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The Association between Phosphatidylinositol Phosphodiesterase Activity and a Specific Subunit of Microtubular Protein in Rat Brain

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1. Supernatant proteins from rat brain were separated into two fractions containing phosphatidylinositol phosphodiesterase activity by chromatography on DEAE-Sephadex A-50. 2. The first fraction sediments in linear sucrose density gradients in two bands corresponding to molecular weights of 66000 and 36000. There was presumptive evidence that the lighter protein constituted the monomeric form of the enzyme. The second fraction sediments predominantly as a single protein of molecular weight 86000. 3. Treatment of rat brain supernatant with [^{3}H]colchicine abolished the second DEAE-Sephadex peak and removed the lighter protein from the first peak. These proteins emerged in the same position as the protein binding [^{3}H]colchicine at high salt concentration; phospholipase activity was recovered from linear sucrose density gradients in positions corresponding to molecular weights 88000 and 43000, together with an aggregate of molecular weight 140000. Electrophoresis on sodium dodecyl sulphate–urea–polyacrylamide gels of this fraction revealed only three proteins: the α and β-subunits of microtubular protein, of molecular weights 56000 and 52000 respectively, and a protein of molecular weight 38000. 4. A sample of microtubular protein from mouse, labelled *in vivo* with [^{3}H]proline and $^{32}P_i$, was added to rat brain supernatant together with an equal amount of the same microtubular protein treated with cyclic AMP and [γ-^{32}P]ATP and the mixture subsequently characterized by ion-exchange chromatography. Some phospholipase activity characteristic of the second peak from DEAE-Sephadex was associated with one fraction of added microtubular protein. This fraction was identified on the basis of the ^3H:^{32}P ratio as the β subunit of the protein treated with ATP and cyclic AMP. The subunit of added microtubular protein untreated with nucleotides was not associated with phospholipase activity.

A selective increase in the incorporation of $^{32}P_i$ into phosphatidylinositol over the other major phospholipid components of a variety of different tissues, often observed within a few minutes of application of appropriate external stimuli, has been described. Thus turnover of phosphatidylinositol phosphorus or [^{3}H]-inositol was increased in lymphocytes treated with phytohaemagglutinin (Fisher & Mueller, 1968, 1971*a*,*b*; Pasternak & Friedrichs, 1970) and in polymorphonuclear leucocytes undergoing phagocytosis (Karnovsky & Wallach, 1961; Sastry & Hokin, 1966). Hormonal stimulation of phosphatidylinositol turnover has been demonstrated in thyroid gland treated with pituitary thyrotrophin (Scott *et al.*, 1968), in rat heart after adrenaline administration (Gaut & Huggins, 1966), and in adipose tissue stimulated with insulin (De Torrentegui & Berthet, 1966). Similar effects have been observed in nervous tissue after electrical stimulation (Larrabee & Leicht, 1965; Pumphrey, 1969) or treatment with acetylcholine (Hokin, 1970). Double-labelling experiments in which radioisotope was incorporated into the glycerol or fatty acid moiety of the phospholipid indicated that degradation was predominantly by a phospholipase *c*-type reaction (Scott *et al.*, 1968; Hokin, 1968).

An enzyme responsible for removing inositol phosphates from phosphatidylinositol has now been reported in a number of tissues (see the Discussion section), including brain (Friedel *et al.*, 1969; Canessa de Scarnati & Rodriguez de Lorez Arnaiz, 1972; Keough & Thompson, 1972). Canessa de Scarnati & Rodriguez de Lorez Arnaiz (1972) reported that acetylcholine in low concentrations stimulated hydrolysis of phosphatidylinositol in isolated nerve endings but at similar concentrations had less effect on the enzyme recovered from the soluble fraction. The incorporation of $^{32}P_i$ into phosphatidylinositol of nerve-ending particles was also stimulated by acetylcholine (Schacht & Agranoff, 1972); however, this observation was not confirmed by Yagihara & Hawthorne (1972), who showed that the specific radioactivity of the phosphatidic acid fraction only increased after acetylcholine treatment.

A purification of the enzyme in the soluble fraction of rat brain preparations has been attempted by using ion-exchange chromatography and identifying the

type of molecular aggregation by preparative density-gradient centrifugation. It was hoped that an approach of this kind might yield information that would help to establish the connection between stimulation of the cell and the specific effect on the turnover of phosphatidylinositol.

Methods

Supernatant preparation and chromatography on DEAE-Sephadex

Brains of Wistar rats were removed immediately *post mortem* and cerebral-cortex tissue was washed in 0.15M-NaCl. The washed tissue was homogenized in 3vol. of 10mM-imidazole–HCl buffer (pH7.0)–10mM-$MgCl_2$–6mM-2-mercaptoethanol at 4°C in a Potter–Elvehjem homogenizer and centrifuged at 150000*g* for 45min. Supernatant was treated with [*methoxy*-^{3}H]colchicine (from New England Nuclear Corp., Boston, Mass., U.S.A.), by incubation for 90min at 37°C in the presence of 5μM-colchicine (Sigma Chemical Co., St. Louis, Mo., U.S.A.) of final specific radioactivity 0.4Ci/mmol.

Column chromatography of supernatant fractions was done with DEAE-Sephadex A-50 at 4°C. The bed volume when equilibrated with the homogenizing buffer was 50ml in a column 2.2cm in diameter. After application of the samples, the column was washed with 50ml of the same buffer and enzyme was eluted with a linear 0–0.8M-KCl gradient in the buffer. Protein concentration of each fraction (2.0ml) was determined from the E_{280}, and phospholipase activities of appropriate column fractions were determined. ^{3}H was assayed by liquid-scintillation counting (Intertechnique SL 30, spectrometer) in a mixture containing 30% (v/v) methanol in toluene and 0.6% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 1,4-bis-(5-phenoxazol-2-yl)benzene. Chloride concentrations of appropriate fractions were measured with an automatic chloride titrator (American Instrument Co., Silver Spring, Md., U.S.A.).

Phospholipase assays

The substrate was phosphatidylinositol prepared from rat liver by a combination of alumina and silicic acid column chromatography (Kai *et al.*, 1966). The phospholipid migrated as a single spot (90% recovery of applied phosphorus) on silicic acid t.l.c. plates. Phosphatidylinositol was labelled with ^{32}P by incubating slices of rat kidney cortex with $Na_2H^{32}PO_4$ (The Radiochemical Centre, Amersham, Bucks., U.K.). At the end of incubation the slices were washed in 0.15M-NaCl to remove exogenous $^{32}P_i$ and homogenized in 19vol. of chloroform–methanol (2:1, v/v). The extract was washed with 0.2vol. of water and twice with 0.5vol. of Folch theoretical upper phase containing $CaCl_2$ (Folch *et al.*, 1957). The organic phase was dried and dissolved in chloroform for spotting on to activated 0.5mm-thick plates of silica gel H (E. Merck A.-G., Darmstadt, Germany). The plates were developed in the first dimension with a solvent of chloroform–methanol–7M-NH_3 (46:18:3, by vol.). The plates were heated to 90°C for 5min and developed in the second dimension with an acid solvent consisting of chloroform–methanol–water–acetic acid (50:50:4:1, by vol.). Radioactive components were detected by radioautography and the appropriate spot was scraped off the plates. Phosphatidylinositol was eluted from the silicic acid by extracting twice with 5ml of ethanol–chloroform–water (10:3:2, by vol.). The extract was dried and the residue was redissolved in chloroform.

Phospholipase assays were done in a final volume of 0.25ml containing 1μg of lipid phosphorus, 20000–30000c.p.m. of ^{32}P as phosphatidylinositol, 2mM-$CaCl_2$ in 0.1M-$\beta\beta$-dimethylglutaric acid buffer, pH5.4. The dried lipids were dispersed in the buffer by shaking for 2min on a vortex mixer and then $CaCl_2$ was added. The substrate was preincubated for 5min at 37°C before addition of the enzyme in a volume of 0.05 or 0.1ml. The reaction was stopped after 30 or 60min by adding 0.4ml of methanol and shaking with 0.8ml of chloroform. After centrifugation, 0.4ml of aqueous phase was removed and washed with 1ml of theoretical lower phase prepared by mixing chloroform, methanol and water in the same proportions as in the reaction. The washed upper layer was counted for radioactivity in 5ml of water by Čerenkov radiation (Schneider, 1971) and blank values were subtracted from the assays to determine the liberation of ^{32}P from the phospholipid. The reaction rate was linear with time provided that less than 15% of the substrate was degraded and all assays were performed under near-zero-order reaction conditions. The water-soluble products were identified by high-voltage ionophoresis (Dawson *et al.*, 1962, 1971) and by ascending chromatography on Whatman 3 MM papers with a developing solvent of 1M-ammonium acetate (pH5)–ethanol (3:7, v/v). Neither system separated D-*myo*inositol 1-phosphate from D-*myo*inositol 2-phosphate, although it is likely that both isomers are present (Dawson *et al.*, 1971).

Density-gradient centrifugation

Pooled phospholipase-active peaks from DEAE-Sephadex chromatography were concentrated in an Amicon ultrafiltration cell (model 10-PA) at 4°C by using a UM-20E membrane. To avoid pressure effects on aggregated microtubular protein, peak III material (Fig. 3*a*) was concentrated after the KCl had been dialysed out by placing the dialysis bag between sheets of Whatman no. 3 paper for 24h. Samples (0.3ml) were layered over linear 5–22.5%

(w/v) sucrose gradients (4.8ml total volume) containing 10mM-imidazole–HCl buffer (pH7)–10mM-$MgCl_2$–0.1M-KCl–6mM-2-mercaptoethanol. The gradients were centrifuged for 16h at 47000rev./min in a Spinco SW 50 rotor and fractionated by puncturing the bottom of the tubes and collecting 130–140μl fractions into 100μg of bovine serum albumin (in 10μl). Gradients were calibrated by determining sedimentation rates of γ-globulin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.), ovalbumin [Sigma (London) Chemical Co., London S.W.6, U.K.] and chymotrypsinogen A (Boehringer, Mannheim, Germany) in gradients run concurrently with the enzyme fractions.

Preparation of microtubular protein

A mouse was injected interperitoneally with 100μCi of [^{3}H]proline and 100μCi of $NaH_2{}^{32}PO_4$. The brain, liver, kidneys, heart and alimentary tract were removed after 20h, minced with scissors and washed three times in 0.15M-NaCl. The washed tissue was homogenized in 2vol. of 0.25M-sucrose–0.1M-KCl–10mM-imidazole–HCl buffer (pH7)–10mM-$MgCl_2$–0.1mM-GTP. The homogenate was centrifuged for 30min at 16000*g* and proteins in the supernatant precipitated by between 38 and 49% saturation with $(NH_4)_2SO_4$ were redissolved in 10mM-imidazole – HCl buffer (pH7) – 10mM-$MgCl_2$ – 0.1mM-GTP – 6mM-2-mercaptoethanol. After dialysis against the same buffer the sample was freeze-dried and stored at –20°C. Microtubular protein was purified by ion-exchange chromatography by the column procedure of Weisenberg *et al.* (1968) (Fig. 1). Only two bands on sodium dodecyl sulphate–urea–polyacrylamide gels were observed, corresponding in molecular weight to the α and β subunits (Eipper, 1972).

Purified microtubular protein was phosphorylated by intrinsic cyclic nucleotide-dependent protein kinase activity (Goodman *et al.*, 1970) by incubating 1mg of the protein with 0.1μmol of [γ-^{32}P]ATP (10mCi/mmol; prepared by the method of Glynn & Chappell, 1964) for 1h at 37°C in the presence of 10μM-cyclic AMP. At the end of the incubation the phosphorylated protein was precipitated by adding solid $(NH_4)_2SO_4$ to give 50% saturation and redissolved in 1ml of 10mM-imidazole–HCl buffer (pH7) – 10mM-$MgCl_2$ – 0.1mM-GTP – 6mM-2-mercaptoethanol. A sample (1mg) of microtubular protein not treated with ATP was added to the phosphorylated sample and dialysed against two changes of 100ml of the same buffer at 4°C.

Gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gels (7% acrylamide–0.19% methylenebisacrylamide) were made to contain 8M-urea and 0.1% sodium dodecyl sulphate in 0.375M-Tris–HCl buffer, pH8.9 (Weber & Osborn, 1969; Davis, 1964). Samples and molecular-weight calibration standards [β-galactosidase (mol.wt. 130 000) and aldolase (mol.wt. 40 000) from Boehringer, phosphorylase, (mol. wt. 94 000), a gift from Dr. M. Iqbal, catalase (mol.wt. 60 000), bovine serum albumin (mol.-wt. 68 000), ovalbumin (mol.wt. 43 000) and cytochrome *c* (mol.wt. 12 000) all from Sigma (London) Chemical Co.] were solubilized in 8M-urea–2% sodium dodecyl sulphate–15mM-2-mercaptoethanol–50mM-Tris–glycine buffer, pH8.9. Electrophoresis was performed at 20°C with a running buffer of 50mM-Tris–glycine (pH8.9, 0.1% sodium dodecyl sulphate included) at 2mA/gel until the marker dye of Bromophenol Blue reached the bottom of the tube. Gels were fixed in 25% (v/v) propan-2-ol in 1.67M-acetic acid and stained for 1h in 0.1% Coomassie Brilliant Blue dissolved in the fixing solvent. Gels were destained by diffusion in 7% (v/v) methanol in 2.34M-acetic acid and microdensitometer

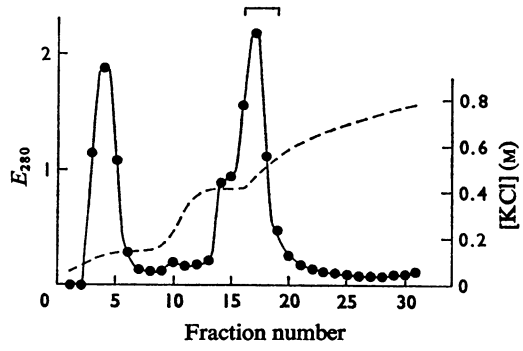


Fig. 1. *Purification of labelled microtubular protein by ion-exchange chromatography*

Ammonium sulphate fraction of mouse protein, precipitated by between 38% and 49% saturation with $(NH_4)_2SO_4$ (12mg) labelled *in vivo* with [^{3}H]proline and ^{32}P was redissolved in 5ml of 10mM-imidazole–HCl buffer (pH7)–10mM-$MgCl_2$–6mM-2-mercaptoethanol–0.1mM-GTP and applied to a DEAE-Sephadex A-50 column (1cm diameter, 5ml) equilibrated with the same buffer. Proteins were eluted by a combination of stepwise and exponential gradients of KCl dissolved in the buffer: 2ml fractions were collected. E_{280} and ^{3}H scintillation counting were coincident. Fractions 16–19 (indicated by bar) were pooled, precipitated by adding $(NH_4)_2SO_4$ to 50% saturation, redissolved in the original buffer and dialysed against the same buffer.

recordings were made with a Joyce–Loebl double-beam recording instrument (Mk III CS).

Results

A 5ml sample of brain supernatant was applied to a DEAE-Sephadex A-50 column equilibrated with buffer of low ionic strength. All the phospholipase activity was adsorbed to the column. Two peaks of phospholipase activity were eluted with increasing KCl concentration (Fig. 2*a*). The first peak (peak I) was eluted at 0.06M-KCl, coincident with a large protein peak measured by E_{280}. The second phospholipase peak (peak II) was eluted at about 0.22M-KCl. There was a shoulder of activity preceding the main peak II fraction which appeared as a consistent feature of the separation. The recovery of applied phospholipase was 103%, suggesting that all the enzyme was present in peaks I and II.

Samples of the water-soluble reaction products recovered from the phospholipase assays of each of the two peaks were pooled and identified by paper chromatography and high-voltage ionophoresis. The results, expressed as a percentage of water-soluble ^{32}P liberated during reaction, are presented in Table 1. Peak I products consisted of inositol phosphates with a ratio of D-*myo*inositol 1:2-cyclic phosphate to D-*myo*inositol 1- and 2-phosphates of approx. 4:1. Nearly half the products of this peak were in the form of glycerylphosphorylinositol, whereas peak II

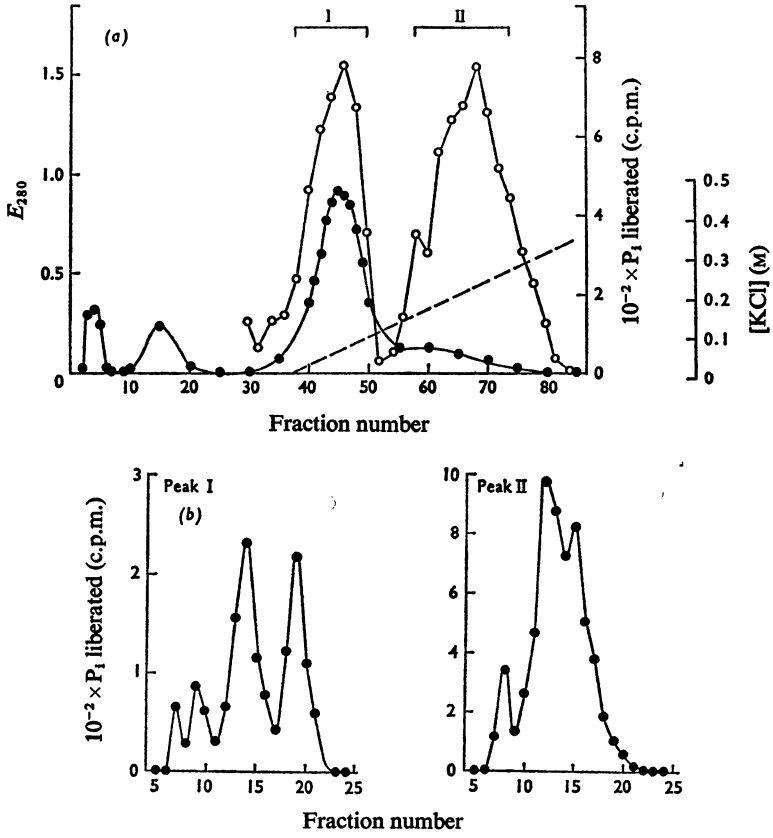


Fig. 2. *Purification and characterization of phosphatidylinositol phosphodiesterase of rat brain supernatant*

(*a*) DEAE-Sephadex ion-exchange chromatography of rat brain supernatant. Details are given in the Methods section. Fractions (2ml) were collected and determinations made of E_{280} (●), and phospholipase activity (○). The broken line indicates the eluting KCl concentration. (*b*) Preparative sucrose-density-gradient sedimentation of pooled peaks I and II from ion-exchange chromatography. Fractions are numbered from the bottom of the gradient. Appropriate fractions were assayed for phospholipase activity. Molecular-weight estimations are given in the text.

Table 1. *Percentage of water-soluble components of recovered phospholipase reaction products*

Percentages are means of recovered radioactivity (c.p.m.) of each compound separated by (1) paper chromatography and (2) by ionophoresis.

Fraction	D-*myo*Inositol 1-phosphate+ D-*myo*inositol 2-phosphate	D-*myo*Inositol 1:2-cyclic phosphate	Glycerylphosphoryl-inositol
Supernatant	26	48	26
Peak I	10	44	46
Peak II	28	60	12

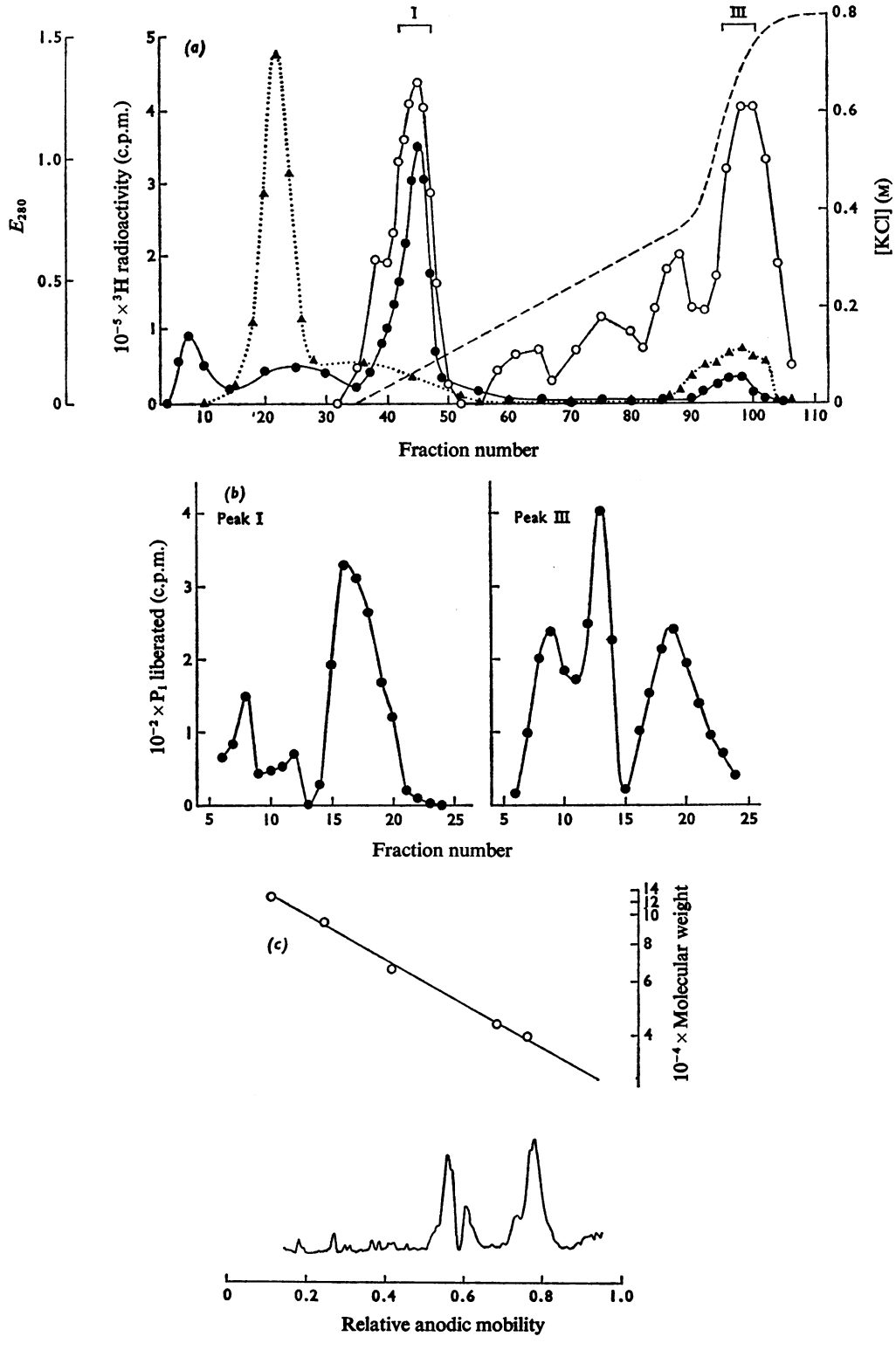
products had only small amounts of this compound. The ratio of D-*myo*inositol 1:2-cyclic phosphate to D-*myo*inositol 1- and 2-phosphates decreased to 2:1 in this peak.

Samples of DEAE-Sephadex fractions from peaks I and II were pooled, concentrated by ultrafiltration and layered over linear sucrose density gradients for preparative ultracentrifugation. Phospholipase assays of gradient fractions of peak I material (Fig. 2*b*) showed two main peaks of activity sedimenting in regions corresponding to molecular weights 66000 and 36000, with minor peaks of activity of higher molecular weight. The peak II fraction (Fig. 2*b*) sedimented in one main band corresponding to molecular weight 86000.

Preliminary experiments with rat brain supernatants treated with colchicine indicated a change in the molecular-weight distribution of phospholipase activity on density-gradient centrifugation. To examine the possible association between colchicine-binding microtubular protein and phospholipase enzyme, brain supernatant proteins labelled with [^{3}H]colchicine were purified by chromatography on DEAE-Sephadex as described above. The elution profile is shown in Fig. 3(*a*). Peak I, which eluted at about 0.06M-KCl, was again observed but peak II was practically abolished. A new peak (peak III) was eluted from the column together with protein-bound [^{3}H]colchicine at salt concentrations greater than 0.5M. The [^{3}H]colchicine not adsorbed to the DEAE-Sephadex was presumably free colchicine, and the trailing of this peak may be the result of a dissociation of protein-bound colchicine. Both main peaks were concentrated and sedimentation rates of components containing phospholipase activity determined as described above (Fig. 3*b*). The 36000-molecular weight component, characteristically observed in control peak I, was not present in the gradients, and most of the activity was recovered in a fraction sedimenting at molecular weight 60000. Peak III phospholipase activity was recovered in three peaks of activity corresponding to molecular weight 140000, 88000 and 43000 respectively. A sample of peak III material containing 0.1mg of protein was subjected to polyacrylamide-gel electrophoresis in sodium dodecyl sulphate–urea gels. There were two main bands recorded on a densitometer scan of the gel (Fig. 3*c*) migrating as proteins of molecular weight 56000 and 38000. A third, less densely staining band, was also present, and this corresponded to molecular weight 52000.

An experiment was designed to determine if there was an association of microtubular subunits with the phospholipase activity of peak II fraction. Two forms of microtubular protein labelled with [^{3}H]proline and $^{32}P_i$ *in vivo* were added to brain supernatant before chromatography on DEAE-Sephadex. The first was protein purified as shown in Fig. 1, which, from the results obtained from the first experiment (Fig. 2*a*), would not be eluted from DEAE-Sephadex in the peak II position. The second form was the same protein as the first but treated previously with cyclic AMP and [γ-^{32}P]ATP so that the amount of ^{32}P incorporated was increased by 63% over the labelling *in vivo*. The ^{32}P labels only serine residues of the β subunit; up to 10mol of P/mol of microtubular dimer prepared from rat brain has been reported (Eipper, 1972). The α and β subunits of microtubular protein possess the same number of proline residues (Bryan & Wilson, 1971; Fine, 1971), and it is reasonable to assume that each subunit incorporates the same amount of [^{3}H]proline during the labelling procedure *in vivo*. Thus the α subunits of the two forms of microtubular protein are indistinguishable from one another, since they have no ^{32}P, but the β subunits from protein treated or untreated with nucleotides can be identified by the ^{3}H/^{32}P ratio.

The elution pattern of phospholipase activity is shown in Fig. 4(*a*). Two peaks of activity were present, corresponding to peaks I and II respectively. Approx. 70% of phospholipase activity recovered from the column was present in the peak II fraction, which differed from untreated supernatant, where activity was more equally distributed between peaks I and II (Fig. 2*a*). Peak II appeared to be composed of two components which were not completely resolved on the salt gradient. The radioactivity of the column fractions (Fig. 4*b*) shows that some of the


(a)
I
III
E_{280}
$10^{-5} \times {}^3H$ radioactivity (c.p.m.)
[KCl] (M)
Fraction number
(b)
Peak I
Peak III
$10^{-2} \times P_i$ liberated (c.p.m.)
Fraction number
(c)
$10^{-4} \times$ Molecular weight
Relative anodic mobility

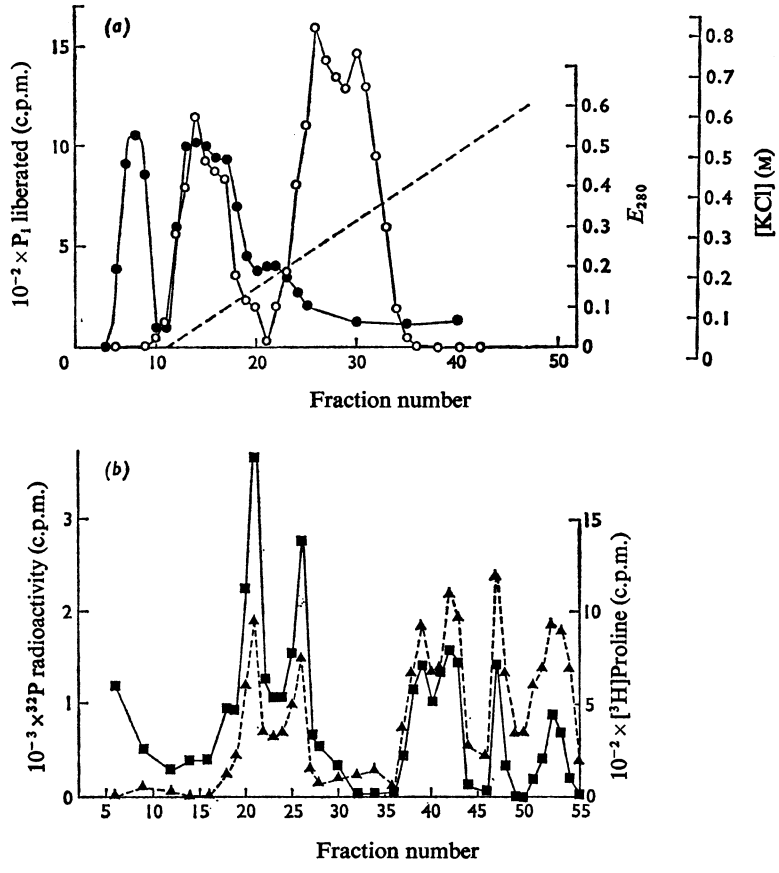


Fig. 4. *Identification of specific microtubular subunit interacting with phospholipase enzyme of rat brain supernatant*

Microtubular protein (mouse, 1 mg) labelled *in vivo* with [^{3}H]proline and $^{32}P_i$ and 1.0 mg of labelled microtubular protein phosphorylated with [γ-^{32}P]ATP in the presence of cyclic AMP were added to 1.0 ml of rat brain supernatant. The sample (2 ml) was applied to a 5 ml column (1 cm diameter) of DEAE-Sephadex A-50 and washed with 10 ml of 10 mM-imidazole–HCl buffer (pH 7)–10 mM-$MgCl_2$–6 mM-2-mercaptoethanol. This was followed by a linear 0–0.8 M-KCl (broken line) gradient of total volume 50 ml: 1.0 ml fractions were collected from the column. (*a*) Elution profile of protein, as determined by E_{280} (●) and distribution of phospholipase activity (○), from the column (*b*) ▲, ^{3}H and ■, ^{32}P radioactivity present in the corresponding fractions.

Fig. 3. *Effect of colchicine on molecular associations of phospholipase enzyme of rat brain supernatant*

(*a*) DEAE-Sephadex chromatography of rat brain supernatant prelabelled with [^{3}H]colchicine. Details are given in the Methods section. Fractions (2 ml) were collected and determinations made of E_{280} (●), phospholipase activity (○) and ^{3}H radioactivity (▲). The broken line indicates the concentration of KCl in the fractions. (*b*) Preparative sucrose-density-gradient sedimentation of pooled peaks I and III from ion-exchange chromatography. Appropriate fractions, numbered from the bottom of the gradient, were assayed for phospholipase activity. Molecular-weight estimations are given in the text. (*c*) Trace taken from a densitometer scan of peak III separated by sodium dodecyl sulphate–urea–polyacrylamide-gel electrophoresis. The mobility of marker proteins in gels run concomitantly is indicated. The two closely migrating bands have estimated molecular weights of 56000 and 52000 corresponding to the α and β subunits of microtubular protein respectively. The fastest-migrating band (mol.wt. 38000) is the phosphodiesterase enzyme. The mobilities are given relative to Bromophenol Blue.

microtubular protein is eluted from the column at much lower KCl concentrations than are required to displace typical microtubular protein from DEAE-Sephadex (see Fig. 1). Of the total [^{3}H]proline applied to the column approx. 25% was recovered in the first two peaks and the remainder was eluted at KCl concentrations greater than 0.4M. The ^{3}H:^{32}P ratio of the microtubular protein eluted at high salt concentration was higher than for the material originally applied to the column. Since only 25% of the [^{3}H]proline is recovered in the two peaks of radioactivity eluted at low salt concentration, then at the most only half of the ^{32}P label *in vivo* could be recovered in these fractions. If this amount of ^{32}P radioactivity is subtracted then the remainder accounts for 92% of the ^{32}P label *in vitro*. This suggests that these two peaks contain only β subunit of microtubular protein which had been previously treated with cyclic AMP and ATP. Of these two peaks of radioactivity only one was associated with phospholipase activity, and this was exclusively with the phospholipase of peak II eluted at lower KCl concentration. No phospholipase activity was associated with β subunit of microtubular protein not treated with nucleotides.

Discussion

A phospholipase C-type enzyme catalysing the specific degradation of phosphatidylinositol (Atherton & Hawthorne, 1968) has been demonstrated in many tissues, including pancreas (Dawson, 1959), liver (Kemp *et al.*, 1961), intestinal mucosa (Atherton *et al.*, 1966), brain (Friedel *et al.*, 1969), and thyroid (Dawson *et al.*, 1971; Jungalwala *et al.*, 1971). Most of the enzyme activity is located in high-speed supernatants, although in brain a considerable proportion of the total activity is found in the particulate fraction, and this can be solubilized to a certain extent by hypo-osmotic shock (Friedel *et al.*, 1969; Harwood & Hawthorne, 1969) or extracted with 2M-KCl (Keough & Thompson, 1972). There were no significant differences between soluble and salt-extracted enzyme with respect to pH optimum, calcium activation and apparent affinity for the substrate. Attempts have been made to purify the soluble enzyme from guinea-pig intestinal mucosa (Atherton & Hawthorne, 1968) by using $(NH_4)_2SO_4$ fractionation, gel filtration and density-gradient centrifugation, but the purified enzyme proved unstable and appeared in a number of molecular-weight species. Recovery of enzyme activity has now been improved by avoiding the initial $(NH_4)_2SO_4$ fractionation and adding bovine serum albumin to purified fractions. Enzyme assays were always performed as soon as practicable after each operation, as the activity decreased rapidly during storage at 4°C.

The soluble enzyme from rat brain is present in two fractions (peaks I and II) separated on DEAE-Sephadex. The phospholipase activity of peak I is resolved into two molecular species on density-gradient centrifugation, and since nearly half of the water-soluble products are in the form of glyceryl-phosphorylinositol it is probable that only one of these species is the phospholipase *c*-type enzyme. Although no analysis of the reaction products of density-gradient fractions was done to identify the enzyme liberating inositol phosphate, the removal of only the lighter component (mol.wt. 36000) from peak I after colchicine treatment suggests that this is the enzyme.

Treatment of the supernatant with colchicine shifts the lighter component of peak I and most of peak II activity to a new position (peak III) on the DEAE-Sephadex fractionation. This peak was coincident with the colchicine-binding protein. The three phospholipase-active species observed on density-gradient centrifugation of peak III (mol.wt. 140000, 88000 and 43000 respectively) were probably formed from association of the phospholipase enzyme with microtubular protein. Sodium dodecyl sulphate–urea–polyacrylamide-gel electrophoresis, which resolves proteins into individual subunits, would support this view, as peak III consisted only of microtubular subunits and a protein of molecular weight 38000. It is not possible to determine the stoicheoimetry of the association from the densitometer scan of the gel, as the β subunit does not stain comparably with the α subunit (Eipper, 1972). The most likely explanation of why phospholipase activity is recovered in peaks I and II and not associated with microtubular protein in untreated supernatant is that the protein is in an aggregated state such as the 30S form reported by Weisenberg *et al.* (1968). Colchicine, which dissociates microtubular protein into dimers (Adelman *et al.*, 1968), may expose binding sites for the phospholipase.

The microtubular protein labelled *in vivo* added directly to supernatant does not interact with phospholipase enzyme (Figs. 4*a* and 4*b*), and could be an aggregated form. Treatment of this protein with cyclic AMP and ATP may dissociate microtubular protein into monomers in which the β subunit can interact with the enzyme to form the 88000-molecular-weight aggregate. Preliminary experiments indicated that phosphorylation of the β subunit may not be essential to this process, as addition of cyclic AMP alone to supernatant caused a similar shift of phospholipase activity from peak I to peak II on DEAE-Sephadex chromatography and resulted in the same heterogeneity of the latter peak. Murray & Froscio (1971) have reported that microtubular protein, phosphorylated *in vitro* under conditions similar to those employed in the present experiments, interacted with an unidentified soluble protein from rat brain. The resulting complex caused a marked decrease in affinity of the labelled protein for DEAE-Sephadex.

The effect of cyclic AMP on microtubular protein and subsequently on the molecular form of the phospholipase enzyme may provide the link between phosphatidylinositol turnover in stimulated tissues and events involving membrane interactions such as secretion, lysosomal fusion etc. This hypothesis would require a demonstration that the activity of the two forms of the enzyme are different in physiological situations. Kemp *et al.* (1961) consistently observed a secondary peak of phospholipase activity about pH7 in addition to the main peak at pH5.7 in a rat liver preparation, and they suggested the possibility of two enzymes acting on the same substrate. Related to the effect of pH on enzyme activity is the change in surface charge of the enzyme when it interacts with the microtubular subunit as judged by affinity for DEAE-Sephadex. It is well established that the electrostatic status of the substrate and enzyme is a major factor in most phospholipase reactions (Dawson, 1968).

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