Biochem. J. (1975) **149**, 199–208
Printed in Great Britain

A Comparison of the Activity of Phosphatidylinositol Phosphodiesterase against Substrate in Dispersions and as Monolayers at the Air–Water Interface

By P. J. QUINN*
Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.

and Y. BARENHOLZ†
Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel

(*Received* 20 *February* 1975)

The activity of phosphatidylinositol phosphodiesterase, purified from rat brain, against substrate in three forms, (*a*) multibilayer liposomes, (*b*) single bilayer vesicles of phosphatidylinositol and (*c*) phosphatidylinositol oriented as monolayers at the air–water interface, was examined. The reaction rate was similar against the two substrate dispersions prepared with the same phospholipid concentration, although there was a large difference in substrate surface area available to the enzyme, and this similarity could not be accounted for by any differences in the microviscosity of the hydrocarbon region of the phospholipid bilayers. The reaction showed apparent zero-order reaction kinetics until about 10% of the substrate had been degraded, whereupon the rate decreased. The reaction against monolayers of phosphatidylinositol was linear throughout the entire digestion of the film, provided that more than 0.25 mg of enzyme was present in the subphase. The pH optimum was 6.6. Bivalent ions (Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+}) facilitated enzyme penetration into substrate monolayers, but the enzyme was only activated by Ca^{2+} (optimal concentration, 1mM) and to a lesser extent by Mg^{2+}. The reaction rate was independent of initial surface pressures of less than about $22\,mN\cdot m^{-1}$ but at higher pressures the rate decreased. This decrease could be prevented by the addition of 10mol of octadecylamine/90mol of phosphatidylinositol to the substrate monolayer; the amine did not increase the rate of reaction in films of less than $22\,mN\cdot m^{-1}$.

Phosphatidylinositol phosphodiesterase is an enzyme found widely distributed among various tissues from a number of different species. The enzyme from mammalian tissues cleaves phosphatidylinositol into diacylglycerol and inositol monophosphate (Dawson, 1959; Kemp *et al.*, 1961; Atherton & Hawthorne, 1968; Friedel *et al.*, 1969). The reaction is usually measured by determining the rate of release of water-soluble products. These have been found to consist of a mixture of one or two isomers of *myo*-inositol monophosphate and inositol 1:2-cyclic phosphate in proportions that depend on the assay pH (Dawson *et al.*, 1971). In some cases the reaction is reported to proceed by zero-order reaction kinetics for up to 70% hydrolysis of the substrate. However, other cases have been reported where this applies only to the initial rate of reaction. Non-linear reaction kinetics would be expected if access of the enzyme to the substrate becomes rate-limiting as the reaction proceeds, since only a proportion of phospholipid molecules are exposed on the surface of particulate dispersions. It is clear also that detergents, some of which are reported to activate the enzyme, will influence the manner of substrate dispersion and probably the rate at which diacylglycerol is removed from the site of enzyme action.

The present study was undertaken to compare the reaction kinetics of the enzyme against dispersions of phosphatidylinositol in aqueous buffers with that against monolayers of the substrate oriented at the air–water interface. The advantages of the monolayer system, apart from the convenience of measuring the reaction rate directly by following the rate of loss of surface radioactivity, is that the packing density of the substrate can be varied over wide limits and all molecules are equally susceptible to attack. The effect of ionic amphipaths on the reaction can also be assessed independently of their effects on substrate dispersion or on the enzyme.

Present addresses: * Department of Biochemistry, Chelsea College, Manresa Road, London SW3 6LX, U.K. † Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Va. 22901, U.S.A.

Materials and Methods

Preparation of enzyme

Rat brains (28.3g) were homogenized in a Teflon Potter homogenizer in 3vol. of 10mM-Tris–HCl buffer, pH7.4. The homogenate was centrifuged for 45min at 150000*g* in a Spinco model L centrifuge and the supernatant removed. A 30ml sample of supernatant was incubated for 1h at 37°C in the presence of 10μM-colchicine [Sigma (London) Chemical Co., London S.W.6, U.K.]. At the end of incubation the colchicine-treated supernatant was applied to a column (12cm×2cm diam.) of DEAE-Sephadex A-50 equilibrated with the homogenization buffer. The column was eluted successively with 150ml of the 10mM-Tris–HCl buffer, 150ml of buffer with 0.15M-KCl added and finally with 100ml of buffer containing 0.8M-KCl. The final eluate from the column yielded three major peptides on discontinuous polyacrylamide-gel electrophoresis run in sodium dodecyl sulphate, phosphatidylinositol phosphodiesterase and the two subunits of microtubular protein. The fraction was dialysed overnight against 8vol. of 10mM-Tris–HCl buffer, pH7.4. The protein concentration of this fraction, determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the protein standard, was 1.3mg/ml. A concentrated preparation of enzyme (7.5mg/ml) for use in some experiments was obtained by diafiltration against a PM 10 membrane (Amicon Corp., Lexington, Mass. 02173, U.S.A.).

Preparation of substrates

[^{32}P]Phosphatidylinositol was prepared by incubating rat kidney-cortex slices in Krebs–Ringer buffer containing $Na_2H^{32}PO_4$ (The Radiochemical Centre, Amersham, Bucks., U.K.) as described by Quinn (1973). The specific radioactivity of the dispersed substrates was decreased by the addition of yeast phosphatidylinositol (Koch–Light Ltd., Colnbrook, Bucks., U.K.) and the dried phospholipid was dispersed in 0.1M-ββ-dimethylglutaric acid–0.1M-NaOH, pH5.4, by (1) shaking for 2min on a vortex mixer, or (2) sonication for 90s with a Braun-Sonic 300 with a 9mm-diam. probe. The flask was gassed with N_2 during sonication to prevent oxidation of fatty acyl groups.

Preparation of diacylglycerol

Diacylglycerol was prepared by digestion of egg phosphatidylcholine dissolved in ether at the interface of an aqueous phase consisting of 50mM-Tris–HCl buffer, pH7.4, 5mM-$CaCl_2$ and 0.5mg of *Clostridium welchii* phospholipase C (Sigma Chemical Co.). After incubation for 24h at 25°C the ether phase was evaporated and the lipids were recovered from the lower phase of chloroform–methanol (2:1, v/v) extract (Folch *et al.*, 1957). When this material was examined by t.l.c. it was found that more than 95% of the phospholipid had been converted into diacylglycerol. This was purified by the extraction procedure of Dole (1956) and stored in a solution of heptane.

Fatty acid composition of the phosphatidylinositol

G.l.c. of methyl ester derivatives of the fatty acyl moieties of the substrate was done on a column of 15% polyethylene glycol succinate. The substituents were identified and quantified by comparison with a standard mixture of derivatives (C_{15} to C_{20}) supplied by Applied Science Laboratories Inc., Philadelphia, Pa., U.S.A. The predominant fatty acyl residues were approximately equal amounts of stearic acid and oleic acid with smaller amounts of linoleic acid and palmitic acid; approx. 55% of the total fatty acids were completely saturated.

Characterization of substrates in aqueous dispersion

The particle Stokes radii of the two substrate preparations were obtained from diffusion coefficients measured by autocorrelation spectroscopy of Rayleigh scattered light; the method is described by Cooper *et al.* (1974). Homogeneity of the particles was also checked by turbidometric measurements of vortex dispersions at wavelengths of 350, 400, 450 and 600nm made with a Spectronic 70 spectrophotometer (Baush and Lomb).

Microviscosity of the hydrophobic regions of sonicated and vortexed dispersions of the substrate containing 160nmol of phosphatidylinositol/ml was assessed by the fluorescence polarization technique described by Cogan *et al.* (1973). The fluorescence calibration curve of the instrument has been published by Shinitzky & Barenholz (1974). The fluorescent probe was 1,6-diphenyl-1,3,5-hexatrien (Aldrich Chemical Co. Ltd., Wembley, Middx., U.K.) and was added to the dispersion as a concentrated solution (1mM) in tetrahydrofuran, yielding a final concentration of 0.3μM with a ratio of 1 probe molecule/500 molecules of phosphatidylinositol. The mixture was kept for 18h at 25°C in the dark before polarization spectroscopy was performed. Particle microelectrophoresis was done at 37°C in an apparatus similar to that described by Bangham *et al.* (1958). Only the vortex dispersion of phosphatidylinositol produced particles of sufficient size for microelectrophoretic-mobility measurements. The concentration of particles was determined by counting in a haemocytometer.

Phospholipase assays with aqueous dispersions of substrate

Assays of sonicated or vortexed dispersions of phosphatidylinositol contained 32nmol of phospholipid (30000c.p.m. of [^{32}P]phosphatidylinositol) in

a final volume of 0.25ml in 0.1M-ββ-dimethylglutaric acid–0.1M-NaOH, pH5.4, and 2mM-$CaCl_2$. After equilibration of enzyme and substrate for 5min at 37°C the reaction was initiated by the addition of 0.3mg of enzyme and, after incubation for specified time-intervals, the reaction was stopped by shaking with 3.2vol. of chloroform–methanol (2:1, v/v). After centrifuging, the upper phase was washed with 1ml of lower phase obtained by partitioning appropriate volumes of buffer, chloroform and methanol. Portions (200μl) of washed upper phase were dispersed in 2.5ml of Instagel (Packard) for radioactivity counting in a Packard Tri-Carb liquid-scintillation spectrometer.

Phospholipase assays of substrate spread as monolayers at the air–water interface

The apparatus consisted of a Teflon trough (16cm ×4.5cm in area and 0.8cm deep) with a capacity of approx. 65ml. The subphase was stirred constantly during reactions by means of a reciprocating Teflon plate moving along the length of the trough. The stirring rate was constant throughout all experiments.

Surface pressure was determined by measuring the vertical force exerted on a glass rod (2mm diam.) dipping into the surface film. The rod was coated with carbon particles to ensure a zero contact angle with the subphase surface by passing it through a benzene flame and subsequently rendering it completely hydrophilic by immersion for several hours in water (Cheesman, 1946). The force was measured continuously with a Beckman LM 601 microbalance operating under null-point conditions and recorded on one channel of a three-channel pen recorder (Rikadenki Kogyo Co. Ltd., Tokyo, Japan). Surface-tension measurements made on a clean water surface at 23°C gave a value of $72.6 mN \cdot m^{-1}$. Surface radioactivity was detected by a gas-flow counter (obtained from a Vangard strip scanner) to which a thin (0.6μm) window of polyethylene terephthalate (Melinex; Imperial Chemical Industries Limited, Welwyn Garden City, Herts., U.K.) was affixed, exposing the aluminized surface on the outside of the counting chamber. With a gas mixture of helium+butane (98.5:1.5) flowing at a rate of 3ml/min a plateau of approx. 1000V centred about the operating voltage of 1160V was obtained. The counter signal was amplified and integrated over 30s by a rate meter (Packard Selectronic model 45-26), the output of which was fed into another channel of the pen recorder. The trough, balance, dipping rod and gas-flow counter were all enclosed in a chamber constructed of copper jackets through which water at the required temperature could be circulated, and there was a removable Perspex front to provide access to the instruments; the box was designed to act essentially as a Faraday cage.

The water used in monolayer experiments was prepared freshly each day and received a final distillation from alkaline $KMnO_4$ solution immediately before use. Interfacial junction potentials measured with a ^{241}Am air-ionizing electrode (The Radiochemical Centre, Amersham, Bucks., U.K.) of a clean water surface were relatively constant with stirring over 30min, indicating virtually complete removal of surface-active contaminants from the water and elimination of any outside sources of contamination. The pH of the water was approx. 6 and was monitored in the bulk phase with a Radiometer pH-meter; where necessary the pH was adjusted by the addition of HCl or NaOH to the subphase. The trough was washed copiously with water after each experiment and filled to the top at the commencement of the next. The surface was cleaned by sweeping several times with a Teflon barrier and aspirating off surface material from in front of the barrier.

Monolayers of phosphatidylinositol were spread on the surface by delivering a portion of a solution of the phospholipid in light petroleum (b.p. 40–60°C)–chloroform (4:1, v/v) on to the surface with an Agla microsyringe (Burroughs Wellcome and Co., Beckenham, Kent, U.K.). Stirring of the subphase was commenced and the film allowed to equilibrate for 10min before injection of the enzyme into the subphase below the monolayer. Mixed monolayers were made by including octadecylamine (Koch–Light), dicetylphosphoric acid (Sigma Chemical Co.) or diacylglycerol in the spreading solvent. All solvents were AnalaR grade and redistilled before use. Bivalent cations added to the suphase were in the form of chloride salts and all were of AnalaR grade as supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. Surface-potential measurements of salt solutions indicated that no surface-active contaminants were present.

Results

Phospholipase activity against substrates in aqueous dispersion

The rate of degradation of phosphatidylinositol in vortexed and sonicated dispersions of the phospholipid is shown in Fig. 1. The reaction rate is slightly greater for substrate in liposome form, but is linear with time in both preparations during the initial 15min of incubation. The reaction rate decreases at longer incubation times and more markedly in the sonicated dispersion; under these conditions more than 10% of the substrate had reacted. In separate incubations of 15min, enzyme concentrations were increased to 1.5 or 3.75mg of protein per assay to check whether the enzyme was rate-limiting. The amount of phospholipid degraded increased with increasing enzyme concentration in liposome dispersions but remained relatively

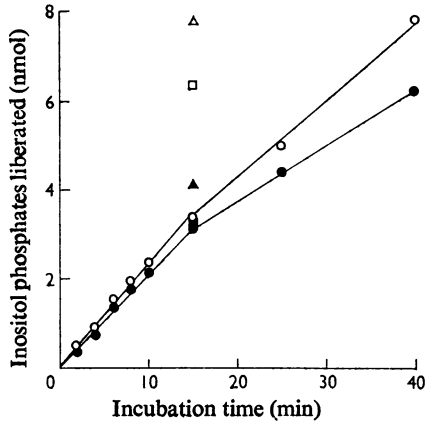

Fig. 1. *Rate of degradation of phosphatidylinositol dispersions by phosphatidylinositol phosphodiesterase*

The conditions of the assay are given in the text. Open symbols, multibilayer liposomes incubated with: ○, 0.33mg; □, 1.5mg; △, 3.75mg of enzyme. Closed symbols, single bilayer vesicles: ●, 0.33mg; ■, 1.5mg; ▲, 3.75mg of enzyme.

constant in the sonicated preparation suggesting that the availability of substrate was limiting in bilayer vesicles but not in multibilayer liposomes.

Measurement of particle size and shape

To compare the surface area of substrate available to the enzyme in dispersions produced by vortex shaking and sonication, Stokes radii of the particles were derived from diffusion coefficients. These measurements were obtained by analysing the train of photon pulses of light scattered at right-angles to an incident beam of a 50mV He–Ne laser source with an autocorrelator. Care was taken to remove extraneous particles from buffer solutions by ultrafiltration through Millipore filters (0.01 μm) and eliminating small gas bubbles formed during dispersions by applying a high vacuum to the samples.

The mean Stokes radius of particles formed by sonication was about 55nm (550Å) and there appeared to be little variation from the mean, since 70% of the scattered-light intensity was within five times the mean intensity. (This was the arbitrary limit accepted by the autocorrelator computer program.) The probable form of the dispersion was as single bilayer vesicles. Phosphatidylinositol dispersions prepared by vortex shaking were significantly larger, with a mean Stokes radius of about 313nm (3130Å); the addition of Ca^{2+} (2mM) decreased the mean particle radius to 274nm (2740Å). The intensity of scattered light was high in these samples and varied greatly, since only about 26% of this light fell within acceptable limits. Heterogeneity of size was also indicated by turbidometric measurements of vortexed preparations in 0.1M buffers of pH4.5, 5.5 and 6.5. There were no significant differences in light-scattering for any of these preparations when measured at several wavelengths, and together with the high intensity of scatter it was concluded that the phospholipid was in the form of large multilayer liposome structures of varying size. These conclusions were verified by electron microscopy of preparations negatively stained with 1% (w/v) phosphotungstic acid or with dispersions fixed with 1% (w/v) osmium tetroxide in 0.1M-sodium cacodylate buffer, pH7, embedded in Epon and sectioned. The vortex dispersion showed liposome structures consisting of a large number of concentric bilayers, whereas the sonicated preparation contained only single bilayer vesicles of uniform size. The maximum surface area of substrate available to the enzyme calculated from Stokes radii was 0.024 and 82cm^2 for the vortexed dispersion and sonicated phospholipid respectively.

Measurement of hydrocarbon-chain mobility of dispersed substrates

An estimation of the microviscosity (fluidity) of the hydrophobic regions of vortexed and sonicated dispersions of phosphatidylinositol at pH5.4 were made by a fluorescence-depolarization technique to determine whether there were any differences in the physical state of the hydrocarbon region of the bilayers which may reflect differences in the packing arrangement of the phospholipid in the two dispersions. Fluorescence depolarization is essentially a measurement of the harmonic mean of the internal viscosities opposing the in-plane and out-of-plane rotations of a non-polar dye confined in the hydrophobic region of the bilayer. A logarithmic plot of microviscosity as a function of temperature between 50° and −2°C for the two preparations is shown in Fig. 2. There is a linear relationship between the co-ordinates at least down to 0°C with correlation coefficients of linearity of 0.997 for both preparations. Since deviation of the $\ln\eta$ versus $1/T$ plots from linearity or a sigmoidal response is indicative of a phase transition or separation it was concluded that the hydrocarbon regions of phosphatidylinositol dispersions were in an isotropic liquid crystalline phase over almost the entire range of temperatures investigated irrespective of the method used to disperse the phospholipid. The logarithmic plots conform to the expression $y = ax+b$ and values of the slope, a, were 48.5 ± 0.7 and 47.9 ± 0.8 and the values of intercept, b, were -16.0 ± 0.2 and -15.8 ± 0.3 for liposomes and bilayer vesicles respectively. Since the dependence of microviscosity (η) on absolute temperature (T) followed the exponential relationship $\eta = Ae^{\Delta E/RT}$ where A represents the fraction of total emission intensity given by the

fluorescent component and R is the gas constant, the fusion activation energies (ΔE) could be calculated. These values were similar for liposomes and bilayer vesicles, 40.1 kJ·mol^{-1} and 39.8 kJ·mol^{-1} respectively. The addition of 2mM-$CaCl_2$ to the dispersions had no effect on the motion of the hydrocarbon chains either immediately after its addition or at intervals during an incubation of 2h.

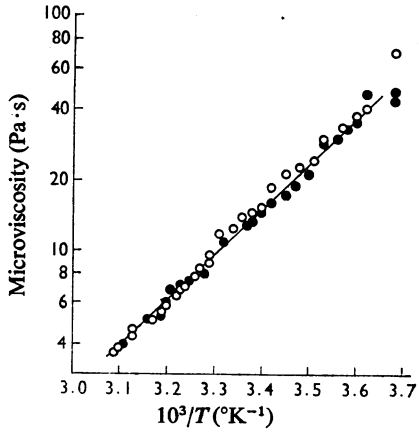

Fig. 2. *Temperature-dependence of microviscosity of phosphatidylinositol dispersions*

The experimental conditions are given in the text. ○, Liposomes; ●, bilayer vesicles.

Electrophoretic determinations of surface charge

To investigate the electrostatic status of dispersed substrate, microelectrophoretic mobilities of vortexed preparations at pH 4.5, 5.5 and 6.5 were determined. Similar values were obtained for each of the three pH values, with a mean mobility of 370 $\mu m^2 \cdot s^{-1} \cdot V^{-1}$. Movement was in an anodic direction and indicated complete ionization of the phosphate group. When $CaCl_2$ was added to the dispersion at pH 5.5 at a final concentration of 2mM the mobility was immediately decreased to 240 $\mu m^2 \cdot s^{-1} \cdot V^{-1}$ and was the same when measured 1h later. This suggested that calcium was adsorbed to the charged phosphate groups on the surface of the liposomes in the form of gegenions and that the position of these ions relative to the surface of the particles was stable with time.

Phospholipase activity against substrate in monolayers

Another series of experiments was performed with substrate presented to the enzyme in the form of monomolecular films oriented at the air–water interface. Changes in surface pressure and radioactivity taken from a recorder tracing of a typical experiment is shown in Fig. 3. The substrate (5nmol) was spread on the subphase of 0.2mM-$CaCl_2$ and maintained at an area of 1.1 nm^2/mol of phosphatidylinositol. After the film had equilibrated for

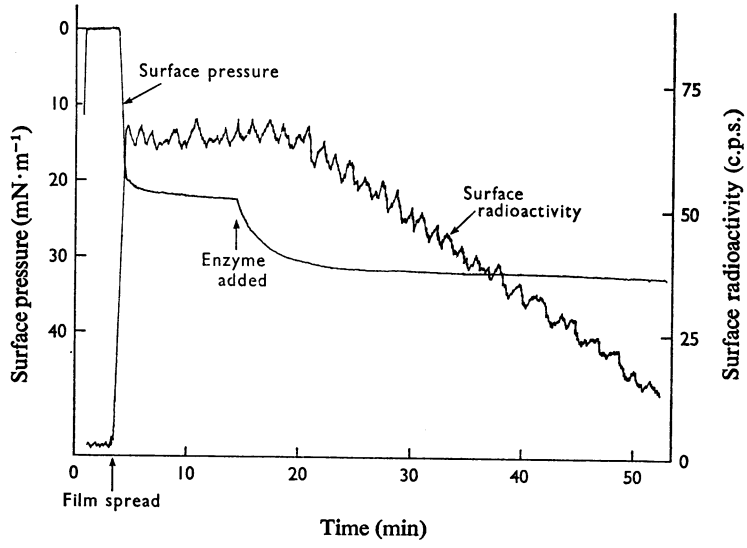

Fig. 3. *Recorder tracing of the time-course of phospholipase digestion of monolayer of phosphatidylinositol*

Substrate (5nmol of [^{32}P]phosphatidylinositol) was spread on a surface area of 31.5 cm^2 of a 0.2mM-$CaCl_2$ subphase and 0.56mg of enzyme added after 10min equilibration of the film. The surface tension of the clean subphase surface was about 72mN·m^{-1}.

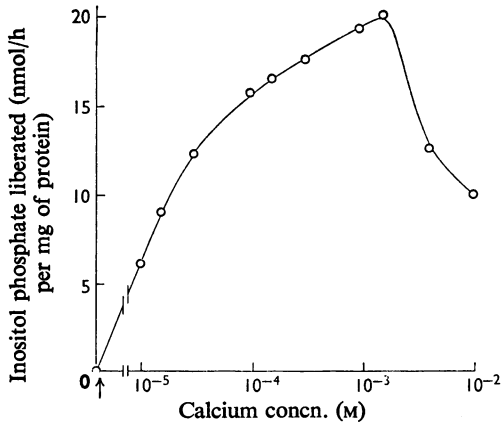

Fig. 4. *Relationship between subphase Ca^{2+} concentration and rate of degradation of monolayers of [^{32}P]phosphatidylinositol*

Substrate (3.2nmol of phospholipid) was spread on a surface area of 31.5cm^2 of subphase containing various amounts of $CaCl_2$ and the reaction was initiated by the addition of 0.26mg of enzyme 10min after spreading the film; 2mM-EDTA was added at the point indicated by the arrow.

10min, 0.56mg of enzyme was injected into the subphase and this caused an increase in surface pressure and a steady decrease in surface radioactivity. To confirm that the loss in surface radioactivity was not due to the removal of intact phospholipid molecules from the surface, samples of the bulk phase were removed at intervals and shaken with 3.2vol. of chloroform–methanol (2:1, v/v); all the radioactivity was recovered in the aqueous phase. At 1h after the addition of the enzyme all of the radioactivity was present in the subphase, indicating that the substrate was completely degraded. The surface pressure increased rapidly from an initial value of 23mN·m^{-1}, reaching a final value of about 32mN·m^{-1} 15min after addition of the enzyme. The magnitude of the increase in surface pressure at constant enzyme concentration depended on the initial pressure of the film since the final equilibrium pressure was always about the same (32mN·m^{-1}), although the rate of increase was related to the concentration of Ca^{2+} in the subphase. When the subphase contained no added ions the addition of enzyme resulted in only a very slow increase in surface pressure over 30min, reaching an apparent equilibrium pressure of 27mN·m^{-1}. If $CaCl_2$ was added to the subphase after this time (final concentration 0.2mM) the pressure increased rapidly to 32mN·m^{-1}. The rate of reaction and increase in surface pressure was the same whether monolayers were spread on subphases containing enzyme or the enzyme was added up to 1h after spreading the monolayer on a subphase of 0.2mM-$CaCl_2$. In all subsequent experiments enzyme was added 10min after spreading the monolayer.

The effect of Ca^{2+} on the reaction rate is shown in Fig. 4. Monolayers containing 3.5nmol of [^{32}P]phosphatidylinositol maintained at an area of 1.5nm^2/mol were spread on subphases containing various concentrations of Ca^{2+}. The rate of decrease in surface radioactivity was determined after the addition of enzyme to the subphase. Increasing concentrations of Ca^{2+} up to 1mM produced an increase in the rate of substrate degradation, but at higher Ca^{2+} concentrations the rate decreased, possibly as a result of an aggregation of the enzyme induced by Ca^{2+}. The enzymic reaction and increase in surface pressure were blocked by the addition of equimolar concentrations of EDTA to the subphase. The reaction rate on a subphase of 2mM-$MgCl_2$ was about one-half that for the same concentration of Ca^{2+}, but there was no loss of surface radioactivity when the subphase was 2mM-$CoCl_2$, 2mM-$NiCl_2$ or 2mM-$MnCl_2$. The rate and magnitude of the change in surface pressure was approximately the same for all the cations tested.

The dependence of reaction rate on enzyme concentration is shown in Fig. 5. The reaction rate increased proportionately with enzyme amount up to 0.6mg and at higher enzyme concentrations there was no further increase in rate. The reaction proceeded

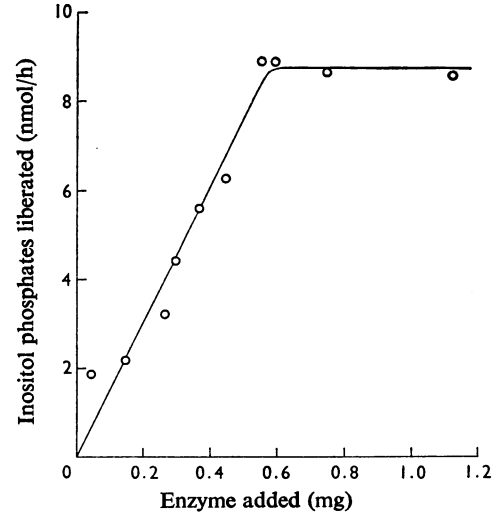

Fig. 5. *Dependence of reaction rate on enzyme concentration*

Substrate (3.2nmol of [^{32}P]phosphatidylinositol) was spread on a surface area of 31.5cm^2 of 0.2mM-$CaCl_2$ subphase, and various amounts of enzyme were injected into the subphase 10min after spreading the monolayer.

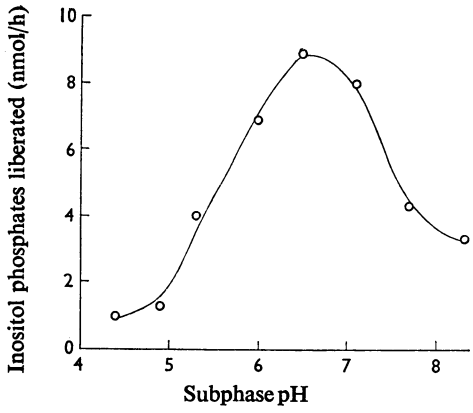

Fig. 6. *Effect of subphase pH on reaction rate*

[^{32}P]Phosphatidylinositol (3.5nmol) was spread on a surface area of 31.5cm^2 of 0.2mM-$CaCl_2$ subphase and adjusted to the required pH value with HCl or NaOH. The reaction was started by the addition of 0.56mg of enzyme to the subphase 10min after spreading the monolayer.

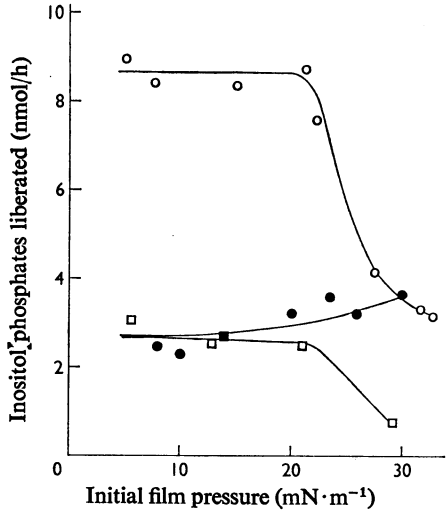

Fig. 7. *Effect of initial pressure of substrate monolayers on the rate of reaction*

Monolayers containing various amounts of substrate were spread on a surface area of 31.5cm^2 of a 0.2mM-$CaCl_2$ subphase. [^{32}P]Phosphatidylinositol monolayers with 0.56mg of enzyme (○) or 0.26mg of enzyme (□); [^{32}P]phosphatidylinositol monolayers containing 10mol of octadecylamine/90mol of phospholipid (●) with 0.26mg of enzyme.

to completion at a constant rate provided that more than 0.25mg of enzyme was present in the subphase (see Fig. 3); at enzyme amounts of less than 0.25mg the reaction rate effectively ceased after about 20% of the substrate had reacted and only initial reaction rates are plotted for these points in Fig. 5.

The effect of subphase pH on reaction rate was determined by spreading monolayers on subphases adjusted to the required pH value with HCl or NaOH. This procedure served to avoid any specific effects of buffers on the reaction. The pH curve (Fig. 6) is fairly symmetrical, with an optimum value of about pH6.6.

The reaction rate was also dependent on the initial pressure of the monolayer (Fig. 7). With 0.56mg of enzyme the rate of reaction was independent of initial pressure up to about 21mN·m^{-1}; at higher pressures the rate decreased substantially, although even at initial pressures of 32mN·m^{-1} the reaction rate was still about 30% of the maximum value. The increase in surface pressure during the reaction against films of initial pressures higher than 30mN·m^{-1} was decreased to 1 or 2mN·m^{-1}. This experiment was repeated with 0.26mg of enzyme for comparison with mixed monolayers containing 10mol of octadecylamine/90mol of phospholipid. The presence of cationic amphipath prevented the decrease of activity observed in monolayers of phospholipid alone when maintained at initial pressures greater than about 22mN·m^{-1}. Increasing the proportion of octadecylamine up to 50mol/50mol of phosphatidylinositol in monolayers at 18mN·m^{-1} had no effect on the reaction rate, nor did the inclusion of 10mol of dicetylphosphoric acid/90mol of phospholipid at the same initial pressure. Although the reaction rate in the presence of sufficient enzyme is linear throughout the entire digestion of the film, the effect of the presence of the reaction product on enzyme activity was checked. The inclusion of up to 65mol of diacylglycerol/35mol of phospholipid in monolayers maintained at a surface pressure of 18mN·m^{-1} did not markedly affect the rate of reaction.

Discussion

Assay of phosphatidylinositol phosphodiesterase enzymes are usually performed by incubating either soluble or particulate preparations of the enzyme with aqueous dispersions of the substrate. The major disadvantage of this system, particularly with regard to measuring enzyme activity in quantitative terms, is that the rate of reaction does not always follow linear reaction kinetics during complete degradation of the substrate. Deviation from the classical Michaelis–Menten model appears to be a general phenomenon common to most phospholipase enzymes

acting on substrates in aqueous dispersion (Gatt & Barenholz, 1973). Constant reaction rates, however, where up to 70% of substrate had reacted have been reported for a purified enzyme from intestinal mucosa (Atherton & Hawthorne, 1968) and particulate preparations from rat brain (Lapetina & Michell, 1973), although high-speed supernatant extracts of ox brain (Keough & Thompson, 1972) and pig thyroid (Jungalwala *et al.*, 1971) do not exhibit linear kinetics. The latter appears to be the case with purified enzyme from rat brain (Fig. 1), in which the reaction rates become non-linear after cleavage of about 10% of the substrate irrespective of whether this is in the form of multibilayer liposomes or as single bilayer vesicles. Why linearity is lost as the reaction proceeds further than this point is not clear. If rearrangement of the substrate molecules in the outer leaflet of the bilayer, which are presumably the only ones readily accessible to the enzyme, is rate-limiting then it would be expected that a greater proportion of substrate would be degraded according to apparent zero-order reaction kinetics in the sonicated preparation where about 60% of the substrate molecules are presented on the outside of the particles, compared with the liposome dispersion where less than 0.2% of the molecules are exposed. Nevertheless, this observation is consistent with that of Atherton & Hawthorne (1968), who found that activity of the rat liver enzyme against brain phosphatidylinositol prepared by dispersion in buffer from an ether suspension was the same as sonicated dispersions of the phospholipid. These authors also found that their enzyme was more active against phosphatidylinositol from corn, and they attributed differences in substrate susceptibility to the packing density of phospholipid molecules in the substrate dispersions arising from differences in the degree of saturation of the fatty acyl constituents. It is noteworthy in the present experiments that the reaction rate depends markedly on the density of substrate molecules at the air–water interface (Fig. 7). Thus at areas/mol approaching that expected for phospholipid molecules in aqueous bilayer dispersions, the reaction rate is very sensitive to small changes in packing density and increased rapidly with expansion of the film up to 1.05 nm^2/mol, beyond which no further increase in rate is observed. It might be anticipated that sonication would decrease the packing density of phospholipid molecules in the bilayer because of the formation of smaller particles with a high radius of curvature (Papahadjopoulos *et al.*, 1973), as clearly seen for phosphatidylcholine dispersions (Horwitz *et al.*, 1973). These effects are likely to be minimized with phosphatidylinositol dispersions because of charge repulsion between neighbouring molecules and the presence of a bulky carbohydrate component, both of which will contribute to a low packing density and allow greater flexibility in the motion of acyl chains in the hydrocarbon region of the bilayer. In this respect no change in microviscosity was observed after subjecting the substrate to ultrasonication. Moreover, spin-label studies have shown that, although Ca^{2+} restricts mobility of the polar region of phosphatidylinositol dispersed in bilayers, the motions of hydrocarbon chains is unhindered by interaction with Ca^{2+} (Schnepel *et al.*, 1974), a factor consistent with our microviscosity measurements.

Ca^{2+} may act to promote the interaction between the enzyme and substrate monolayer by decreasing the negative charge differential between the reactants. The electrophoretic mobility of phosphatidylinositol dispersions indicates that the net negative charge is decreased on addition of 2mM-Ca^{2+} and it is likely that monolayers retain a net negative charge when spread on subphases containing less than 2mM-$CaCl_2$. The enzyme preparation also carries a net negative charge in the presence of 10mM-$MgCl_2$, since it adsorbs strongly to DEAE-Sephadex even when it is not complexed with microtubular protein (Quinn, 1973) and, as expected, the rate and extent of penetration is low in the absence of Ca^{2+} from the bulk phase and increases as the Ca^{2+} concentration is increased. It should be stressed, however, that the enzyme preparation contains microtubular protein (Quinn, 1973), which probably also penetrates the monolayer. The effect of Ca^{2+} therefore may not be confined to lowering the surface charge of the substrate, and there are several lines of evidence to suggest that Ca^{2+} binding to the enzyme rather than adsorption to the substrate surface is the specific function of this cation. First, charge-reversal experiments indicate that although a number of other bivalent cations, including Co^{2+}, Ni^{2+} and Mn^{2+}, are capable of decreasing the negative potential of phospholipid dispersions to about the same extent as Ca^{2+} (Barton, 1968), none of these activate the enzyme. This non-specific effect of charge is noted by the observation that the extent and rate of increase in surface pressure can be duplicated by other bivalent ions, but the enzyme remains inactive. The failure of other bivalent ions to activate the enzyme to any significant extent is a consistent feature of the reaction in dispersed substrates (Atherton & Hawthorne, 1968), and with a number of other preparations, Mg^{2+} appears to be the best substitute for Ca^{2+} (Kemp *et al.*, 1961; Thompson, 1967). It should be noted also that EDTA prevents enzyme penetration if all the Ca^{2+} is complexed, but in this instance any specific effects of the cation on the enzyme will also be blocked. The second factor suggesting the binding of Ca^{2+} to the enzyme was reported by Thompson (1967), who showed that an enzyme purified from rat brain had a certain amount of tightly bound Ca^{2+} which could not be removed readily by dialysis. This Ca^{2+} gave rise to a small residual enzyme activity

against substrate prepared essentially free of Ca^{2+}, and this activity could be blocked by EDTA. Activation of the enzyme by Ca^{2+} appears to be analogous to that reported for phospholipase A_2 (De Haas *et al.*, 1971).

The effect of enzyme concentration on the reaction rate against substrate monolayers showed two anomalies. At low enzyme concentration the reaction ceased after partial digestion of the film. This could be due to inactivation of the enzyme, which is relatively unstable in dilute solution (protein concentration in the bulk phase is less than 4μg/ml on addition of 0.25mg of enzyme). Secondly, at higher concentrations of enzyme (higher than 0.5mg) the reaction rate becomes constant. This could be the limiting rate of diffusion of product through the unstirred layer immediately below the film or, more likely, a complete coverage of the substrate surface with protein.

The dependence of reaction rate on the surface pressure of monolayers is a feature common to most phospholipase enzymes. Phospholipase A, for example, will not hydrolyse phosphatidylcholine monolayers when maintained at high pressure, the limit of which is determined to a certain extent by the nature of the fatty acyl residues (Dawson, 1966; Colacicco & Rapport, 1966; Shah & Schulman, 1967). Similarly, phospholipases B, C and D are inactive against monolayers of yeast phosphatidylcholine at pressures greater than about $30 mN \cdot m^{-1}$ (Bangham & Dawson, 1960, 1962; Quarles & Dawson, 1969) unless certain agents are introduced into the system to modify the physical state of the substrate surface. These effects are usually explained in terms of an insufficient space between the substrate molecules to permit penetration of the enzyme into the monolayer and orientation of the active site about the bond susceptible to attack (Dawson & Quinn, 1971).

There are a number of conflicting reports on the effects of addition of detergents to phosphatidylinositol dispersions on phospholipase activity (Thompson, 1967; Atherton & Hawthorne, 1968; Keough & Thompson, 1972; Lapetina & Michell, 1973). The action of cationic amphipaths is thought to reside in their ability to decrease the negative surface potential of the substrate (and incidentally to displace adsorbed Ca^{2+}), although these and other detergents as well probably influence the reaction in other ways. Ancillary effects of detergents on the reaction are eliminated by the use of insoluble amphipaths in mixed monolayers with the substrate, and the effects of the cationic amphipath, octadecylamine, are clearly shown in Fig. 7. In expanded monolayers where the enzyme is able to penetrate freely into the monolayer no increase in enzyme activity is observed, but the decrease in activity at higher pressures is prevented by the amine, which may facilitate the penetration of enzyme into the film.

We are indebted to Dr. V. G. Cooper and Dr. M. Shinitzky for their assistance in measuring physical parameters and to Dr. S. Knutton for preparing electron micrographs of substrate dispersions. The monolayer apparatus was constructed under the skilled supervision of Mr. E. Yecheskeli, of the Mechanical Workshop of the Hadassah Medical School. Mrs. E. M. Naples provided invaluable technical assistance. Financial aid from the European Molecular Biology Organisation and the Medical Research Council is gratefully acknowledged by P. J. Q.

References

Atherton, R. S. & Hawthorne, J. N. (1968) *Eur. J. Biochem.* **4**, 68–75

Bangham, A. D. & Dawson, R. M. C. (1960) *Biochem. J.* **75**, 133–138

Bangham, A. D. & Dawson, R. M. C. (1962) *Biochim. Biophys. Acta* **59**, 103–115

Bangham, A. D., Flemans, R., Heard, D. H. & Seaman, G. V. F. (1958) *Nature (London)* **182**, 642–644

Barton, P. (1968) *J. Biol. Chem.* **243**, 3884–3890

Cheesman, D. F. (1946) *Ark. Kemi Mineral. Geol.* **B22**, no. 1, 1–8

Cogan, U., Shinitzky, M., Weber, G. & Nishida, T. (1973) *Biochemistry* **12**, 521–528

Colacicco, G. & Rapport, M. M. (1966) *J. Lipid Res.* **7**, 258–263

Cooper, V. G., Yedgar, S. & Barenholz, Y. (1974) *Biochim. Biophys. Acta* **358**, 262–274

Dawson, R. M. C. (1959) *Biochim. Biophys. Acta* **33**, 68–77

Dawson, R. M. C. (1966) *Biochem. J.* **98**, 35c–37c

Dawson, R. M. C. & Quinn, P. J. (1971) *Adv. Exp. Med. Biol.* **14**, 1–18

Dawson, R. M. C., Freinkel, M., Jungalwala, F. B. & Clarke, N. (1971) *Biochem. J.* **122**, 605–607

De Haas, G. H., Bonsen, P. P. M., Pieterson, W. A. & Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* **239**, 252–266

Dole, V. P. (1956) *J. Clin. Invest.* **35**, 150–154

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509

Friedel, R. D., Brown, J. D. & Durrell, J. (1969) *J. Neurochem.* **16**, 371–378

Gatt, S. & Barenholz, Y. (1973) *Annu. Rev. Biochem.* **42**, 61–90

Horwitz, A. F., Michaelson, D. & Klein, M. P. (1973) *Biochim. Biophys. Acta* **298**, 1–7

Jungalwala, F. B., Freinkel, N. & Dawson, R. M. C. (1971) *Biochem. J.* **123**, 19–33

Kemp, P., Hubscher, G. & Hawthorne, J. N. (1961) *Biochem. J.* **79**, 193–200

Keough, K. M. W. & Thompson, W. (1972) *Biochim. Biophys. Acta* **270**, 324–336

Lapetina, E. G. & Michell, R. H. (1973) *Biochem. J.* **131**, 433–442

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

Papahadjopoulos, D., Jacobson, K., Nir, S. & Isac, T. (1973) *Biochim. Biophys. Acta* **311**, 330–348

Quarles, R. H. & Dawson, R. M. C. (1969) *Biochem. J.* **113**, 697–705
Quinn, P. J. (1973) *Biochem. J.* **133**, 273–281
Schnepel, G. H., Hegner, D. & Schummer, U. (1974) *Biochim. Biophys. Acta* **367**, 67–74
Shah, D. O. & Schulman, J. H. (1967) *J. Colloid Interface Sci.* **25**, 107–119
Shinitzky, M. & Barenholz, Y. (1974) *J. Biol. Chem.* **249**, 2652–2657
Thompson, W. (1967) *Can. J. Biochem.* **45**, 853–861