9.2 for this enzyme in rat kidney.

Activation by bivalent metal ions

EDTA (1.2mm) completely suppressed the endogenous activity of a pancreas particulate fraction observed in the absence of added bivalent metal ions. The hydrolysis was markedly stimulated by the addition of Mg^{2+} , being near maximum at 10mm (Fig. 2), and a somewhat lesser stimulation occurred with Mn^{2+} . Ca^{2+} did not stimulate activity, and, at higher concentrations, inhibited the basic hydrolysis (Fig. 2). Little $[\text{P}]_i$ release from glycerophosphate was observed with either Mg^{2+} or Ca^{2+} added (Fig. 2). Co^{2+} and Fe^{3+} (0.64mm) neither stimulated nor inhibited the endogenous hydrolysis of glycerophosphoinositol.

Inhibition by substrate and reaction products

Fig. 3 shows that concentrations of substrate

glycerophosphoinositol above 3 mM inhibited the reaction; below 3 mM Michaelis–Menten kinetics applied. The cause of this inhibition is unknown, although it could be that the excess substrate is competing with the enzyme for Mg^{2+} , as has been suggested for a similar phenomenon occurring with inorganic pyrophosphatase (Heppel & Hilmore, 1951). The reaction was markedly inhibited by the reaction product glycerophosphate (Fig. 4), but not by inositol (15 mM).

Distribution in tissues

The activity of the enzyme was assayed in six tissues of the rat (Table 1). Under the incubation conditions used with an absence of added Ca^{2+} and cysteine, any hydrolysis of the substrate by glycerophosphoinositol inositol phosphohydrolase is likely to be at a very low level (Dawson & Hemington, 1977). It can therefore be assumed that the P_i liberated by the

Fig. 3. Effect of substrate concentration on rat pancreas glycerophosphoinositol glycerophosphohydrolase. The enzyme activity was measured by using standard conditions (see the Experimental section), but with the substrate concentration being varied between 1 and 6.5 mM.

kidney and intestinal particulate fractions is derived from glycerophosphoinositol by glycerophosphohydrolase action followed by alkaline phosphatase degradation of the glycerophosphate. If this is so, then these two tissues are the richest source of the enzyme of the organs examined.

Fig. 4. The inhibition of pancreas glycerophosphoinositol glycerophosphohydrolase by glycerophosphate. The enzyme was assayed under standard conditions (see the Experimental section) with added glycerophosphate being present from the commencement of incubation. Closed and open circles represent independent experiments.

Table 2. Distribution of glycerophosphoinositol glycerophosphohydrolase in subcellular fractions from rat pancreas. Portions of subfractions obtained as stated in the Experimental section were incubated under standard conditions with glycerol- $[^{32}P]$ phosphoinositol. Radioactive products were analysed by ionophoresis.

Fraction	Specific activity (nmol of P liberated/mg of protein per h)	
	Expt. 1	Expt. 2
Total homogenate	5.6	5.6
Nuclear fraction	7.3	5.4
Zymogen granules	1.8	1.9
Microsomal fraction	18.6	13.4
Mitochondria	7.3	4.7
Supernatant	0.4	1.0
Recovery (%)	81	89

Fig. 5. Temperature stability of various phosphodiesterases in a rat pancreas particulate preparation

Portions of the particulate preparations were kept in water baths at 50°C, 55°C, 60°C and 65°C for 5 min, cooled and then the enzymes were assayed at 37°C by the methods given in the Experimental section. Results are means for two independent experiments. Symbols: ○ and ●, glycerophosphoinositol glycerophosphohydrolase activity; △ and ▲, glycerophosphoinositol inositolphosphohydrolase activity; □, glycerophosphocholine diesterase activity; △ and ▲, phosphodiesterase I activity.

Fig. 6. Temperature stability of rat kidney phosphodiesterase

Conditions are as stated in the legend to Fig. 5. Symbols: ○ and ●, glycerophosphoinositol glycerophosphohydrolase (filled and open symbols represent independent experiments); △ and ▲, inositol 1:2-cyclic monophosphate 2-phosphodiesterase (filled and open symbols represent independent experiments).

Subcellular distribution

In two experiments in which subcellular fractions of the pancreas were isolated, the enzyme was mainly located in the microsomal fraction (Table 2). It is likely that much of the activity in the nuclear and mitochondrial fractions was due to microsomal contamination. Low activities were present in the zymogen granules and supernatant. In one experiment the microsomal fraction was subfractionated into smooth and rough fractions; activity was found in both fractions with a somewhat higher specific activity in the rough fraction. This suggests that the enzyme is really in the endoplasmic reticulum and not in plasma or Golgi membranes, which would largely contaminate the smooth microsomal fraction.

Temperature stability

To distinguish the enzyme from other membrane-bound phosphodiesterases that are active at an alkaline pH, and particularly those activated by Mg^{2+} , use was made of the temperature stability of the enzymes when the particulate fraction was stored at elevated temperatures for 5 min. Glycerophosphoinositol glycerophosphohydrolase proved to be very sensitive to temperature: over 90% of its activity was lost on keeping the preparation for 5 min at 60°C (Fig. 5). The stability characteristics were clearly quite different from those of glycerophosphoinositol inositolphosphohydrolase, glycerophosphocholine diesterase and phosphodiesterase I (Fig. 5).

Glycerophosphoinositol glycerophosphohydrolase has a similar pH optimum to that reported for the Mg^{2+} -activated inositol 1:2-cyclic monophosphate 2-phosphodiesterase (Dawson & Clarke, 1972), an enzyme that like the present one is most active in

kidney. However, we were unable to detect this latter phosphodiesterase in the present pancreas particulate fraction. In addition, when we examined the temperature stability of kidney particulate glycerophosphoinositol glycerophosphohydrolase, it was very similar to that of pancreas glycerophosphoinositol glycerophosphohydrolase, but quite different from that of kidney inositol 1:2-cyclic monophosphate 2-phosphodiesterase (Fig. 6).

Discussion

It is really rather surprising that mammalian tissues should have two distinct phosphodiesterases for catabolizing glycerophosphoinositol, which is itself a product of the catabolism of phosphatidylinositol. However, there are a number of reasons for considering that the present enzymes cleaving free inositol from glycerophosphoinositol is quite different from the phosphodiesterase previously described, where free glycerol is the primary product (Dawson & Hemington, 1977). Thus the latter enzyme had an absolute requirement for Ca^{2+} , and was inhibited by Mg^{2+} and markedly stimulated by the inclusion of thiol reagents. By contrast the present glycerophosphohydrolase has an absolute requirement for Mg^{2+} or Mn^{2+} , is inhibited by Ca^{2+} and is unaffected by cysteine addition. In addition, the two enzymes show an entirely different temperature stability with a much slower inactivation of the inositolphosphohydrolase at 60°C.

The route by which glycerophosphoinositol is metabolized *in vivo* has not been established. Preliminary experiments have shown that in homogenates of pancreas inositol phosphate is only liberated if a reducing agent such as cysteine or glutathione is added. The membranes themselves seem to contain adequate supplies of the necessary bivalent metal ion to catalyse both reactions and the redox potential is of greater importance in directing the pathway. It is noteworthy that during stimulated catabolism of phosphatidylinositol in this tissue, the predominant inositol-containing water-soluble product is free inositol (Hokin-Neaverson *et al.*, 1975; however, glycerophosphoinositol was not measured in these experiments). As a major route of phosphatidylinositol catabolism in pancreas may be by deacylation (White *et al.*, 1971) there is a possibility that this free inositol was derived from the activity of the present glycerophosphoinositol glycerophosphohydrolase.

Although a glycerophosphoinositol glycerophosphohydrolase has not previously been described in mammalian tissues, an enzyme catalysing this reaction has been reported in housefly larvae (Hildenbrandt & Bieber, 1972). However, its properties seem quite different from the present enzyme. First, it is a soluble enzyme, whereas the present

phosphodiesterase is firmly bound to the microsomal fraction. Secondly, the insect enzyme's pH optimum is lower (7.2) and unlike the mammalian enzyme it is not inhibited by the reaction product, glycerophosphate. Thirdly, evidence was obtained by Hildenbrandt & Bieber (1972) that the insect glycerophosphoinositol glycerophosphohydrolase could also attack glycerophosphocholine, which is not true of the mammalian enzyme. Thus although the rat tissue particulate preparations used in the present investigation would hydrolyse glycerophosphocholine, the pH optimum of the hydrolysis was much higher (9.0) and the temperature stability of the enzyme greater than that of glycerophosphoinositol glycerophosphohydrolase. In addition, it was shown by Baldwin & Cornatzer (1968) and confirmed by Dawson & Hemington (1977) that purified preparations of mammalian glycerophosphocholine diesterase do not release glycerophosphate from a glycerophosphoinositol substrate.

The present enzyme can also be distinguished from other previously described membrane-bound phosphodiesterases that are active at an alkaline pH. Thus the general-purpose phosphodiesterase I that has a fairly wide substrate specificity has a much higher pH optimum (9.3) and a greater temperature stability than glycerophosphoinositol glycerophosphohydrolase. Although in some ways the present phosphodiesterase is similar to inositol 1:2-cyclic monophosphate phosphodiesterase (pH optimum, Mg^{2+} requirement), it can be distinguished in a number of ways. Thus the latter enzyme appears to be largely absent from pancreas and is grossly enriched in rat kidney (Dawson & Clarke, 1972) compared with glycerophosphoinositol glycerophosphohydrolase. In addition, present measurements show that glycerophosphoinositol glycerophosphohydrolase in rat kidney is much more labile at higher temperatures than is inositol 1:2-cyclic monophosphate phosphodiesterase (Fig. 6).

The absolute differentiation of the various tissue phosphodiesterases requires the isolation of the pure enzyme and the determination of their substrate specificities. To solubilize and purify each membrane-bound phosphodiesterase would be a very major undertaking. Nevertheless, the present method of investigating differential temperature inactivation of each enzyme is simple to perform and gives consistent results from enzyme preparation to enzyme preparation. Its use, coupled with other evidence, gives strong support for the separate identity of the various Mg^{2+} -stimulated membrane-bound phosphodiesterases with alkaline pH optimal.

These experiments were performed while D.E.R. was a British Council Scholar, and R.F.I. was a Beit Memorial Fellow.

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