Phosvitin Kinase from Brain: Activation by Ions and Subcellular Distribution

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Protein kinases (Rabinowitz, 1962) catalysing the transfer of the terminal phosphate of ATP to phosvitin, casein and possibly other phosphoproteins have been found in mammalian liver (Burnett & Kennedy, 1954), mammary gland (Sundararajan, Sampath-Kumar & Sarma, 1960), erythrocyte membranes (Judah, Ahmed & McLean, 1962) and brain (Rabinowitz & Lipmann, 1960). The following study of a protein kinase from cerebral cortex was prompted by a number of observations suggesting that a phosphoprotein may be involved in the transport of cations across the cell membrane. For example, on the application of electrical pulses to slices of cerebral cortex *in vitro*, the incorporation of added [^{32}P]phosphate into a tissue phosphoprotein increases concomitantly with a breakdown of energy-rich phosphates and changes in ionic flux across the neuronal membrane (Heald, 1957*a*; Bachelard, Campbell & McIlwain, 1962; Trevor & Rodnight, 1963). Further, the increase in phosphoprotein metabolism induced by stimulation is prevented by the inclusion of ouabain in the medium or by substitution of the Na^+ ions by Li^+ ions (Ahmed, Judah & Wallgren, 1963). Evidence for a role for phosphorylated proteins in ion transport has also been reported in liver (Ahmed & Judah, 1962), erythrocytes (Judah *et al.* 1962) and kidney (Charnock & Post, 1963).

The main purpose of the present work has been to study the effect of univalent cations on the protein-kinase system in ox brain described by Rabinowitz & Lipmann (1960). We prefer to refer to the system as phosvitin kinase rather than protein kinase, as throughout the investigation we have used only phosvitin as a phosphate acceptor. A preliminary report on the work has already appeared (Rodnight & Lavin, 1963).

EXPERIMENTAL

Materials

Nucleotides. The disodium salt of ATP and the sodium salt of GTP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Where required, the tris salts were prepared by ion-exchange on micro-columns of Dowex 50 (H^+ form; X 12; 200–400 mesh).

Phosvitin. For most experiments phosvitin purchased from Nutritional Biochemicals Corp., Cleveland, Ohio,

U.S.A., was used. After being dissolved in water and dialysed against EDTA and water (Rose & Heald, 1961), the solution was adjusted to a concentration of 10 or 20 mg./ml. and stored at -20° . For one series of experiments a sample of phosvitin was prepared from egg yolk by the method of Joubert & Cook (1958). A yield of 200 mg. from 14 eggs was obtained. The behaviour of this product in the kinase system was indistinguishable from that of the commercial product.

Methods

General. Dispersions of tissues, acetone-dried powders or protein fractions were made in glass homogenizers fitted with Teflon pestles (A. H. Thomas Inc., Springfield, Ill., U.S.A.).

Centrifuging at high speed was carried out either in the 'superspeed' head of a Measuring and Scientific Equipment (MSE) refrigerated centrifuge, in a Spinco model L centrifuge, or for a few experiments in a MSE '25 000' centrifuge. As the performance of these machines differed, centrifugal forces are expressed throughout as $\text{g}\cdot\text{min.}$, i.e. $\text{g}\cdot\text{av.}$ multiplied by the time of centrifuging in minutes. In general, the times employed depended on the machine used and were as short as possible.

Analytical methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and inorganic phosphate by a micro-modification of the method of Martin & Doty (1949), the silicotungstate reagent being omitted.

Preparation of labelled nucleotides. (a) [^{32}P]ATP. The tris salt of ATP labelled with ^{32}P in the β - and γ -positions was prepared with rat-liver mitochondria essentially by the method of Pressman (1960). The same reaction mixture was used, except for the addition of only 1 mc of [^{32}P]phosphate (carrier-free, neutralized with NaHCO_3), 2 μmoles of Na_2HPO_4 and 1.5 μmoles of AMP. The best results were obtained by incubating at 30° for 0.5 hr.; the reaction was then stopped by adding 7 ml. of 0.25M-sucrose. After centrifuging for $2.5 \times 10^3 \text{g}\cdot\text{min.}$ the ATP was separated from the ADP and inorganic phosphate by chromatography on Dowex 1 (X 12), as described by Pressman (1960). The ATP fraction was further purified either by precipitation of the ATP as its barium salt (for details see McIlwain & Rodnight, 1962) or, after freeze-drying, by adsorption on deactivated charcoal as described below for [^{32}P]GTP. The latter method was found preferable and was eventually adopted for all preparations. Before precipitation or adsorption cold ATP (25 μmoles) was added. The ATP (barium salt) precipitate was suspended in 2 ml. of water and treated in a centrifuge tube with 1.5 g. of moist Dowex 50 (X 12; H^+ form; 200–400 mesh). After centrifuging and

washing the resin twice with 1 ml. volumes of water, the combined supernatants were similarly treated in a second tube with 0.3 g. of Dowex resin. The resulting solution was brought to pH 7.4 with *m*-tris and made up to 10 ml. with water. Typical preparations contained 1–3 μ moles of ATP/ml., less than 0.5 μ mole of inorganic phosphate/ml., and less than 0.2 μ mole of Na^+ ion/ml. The specific radioactivity of the [^{32}P]ATP ranged from 2×10^5 to 7×10^5 counts/min./ μ mole of acid-labile phosphate (10 min. at 100° in *N*-HCl). The solution was stored at -20° and before use samples were diluted with 10 mM-ATP (tris salt) to give the required radioactivity.

(b) [^{32}P]GTP. The same reaction mixture was used but containing in addition 10 μ moles of GTP. After the addition of sucrose solution and centrifuging as above, the supernatant was washed into a column (0.9 cm. $\times$ 15 cm.) of Dowex 1 (formate form; X 8; 200–400 mesh). The following volumes of 4*M*-formic acid, containing ammonium formate to the molarity stated (see Hurlbert, Schmitz, Brumm & Potter, 1954), were then applied to the column: (a) 350 ml., 0.05*M*; (b) 100 ml., 0.2*M*; (c) 100 ml., 0.4*M*; (d) 100 ml., 0.8*M*. Eluent (c), containing ATP, and eluent (d), containing GTP, were freeze-dried and the bulk of the ammonium formate was removed by sublimation. The residues were dissolved in 4 ml. of water and run through a charcoal–Celite column consisting of 500 mg. of Norite SX 2 (Harrington Brothers Ltd., City Rd., London) mixed with 500 mg. of Celite 535. The column was deactivated with 25 ml. of 8% (v/v) octan-2-ol in ethanol and washed with 2.5 ml. of water before use (Threlfall, 1957). After adsorption the columns were washed with 2 ml. of water and the nucleotides eluted with 15 ml. of aq. 20% (v/v) pyridine. The pyridine was removed by freeze-drying. To the appropriate residues 25 μ moles of cold ATP or GTP were added and the tris salts prepared as described above. In one such preparation the [^{32}P]ATP gave a specific radioactivity of 3.5×10^5 and the [^{32}P]GTP 1.0×10^5 counts/min./ μ mole of acid-labile phosphate. The identity of the GTP fraction was confirmed by chromatography for a second time on Dowex 1 (formate form).

Enzyme preparations from acetone-dried powders. Ox brain, obtained from the slaughter house within 2 hr. of death, was used to make acetone–butanol-dried powders essentially according to the method of Morton (1955). Approx. 45 g. of grey matter from the cerebral cortex was used for each preparation. The acetone-dried powders, when stored under vacuum at -20° , were stable for at least 4 months.

Phosvitin kinase was extracted from acetone-dried powders by a modification of the procedure of Rabinowitz & Lipmann (1960). The powder (2 g.) was dispersed in 50 ml. of 0.1*M*-phosphate buffer, pH 6.8 (adjusted with HCl), containing EDTA (0.1 mM). After being stirred at 2° for 1–2 hr., the dispersion was centrifuged for 4×10^5 g.-min. To the supernatant, while stirred vigorously at 2° , 12.5% of cold *N*-acetic acid was slowly added; the final pH was 4.6–4.8. The resulting precipitate was separated by centrifuging for 10^5 g.-min. and was dispersed in 45 ml. of 0.05*M*-phosphate buffer, pH 7.2 (adjusted with HCl), containing EDTA (0.1 mM). The dispersion was stirred for 1 hr. at 2° and adjusted, if necessary, to pH 7 with *N*-NaOH. A certain amount of lipoprotein always failed to redissolve at this stage and was usually removed by centrifuging for 10^5 g.-min. In some instances, however, this operation was

omitted and the insoluble proteins were then carried down with the precipitate that formed at 0.3 saturation with $(\text{NH}_4)_2\text{SO}_4$ in the next stage. The final yield of protein in the subsequent phosvitin-kinase-rich fraction was the same in either procedure.

Fractionation of the extract with $(\text{NH}_4)_2\text{SO}_4$ was carried out at 2° . After removal of the precipitate that formed on saturation to 0.3 by low-speed centrifuging, the supernatant was raised to 0.5 saturation, set aside at 2° for several hours, and the precipitate isolated by centrifuging for 10^5 g.-min. In some experiments a further fraction was isolated from the supernatant by full saturation with $(\text{NH}_4)_2\text{SO}_4$.

The protein precipitates were dissolved in a minimal volume of 0.05*M*-tris–HCl buffer, pH 7.0, containing EDTA (0.1 mM), and dialysed against two changes (100 vol. each) of the same buffer. Unless otherwise indicated, the first and last precipitates were discarded before dialysis. All the phosvitin-kinase studies were carried out on the precipitate collected between 0.3 and 0.5 saturation. Dialysis did not affect enzyme activity. The protein solutions were stored at -20° . For purposes of identification, each acetone-dried powder as prepared was given a number and the extracts prepared from it were given a letter.

Subcellular fractions. Dispersions of guinea-pig cerebral cortex were made in 10 vol. (w/v) of 0.32*M*-sucrose containing EDTA (0.5 mM) adjusted to pH 7.0 with *N*-NaOH. The nuclear fraction was isolated by centrifuging for 8000 g.-min. and was washed once with a further 10 vol. of sucrose solution by resuspension. A mitochondrial fraction was obtained at 2×10^5 g.-min. and was washed once with 25 vol. of sucrose solution. The microsomes were then isolated at 9×10^5 g.-min. Each particulate fraction was suspended in 0.32*M*-sucrose and stored at 2° pending analysis the following day.

Enzyme assays. (a) Phosvitin kinase. The reaction mixture contained 2 mg. of phosvitin, 1 μ mole of [^{32}P]ATP containing up to 5×10^4 counts/min./ μ mole of acid-labile phosphate, 50 μ moles of tris–HCl buffer, pH 7.4, and cations as stated. In some early experiments tris–phosphate buffer was used. After preincubation at 37° for 10 min., 0.1–0.2 ml. of enzyme was added to make a final volume of 1 ml. The reaction was stopped, usually after 10 min., by the addition of 4–5 ml. of ice-cold 10% (w/v) trichloroacetic acid. The precipitated protein was separated by centrifuging and washed three times in the centrifuge with 5 ml. of 10% trichloroacetic acid. Finally, the protein was dissolved in 1 ml. of *N*-NaOH, diluted to 10 ml. with water, and the radioactivity determined in a M6H liquid counter (20th Century Electronics Ltd.). Controls consisting of the complete reaction mixture, but without the addition of enzyme, were always carried through the whole procedure and the resulting counts/min. due to adsorbed radioactivity (never more than twice background) were subtracted from the counts/min. incorporated in the presence of enzyme; the latter generally ranged from 10 to 100 times background.

(b) Adenosine triphosphatase. The reaction mixture and procedure were as indicated above except that the phosvitin was omitted. Inorganic phosphate was determined in a fraction of the first trichloroacetic acid supernatant.

(c) Phosvitin phosphatase. The basic procedure was the same, except that ATP was omitted and the reaction was buffered with 60 μ moles of sodium succinate, pH 5.5.

RESULTS

Enzymes from acetone-dried powders

General observations. The extraction procedure gave an eight- to ten-fold increase in specific activity in the fraction precipitated by 0.5 saturation with ammonium sulphate, representing approx. 30 % of the activity present in the original acetone-dried powder extract. This degree of purification is lower than that of 15–20-fold reported by Rabinowitz & Lipmann (1960), who apparently used whole calf brain; the total recovery of activity in our experiments was also lower. Losses occurred partly while precipitating the extracted proteins with acetic acid at pH 4.7 and partly during fractionation with ammonium sulphate. We are unable to explain this discrepancy, as every precaution was taken to minimize losses of enzyme activity, including working at near 0° and using pure reagents at all stages. No loss of activity occurred on dialysis.

An analysis for phosphoprotein, phospholipid and phosphate-transferring enzymes in the fractions obtained with ammonium sulphate fractionation is given in Table 1. Little variation is seen in phosphoprotein content, but phospholipids tend to be higher in the first fraction, particularly in the material that failed to redissolve after precipitation at pH 4.7. Some 75 % of the phosvitin kinase was found in the fraction precipitated by 0.5 saturation with ammonium sulphate. The value of 0.73 μ mole of phosphate transferred/mg. of enzyme protein/hr. is typical for the specific activity of this fraction in the present work. The fact that it is lower than the value of 10 quoted for this fraction by Rabinowitz & Lipmann (1960) may be due to our use of untreated phosvitin as an acceptor, rather than dephosphophosvitin, which according to these authors yields a higher rate of transfer.

All fractions contained some adenosine-triphosphatase and phosvitin-phosphatase activities; these were approx. 5–10 % and 50 % respectively of the rates found in fresh tissue. It is known that both

these enzymes are sensitive to dialysis (Rose & Heald, 1961), and at least one of the several cerebral adenosine triphosphatases is not extracted from acetone-dried powders by aqueous solvents (Schwartz, Bachelard & McIlwain, 1962).

Characteristics of reaction. The specific activity, expressed as μ moles of phosphate transferred/mg. of protein/hr., of the soluble enzyme was about ten times that of fresh dispersions of tissue. Enzyme activity was linear in the range 50–400 μ g. of protein. For each enzyme preparation the quantity of protein added was adjusted so as to give an incorporation at least five times the control value; with most preparations about 200 μ g. of protein was added. During an incubation time of 10 min. 1–5 % of the [γ - 32 P]phosphate of [32 P]ATP was incorporated into the phosvitin. The time-course of the reaction with a typical enzyme preparation is shown in Fig. 1. With tris and Mg^{2+} ions as the only cations present, the transfer of [32 P]phosphate to the phosvitin proceeded linearly for at least 40 min. However, with the addition of potassium chloride (200 mM) the stimulated rate (see below) declined after 10 min. Increasing the concentration of phosvitin from 2 to 3 mg./ml. did not affect the stimulated rate over the first 10 min., but decreased by approx. 20 % the degree to which the rate declined over the subsequent 10 min. period. Thus, for measurements taken over the first 10 min., 2 mg. of phosvitin/ml. was considered a satisfactory concentration of substrate.

Increasing the ATP concentration twofold in the medium, while maintaining the same specific radioactivity, decreased the reaction rate. Two experiments illustrating this finding are shown in Table 2. In the first experiment, the concentration of magnesium chloride in the reaction mixture was also increased with that of the ATP to maintain the same molar ratio of the two substances, whereas in the second experiment the Mg^{2+} ion concentration was 5 mM throughout. In both cases, an increase in the ATP concentration decreased the rate of transfer.

Table 1. *Composition of acetone-dried-powder extracts fractionated with ammonium sulphate*

An extract of acetone-dried powder from ox brain was fractionated and dialysed as described in the Experimental section. Material failing to redissolve after precipitation at pH 7.4 was not separated before adding $(NH_4)_2SO_4$ to 0.3 saturation; it was therefore recovered as insoluble material in this fraction and analysed separately. All enzyme assays were carried out with Mg^{2+} ions at 3 mM concentration; further details are given in the Experimental section. Values marked with an asterisk were obtained with 200 mM-NaCl present in the reaction mixture.

Fraction obtained with $(NH_4)_2SO_4$ at saturation stated	Total protein (mg.)	Protein P and lipid P (μ m-moles of phosphate/mg. of protein)		Enzyme activities (μ mole of phosphate transferred to or released from phosvitin/mg. of enzyme protein/hr.)		
		Phosphoprotein	Phospholipid	Phosvitin kinase	Adenosine triphosphatase	Phosvitin phosphatase
0.3 (insoluble)	110	6.8	178	—	—	—
0.3 (soluble)	204	7.7	75	0.04, 0.12*	0.88, 0.69*	0.31
0.5	119	7.0	42	0.20, 0.73*	0.48, 0.35*	0.35
1	47	7.4	6	0.01, 0.03*	0.91, 0.11*	0.31

Effect of cations on enzyme activity. The requirement for Mg^{2+} ions reported by Rabinowitz & Lipmann (1960) was confirmed, the optimum concentration lying in the range 4–6 mM, with ATP at 1 mM concentration (Table 3). The Ca^{2+} ions in the same range either had no effect or, in the presence of Mg^{2+} ions, were inhibitory.

The percentage stimulation of enzyme activity given by a variety of univalent cations and also by tris, over and above that given by Mg^{2+} ions alone,

is shown in Table 4 (see also Table 7). These results are representative of a number of experiments with different enzyme preparations; the results from each preparation were obtained in the same experiment. All the univalent cations, with the exception of Li^+ ions, stimulated activity. The percentage stimulation was dependent on the concentration of

Fig. 1. Time-course of phosvitin-kinase reaction in acetone-dried-powder extract of ox brain. The basic reaction mixture (final vol. 1 ml.) contained phosvitin (2 mg.), $MgCl_2$ (3 mM), the tris salt of $[^{32}P]ATP$ (10^4 counts/min./ μ mole of acid-labile phosphate) (1 mM) and tris-HCl buffer, pH 7.4 (50 mM). At zero time 320 μ g. of enzyme protein was added. After incubation the reaction was stopped by the addition of 10% trichloroacetic acid and the precipitated protein was washed and counted as described in the text. All points are corrected for a zero-time control. ○, Basic reaction mixture; ●, with 200 mM-KCl.

Table 2. *Effect of adenosine triphosphate concentration on phosvitin-kinase reaction*

The reaction mixture (final vol. 1 ml.) contained 2 mg. of phosvitin (2 mg.), tris-HCl buffer, pH 7.4 (50 mM), NaCl (200 mM), KCl (30 mM) and enzyme protein (130 μ g.). The tris salt of $[^{32}P]ATP$ (10^4 counts/min./ μ mole of acid-labile phosphate) and $MgCl_2$ were incorporated as stated. The reaction time was 10 min. at 37°. Other details are given in the Experimental section.

Concn. of ATP (mM)	Concn. of $MgCl_2$ (mM)	Phosphate transferred (μ mole/mg. of enzyme protein/hr.)
1	5	0.94
1.5	7.5	0.81
2	10	0.76
1	5	0.88
1.5	5	0.77
2	5	0.70

Table 3. *Effect of magnesium and calcium ions on phosvitin-kinase activity*

The basic reaction mixture (final vol. 1 ml.) contained phosvitin (2 mg.), the tris salt of $[^{32}P]ATP$ (10^4 counts/min./ μ mole of acid-labile phosphate) (1 mM), tris-HCl buffer, pH 7.4 (50 mM), and bivalent cations as stated. The reaction time was 10 min. at 37°. Other details are given in the Experimental section.

Enzyme preparation (protein added)	Concn. of Mg^{2+} or Ca^{2+} ions (mM)	Phosphate transferred (μ mole/mg. of enzyme protein/hr.)
2F (152 μ g.)	Mg^{2+} , 0; Ca^{2+} , 0	0
	Mg^{2+} , 5	0.47
	Ca^{2+} , 3	0
	Ca^{2+} , 7	0
	Mg^{2+} , 5; Ca^{2+} , 5	0
4A (230 μ g.)	Mg^{2+} , 1	0.06
	Mg^{2+} , 2	0.14
	Mg^{2+} , 3	0.27
	Mg^{2+} , 5	0.39
	Mg^{2+} , 7	0.35
	Mg^{2+} , 8	0.26

Table 4. *Effect of univalent cations on phosvitin-kinase activity*

The basic reaction mixture (final vol. 1 ml.) contained phosvitin (2 mg.), the tris salt of $[^{32}P]ATP$ (10^4 counts/min./ μ mole of acid-labile phosphate) (1 mM), tris-HCl buffer, pH 7.4 (50 mM), and cations as stated at 200 mM concentration. The reaction time was 10 min. at 37°. Other details are given in the Experimental section.

Enzyme preparation (protein added)	Univalent cation added	Concn. of $MgCl_2$		
		1 mM	3 mM	5 mM
		Phosvitin-kinase activity (% of control with $MgCl_2$ at concn. stated)		
4A (260 μ g.)	Na^+	870	—	—
	K^+	910	—	180
4B (180 μ g.)	K^+	—	380	156
	NH_4^+	—	350	120
	Li^+	—	—	50
4C (250 μ g.)	Na^+	740	435	160
	K^+	760	490	180
5B (230 μ g.)	K^+	—	—	215
	Rb^+	890	310	210
	Cs^+	720	270	195
	Li^+	101	120	90
	Choline	—	172	137
9A (277 μ g.)	Tris	—	190	—

Mg²⁺ ions in the medium, decreasing markedly as this was increased to 5 mM. Thus at 1 mM-Mg²⁺ ion both Na⁺ and K⁺ ions at 200 mM concentration caused a seven- to ten-fold increase in activity, whereas at 5 mM-Mg²⁺ ion this was decreased to an increase of less than twofold. The same effect was seen when NH₄⁺, Rb⁺, Cs⁺, choline or tris ions were added, also at 200 mM concentration. Further, for maximal stimulation the optimum concentration of Na⁺ and K⁺ ions was found to be 200 mM at several concentrations of Mg²⁺ ions (Fig. 2). The addition of Na⁺ and K⁺ ions together gave no further activation than could be accounted for by the sum of their individual effects (Table 5).

Effect of phosphate. A stimulation of the enzyme by phosphate ions, maximal at 60 mM concentration, was reported by Rabinowitz & Lipmann (1960). No details were given of the corresponding cation used with the phosphate. In an early stage of the present investigation, we found that the

addition of 60 mM-tris, brought to pH 7.4 with phosphoric acid, instead of the usual 50 mM-tris-hydrochloric acid buffer, had no effect on enzyme activity, but that stimulation occurred when the tris-phosphate concentration (as tris) was increased to 100–200 mM, giving 30–70 mM-phosphate ion in the medium. At first this was taken as indicating a stimulation by phosphate ions, as we were then unaware that tris stimulates the enzyme system. Later it was shown that the stimulation given by 100–200 mM-tris-phosphate was approximately matched by the affect of equimolar amounts of tris ion added as the hydrochloride (Fig. 3). It would appear likely, therefore, that the stimulation reported by Rabinowitz & Lipmann (1960) was due to the counter-cation used by them, although this was not specified. In fact, Li⁺ is the only cation we have found that does not stimulate the enzyme, but, as lithium phosphate is insoluble, it cannot therefore be used for examining the effect of the phosphate ion on the system.

Guanosine triphosphate as precursor. An increase in the specific activity of GTP in cerebral-cortex slices after electrical stimulation has been reported (Heald, 1957*b*). A further study (Heald & Stancer, 1962) gave support to these findings, and was held to support the hypothesis that GTP is a more immediate precursor of the tissue phosphoprotein phosphate than is ATP. Experiments were therefore carried out to determine whether [³²P]GTP is a more effective donor than [³²P]ATP in the phospho-kinase system. No difference in the efficacy of

Fig. 2. Effect of different concentrations of Na⁺ ions (a) and K⁺ ions (b) on phosvitin kinase at three concentrations of Mg²⁺ ions. The basic reaction mixture (final vol. 1 ml.) contained phosvitin (2 mg.), the tris salt of [³²P]ATP (10⁴ counts/min./μmole of acid-labile phosphate) (1 mM) and tris-HCl buffer, pH 7.4 (50 mM). The reaction was started by the addition of 250 μg. of enzyme protein. ●, 1 mM-MgCl₂; ○, 3 mM-MgCl₂; ■, 5 mM-MgCl₂.

Table 5. *Effect of sodium and potassium ions added together on phosvitin kinase*

The basic reaction mixture and conditions were the same as given in Table 4.

Enzyme preparation	Cations added (mM)	Phosphate transferred to phosvitin (μmole/mg. of enzyme protein/hr.)
2C	Mg ²⁺ , 5; Na ⁺ , 100	0.54
	Mg ²⁺ , 5; Na ⁺ , 200	0.69
	Mg ²⁺ , 5; Na ⁺ , 100; K ⁺ , 30	0.60
	Mg ²⁺ , 5; Na ⁺ , 200; K ⁺ , 30	0.68
6A	Mg ²⁺ , 4; K ⁺ , 200	0.63
	Mg ²⁺ , 4; K ⁺ , 100; Na ⁺ , 100	0.60

Fig. 3. Stimulating effect of tris salts on phosvitin kinase. The basic reaction mixture (final vol. 1 ml.) contained phosvitin (2 mg.), the tris salt of [³²P]ATP (10⁴ counts/min./μmole of acid-labile phosphate) (1 mM) and MgCl₂ (3 mM); the final pH after the addition of tris salts was 7.4. The reaction was started by the addition of 277 μg. of enzyme protein. ●, Tris-HCl buffer; ○, tris-phosphate buffer.

the two nucleotides was found; a representative experiment is shown in Table 6.

Effects of added agents on enzyme activity. The following agents, examined at the concentrations stated under standard conditions both with and without the addition of sodium chloride or potassium chloride (200 mM), had no effect on enzyme activity: 0.1 mM-ouabain; 0.1 mM-dinitrophenol; 1 mM-acetylcholine. The lack of effect of ouabain is

shown in Table 7. In one experiment serotonin creatinine sulphate (1 mM) caused a small (20–30 %) inhibition of activity both with and without added K⁺ ions; this effect was not investigated further. In agreement with Rabinowitz & Lipmann (1960) enzyme activity was unaffected by the thiol reagent *p*-chloromercuribenzoate (10 μM).

Subcellular distribution of enzyme

When increasing amounts of a dispersion of guinea-pig cerebral cortex in 0.32M-sucrose were assayed for phosvitin-kinase activity, the incorporation of [³²P]phosphate into phosvitin increased linearly up to 500 μg. of protein and then fell off. In examining enzyme activity in subcellular fractions, therefore, the quantity of protein added to the assay system was limited to 200 μg. The results of two experiments are given in Table 8. These experiments were carried out before the stimulating effect of univalent cations on activity had been studied in detail, and both Na⁺ and K⁺ ions were included in the reaction mixture. Under these conditions the soluble cytoplasmic proteins were highest in activity, both in absolute amount and in terms of unit quantity of protein. Enzyme activity was present in all other fractions and was particularly high in the microsomal fraction. In fact, in subsequent experiments rather more activity was usually found in the microsomal fraction than in the soluble proteins.

The presence of a protein kinase in the microsomal fraction was of interest in view of the possible role of phosphoproteins in the transport of univalent cations. However, Na⁺ and K⁺ ions stimulated the enzyme in similar fashion in both the microsomal membrane-rich fraction and in the soluble cytoplasmic protein fraction (Table 9). Similar results were obtained with 2 mM- and 4 mM-magnesium chloride, instead of 3 mM, in the medium.

Table 6. *Comparison of the efficacy of adenosine [³²P]triphosphate and guanosine [³²P]triphosphate as phosphate donors in the phosvitin-kinase system*

The basic reaction mixture and conditions were the same as given in Table 4, except that the specific radioactivity of the [³²P]ATP (tris salt) was 0.9 × 10⁴ counts/min., and that of the [³²P]GTP (tris salt) was 1.2 × 10⁴ counts/min./μmole of acid-labile phosphate. The final concn. of both nucleotides was 1 mM.

Cations added (mM)	Phosphate transferred to phosvitin (μmole/mg. of enzyme protein/hr.)	
	[³² P]ATP	[³² P]GTP
Mg ²⁺ , 3	0.22	0.21
Mg ²⁺ , 3; Na ⁺ , 200	0.64	0.69
Mg ²⁺ , 3; K ⁺ , 200	0.78	0.71

Table 7. *Lack of effect of ouabain on phosvitin kinase*

The basic reaction mixture and conditions were the same as given in Table 4.

Cations added (mM)	Phosphate transferred to phosvitin (μmole/mg. of enzyme protein/hr.)	
	Without ouabain	With ouabain (0.1 mM)
Mg ²⁺ , 3	0.26, 0.28	0.28
Mg ²⁺ , 3; Na ⁺ , 200	0.63, 0.64	0.68, 0.66
Mg ²⁺ , 3; K ⁺ , 200	0.67, 0.70	0.72, 0.71

Table 8. *Subcellular distribution of phosvitin kinase in dispersions of guinea-pig cerebral cortex*

Subcellular fractions were prepared in 0.32M-sucrose as described in the Experimental section. The reaction mixture (final vol. 1 ml.) consisted of phosvitin (2 mg.), the tris salt of [³²P]ATP (10⁵ counts/min./μmole of acid-labile phosphate) (1 mM), tris-phosphate buffer, pH 7.6 (50 mM), MgCl₂ (5 mM), NaCl (200 mM) and KCl (30 mM). The quantities of protein added for each fraction were 250 μg. in Expt. 1 and 150 μg. in Expt. 2. After 15 min. at 37° the reaction was stopped with 10 % trichloroacetic acid and the ³²P incorporated into phosvitin determined as described in the text.

Fraction	Expt. 1			Expt. 2		
	10 ⁻³ × Counts/ min./g. of tissue	Counts/min./μg. of protein	Recovery (%)	10 ⁻³ × Counts/ min./g. of tissue	Counts/min./μg. of protein	Recovery (%)
Original dispersion	246	1.89	—	136	1.58	—
Nuclear	12	1.74	5	10	1.58	—
Mitochondrial	67	1.07	25	34	0.82	25
Microsomal	52	2.36	21	27	1.66	20
Soluble proteins	94	3.69	38	75	4.50	55
		Total recovery	89		Total recovery	107

Table 9. *Effect of sodium and potassium ions on phosphatase in microsomal and soluble protein fractions*

The basic reaction mixture (final vol. 1 ml.) contained phosphatase (2 mg.), the tris salt of [32 P]ATP (10^5 counts/min./ μ mole of acid-labile phosphate) (1 mM), tris-HCl buffer, pH 7.4 (50 mM), $MgCl_2$ (3 mM), and univalent cations as stated. For both fractions approx. 200 μ g. of enzyme protein was added. The reaction time was 10 min. Other details are given in the Experimental section. The results quoted are from two independent experiments, A and B.

Univalent cation (mM)	Phosphate transferred to phosphatase (μ m-moles/mg. of enzyme protein/hr.)			
	Enzyme in microsomes		Enzyme in soluble proteins	
	Expt. A	Expt. B	Expt. A	Expt. B
None	55	29	45	13
Na^+ , 200	127	58	92	36
K^+ , 200	144	58	110	47

DISCUSSION

Stimulation by cations. Requirements for univalent cations, similar to those demonstrated above for the phosphatase of brain, have been found for certain other Mg^{2+} ion-dependent enzymes transferring phosphate from ATP. Particular examples are pyruvate kinase (Kachmar & Boyer, 1953), fructokinase (Hers, 1952; Parks, Ben-Gershom & Lardy, 1957) and a protein kinase found in erythrocyte membranes (Judah *et al.* 1962). The specificity of the stimulation by univalent cations is also similar for these enzymes and for phosphatase kinase, K^+ being the most effective ion, whereas Li^+ ions are almost without action. The Na^+ ion appears to be a relatively more effective activator of the two protein kinases than of the other enzymes, where Na^+ ions are less effective than Rb^+ or NH_4^+ ions; this is particularly so with pyruvate kinase. However, the low degree of purification at present achieved with the protein kinases makes a definitive assessment of their cation requirements premature. Finally, it should be emphasized that the specificity and pattern of stimulation of cerebral phosphatase kinase by univalent cations is different from that found with the membrane adenosine triphosphatases, where both Na^+ and K^+ ions (or certain other cations) are required for maximum activity (McIlwain, 1963).

A relation between the Mg^{2+} ion concentration and the magnitude of the stimulation by univalent cations similar to that found in the present study (Table 4) was observed for liver fructokinase by Hers (1952), although this finding was not confirmed in a later study of a purer form of this enzyme by Parks *et al.* (1957). In the work of Hers the optimum concentration of Mg^{2+} ions depended on both the ATP and the univalent cation concen-

trations, whereas with phosphatase kinase maximal activity occurred at 5 mM- Mg^{2+} ion in the range 1–2 mM-ATP (Table 2) and with 200 mM- Na^+ or K^+ ion (Fig. 2). The fact that the molar concentration of Mg^{2+} ions for maximal activity of the protein kinase is five times the ATP concentration suggests that Mg^{2+} ions may modify the conformation of the phosphatase acceptor as well as combining with an enzyme-ATP complex.

A stimulation of cerebral phosphatase kinase by phosphate ions, reported by Rabinowitz & Lipmann (1960), could not be demonstrated in our study because all the counter-cations available for neutralizing the added phosphate ions stimulated the enzyme. If stimulation by an unspecified cation is the explanation for the 'phosphate' effect described by Rabinowitz & Lipmann (1960), then it is reasonable to surmise that the protein kinase of yeast, found by these workers not to require phosphate for maximum activity, would be insensitive to univalent cations.

Subcellular distribution and significance of phosphatase kinase. The wide subcellular distribution of cerebral phosphatase kinase suggests that the phosphorylation of certain proteins can occur in all parts of the cell. The identity of the native proteins that may accept phosphate from ATP through this enzyme is unknown, but the evidence of Rabinowitz & Lipmann (1960) suggests that they possess structures analogous to the classical phosphoproteins lacking enzyme function such as phosphatase and casein, rather than the phosphotransferases. However, there is no direct evidence, as yet, for the occurrence of the classical type of phosphoprotein in cerebral tissue. Evidence has been presented (Heald, 1961) suggesting the presence in brain proteins of polyphosphorylserine peptides similar to those found by Williams & Sanger (1959) in phosphatase and casein, but their isolation and characterization was not attempted owing to shortage of starting material. By contrast, attempts to isolate phosphoproteins from brain by chromatographic methods (K. Kothary & R. Rodnight, unpublished work) have given no evidence for a protein species greatly enriched in phosphorus, such as casein, but have rather seemed to indicate that the tissue contains a wide range of phosphorylated proteins containing relatively few phosphorus atoms/molecule.

The present results do, however, make it unlikely that a phosphatase kinase is a component of a cation-transport mechanism. The enzyme in the soluble protein fraction is as active and shows the same cation requirements as that in the membrane-rich microsomal fraction; moreover, the molar rate of the reaction is many times less than that of the Na^+ ion-stimulated adenosine triphosphatase of cerebral microsomes.

SUMMARY

1. The Mg^{2+} ion-dependent phosvitin kinase prepared from ox-brain grey matter was further activated by a variety of univalent cations. At 200 mM concentration, K^+ was the most effective cation, but Na^+ , Rb^+ , Cs^+ and NH_4^+ ions were only slightly less effective. Choline and tris also stimulated the enzyme system, but Li^+ ions were altogether without effect.

2. The percentage stimulation by univalent cations was markedly dependent on the concentration of Mg^{2+} ions present: for instance, at 1 mM-magnesium chloride, 200 mM-potassium chloride increased activity approximately eightfold, whereas at 5 mM-magnesium chloride (the optimum Mg^{2+} ion concentration in the absence of univalent cation) stimulation was only twofold.

3. Previous reports that this enzyme is stimulated by phosphate ions were not confirmed.

4. GTP was approximately as effective as ATP in donating phosphate to phosvitin.

5. The enzyme was present in the nuclear, mitochondrial, microsomal and soluble protein fractions of the cell. Both in total activity and activity per unit quantity of protein the soluble proteins were highest. The effect of Na^+ and K^+ ions on the enzyme of the soluble fraction was compared with their effect on the microsomal enzyme: approximately equal stimulation was found in both cases.

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