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The Enzymic Formation of *myo*Inositol 1:2-Cyclic Phosphate from Phosphatidylinositol

By R. M. C. DAWSON, N. FREINKEL, F. B. JUNGALWALA and N. CLARKE
Department of Biochemistry, Institute of Animal Physiology, Babraham, Cambridge, U.K.

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Previous studies (Dawson, 1959; Kemp, Hübscher & Hawthorne, 1961; Thompson, 1967; Atherton & Hawthorne, 1968; Friedel, Brown & Durell, 1969) have indicated that mammalian tissues contain an enzyme that catalyses the degradation of phosphatidylinositol, producing a diglyceride and inositol monophosphate. Recent investigations have shown that with washed ram spermatozoa (Scott & Dawson, 1968) and extracts of pig thyroid tissue (Jungalwala, Freinkel & Dawson, 1971) the major water-soluble phosphate ester produced from phosphatidylinositol could be separated from inositol monophosphate by paper ionophoresis. This substance has now been identified as inositol 1:2-cyclic phosphate, and provided that precautions are taken to prevent its further breakdown its release can be demonstrated as a general property of the catabolic enzyme in all tissues.

Phosphatidylinositol was isolated from pig liver or baker's yeast, by using a combination of alumina columns (Dawson, 1958) and preparative t.l.c. (Scott & Dawson, 1968). ^{32}P-labelled phosphatidylinositol was isolated either from rat liver slices incubated with [^{32}P]P_i (Jungalwala *et al.* 1971) or baker's yeast grown in a ^{32}P-labelled medium (Hauser & Dawson, 1967).

The phosphatidylinositol (15μg of P/ml) was incubated at 38°C with the enzyme in 0.05M-sodium acetate–acetic acid buffer, pH 5.3, containing 0.2mM-calcium chloride. The enzyme preparation was usually a pig thyroid 0.25M-sucrose supernatant or a partially purified rat liver 'phosphatidylinositol hydrolase' [25–40%-satd.-$(NH_4)_2SO_4$ fraction; Kemp *et al.* 1961]. After incubation (20–40min) the mixture was shaken with methanol (1.6vol.) followed by chloroform (3.2vol.). The upper phase separated by centrifuging was removed and rewashed with an equal volume of lower phase (prepared by shaking the solvents and water in the same proportion). This mild solvent fixation avoids any decomposition of inositol 1:2-cyclic phosphate. When the procedure of Kemp *et al.* (1961) employing trichloroacetic acid was used, complete decomposition of the cyclic phosphate occurred. Paper ionophoresis (Jungalwala *et al.* 1971) of the water-soluble phosphate esters revealed two components, a minor one (M_{P_i} 0.70), which migrated to the same position as inositol 1-phosphate (prepared by the procedure of Brown, Hall & Letters, 1959), and an unknown major spot (M_{P_i} 0.76). The inositol monophosphate was identified as the 1-isomer by paper chromatography in the ammoniacal solvent used by Pizer & Ballou (1959) and in ethanol–13M-NH_3 (3:2, v/v).

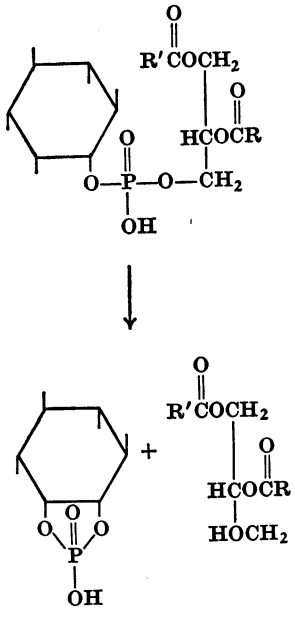
Both components were isolated by preparative paper ionophoresis and analysed for P (Bartlett, 1959), inositol and glycerol (Dawson, 1958). Inositol was also measured by g.l.c. of the trimethylsilyl derivatives (Roberts, 1967). Neither product contained glycerol but both contained inositol and phosphorus in equimolar proportions (inositol/P ratios: unknown component 1.01, 1.05; inositol monophosphate 0.87, 1.11). Brief acid hydrolysis (3min, M-HCl, 80°C) of the unknown component quantitatively converted it into a phosphate ester with the ionophoretic properties of an inositol monophosphate. Paper chromatography in the ethanol–NH_3 solvent indicated that the inositol monophosphate produced was largely the 1-isomer ($R_{2\text{-phosphorylglycerol}}$ 0.51), but with some inositol 2-phosphate ($R_{2\text{-phosphorylglycerol}}$ 0.66). Complete hydrolysis of the unknown in 0.25M-NaOH (5min, 100°C), which would not allow phosphoryl-group migration round the inositol ring, formed a mixture of the 1- (66%) and 2- (34%) isomers of inositol monophosphate. This suggested that the unknown metabolic product was an inositol cyclic phosphate. DL-*myo*Inositol 1:2-cyclic phosphate synthesized by the method of Pizer & Ballou (1959) showed identical paper-ionophoretic behaviour to the unknown (M_{P_i} 0.76) and both ran to the same position in ethanol–NH_3 ($R_{2\text{-phosphorylglycerol}}$ 1.28) and in the propan-2-ol–NH_3 solvent used by Pizer & Ballou (1959) ($R_{2\text{-phosphorylglycerol}}$ 1.08).

Since in phosphatidylinositol the phosphate moiety is linked to the 1-position of the inositol (Pizer & Ballou, 1959) and as inositol 1-phosphate is released after a brief acid and base hydrolysis of the cyclic compound, it seems reasonable to believe that the phosphate moiety is linked to the 1-position of the ring. Further, since the acid and base instability is characteristic of a *cis* five-membered phosphate ring (Khorana, Tener, Wright & Moffatt, 1957) and as inositol 2-phosphate is released on acid and base

hydrolysis the second linkage is presumably in the 2-position.

T.l.c. of the lipid fraction from the liver-enzyme digest indicated that diglyceride was the major product but some monoglyceride was also formed. Since the substrate did not contain lysophosphatidylinositol the obvious inference is that the latter originates through secondary lipase action. However, when the dilgyceride, formed in the primary incubation, was isolated and reincubated with the enzyme, monoglyceride liberation was minimal, although, of course, this may be greater in the presence of phosphatidylinositol. Both inositol 1-phosphate and the inositol cyclic phosphate are released from the substrate simultaneously and there is no subsequent interconversion of the cyclic phosphate into inositol phosphate or vice versa.

These results suggest therefore that the hydrolytic enzyme which attacks phosphatidylinositol functions primarily as a transferase by using part of the substrate molecule as an acceptor:



The transferase reaction is analogous to the enzymic reaction by which cyclic AMP is formed from ATP (adenylate cyclase).

The optimum pH for substrate breakdown was 5.3–5.5 (Fig. 1) but there was a small secondary peak at pH 6.6, as also observed by Kemp *et al.* (1961). However, as the pH was raised from 4.4 to 6.8 the percentage of the cyclic phosphate formed decreased progressively from 87% to 45% (Fig. 1). Once released there was no further hydrolysis of the inositol 1:2-cyclic phosphate to inositol monophosphate at any pH value. It is possible that this change in the product ratio could be connected with

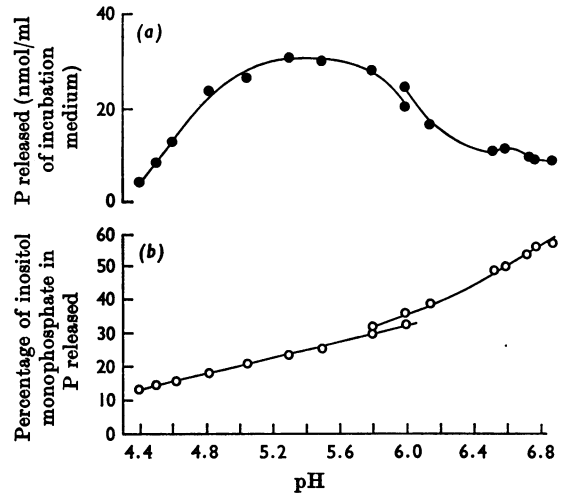


Fig. 1. Effect of pH on the release of water-soluble inositol phosphate esters from phosphatidylinositol. Incubation was carried out as described in the text (45 min; final concentration of purified enzyme protein 1.36 mg/ml) and the inositol monophosphate and inositol cyclic phosphate released were separated by ionophoresis and measured by their ^{32}P content as determined by Čerenkov counting (Jungalwala *et al.* 1971). The two curves (pH 4.4–6.0 and pH 5.8–6.8) represent separate experiments carried out 3 days apart. (*a*) Total P released; (*b*) percentage of inositol monophosphate in P released.

the greater concentration of hydroxyl ions in the aqueous medium as the pH is increased. This may encourage hydrolysis, with the formation of inositol 1-phosphate rather than transfer to the *cis*-2-hydroxyl group on the inositol ring. The formation of inositol 1-phosphate is not directly proportional to the concentration of free hydroxyl ions in the water, but the change in pH would also have effects on the enzyme protein as well as on the minimal ionization of the 2-hydroxyl group of the inositol.

Since at the optimum pH of the enzyme the major product is inositol 1:2-cyclic phosphate it is suggested that the enzyme should be called phosphatidylinositol–2-inositol phosphotransferase (cyclizing) rather than, as hitherto, phosphatidylinositol inositolphosphohydrolase. The implications of inositol 1:2-cyclic phosphate release in tissues should be examined, but perhaps it is not without relevance that the turnover of phosphatidylinositol has often been observed to increase specifically when secretory cells are stimulated into activity.

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