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PHOSPHORYLATION OF 9-(2-PHOSPHONOMETHOXYETHYL)ADENINE AND 9-(*S*)-(3-HYDROXY-2-PHOSPHONOMETHOXYPROPYL)ADENINE BY AMP(dAMP) KINASE FROM L1210 CELLS

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Abstract—Acyclic nucleotide analogues 9-(2-phosphonomethoxyethyl)adenine (PMEA) and 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine ((*S*)-HPMPA) which display potent antiviral activity are transformed in the cells to their mono- and disphosphoryl derivatives. We purified from mouse L1210 cells the enzyme that in two steps phosphorylates PMEA and (*S*)-HPMPA to their diphosphoryl derivatives and found that it co-purifies with AMP(dAMP) kinase activity; the best substrates of this enzyme were AMP, ADP and dAMP. Other nucleoside 5′-triphosphates or creatine phosphate could not be substituted for ATP as a phosphate donor. Our results also indicated that at least one other enzyme (creatine kinase) is capable of transforming the monophosphoryl derivatives of the studied compounds to their respective diphosphates.

The phosphonomethoxyalkyl nucleotide analogues 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)-adenine [(*S*)-HPMPA†] and 9-(2-phosphonomethoxyethyl)adenine (PMEA) (Fig. 1) and several other related compounds exhibit a potent and selective activity against a great variety of viruses, mainly DNA viruses and retroviruses [1–4]. (*S*)-HPMPA and PMEA are transported both into infected and non-infected cells where they are converted to their respective mono- and diphosphoryl derivatives [5–7] and bring about selective inhibition of viral DNA synthesis [7]. Of the viral enzymes HSV-1 DNA polymerase [8], HIV and AMV reverse transcriptase [5, 9] and HSV-1 ribonucleotide reductase [10] were shown to be inhibited by phosphorylated derivatives of (*S*)-HPMPA, PMEA and related compounds and are considered as possible targets for virus chemotherapy. The antiviral activity of (*S*)-HPMPA and PMEA apparently depends on their phosphorylation to active compounds by cellular enzymes.

We have demonstrated that the transformation of (*S*)-HPMPA and PMEA to their respective mono- and diphosphoryl derivatives takes place in crude extracts from mouse L1210 cells [11]. This phosphorylation is slow, proceeds in two steps, and in addition to ATP, also CTP, UTP and GTP can serve as phosphate donors. When ATP regenerating systems are used, the extent of phosphorylation is increased significantly, and ATP appears as the most efficient phosphate donor [7, 11]. The enzyme(s) responsible for (*S*)-HPMPA and PMEA phosphorylation has not been isolated yet. Recently Balzarini *et al.* [5, 12, 13] demonstrated one-step phosphorylation of PMEA and 9-[(2*RS*)-3-fluoro-2-phosphonomethoxypropyl]adenine (FPMPA, Fig. 1) to PMEApp and FPMPApp by diphosphate transfer from 5-phosphoribose-1-diphosphate (PRPP) catalyzed by 5-phosphoribose-1-diphosphate synthetase (PRPP-synthetase) from *Escherichia coli.*

Our investigation has been undertaken to gain a better insight into the metabolism of (*S*)-HPMPA and PMEA; we decided to purify the enzyme(s) that is responsible for phosphorylation of these compounds. Mouse L1210 cells were chosen as the source of enzymes because they are known to possess high activities of various kinases [14]. It turned out that the PMEA phosphorylating enzyme co-purified with AMP(dAMP) kinase; we also provide evidence that this enzyme can catalyze a two-step transformation of PMEA [(*S*)-HPMPA] to PMEA [(*S*)-HPMPA] diphosphate.

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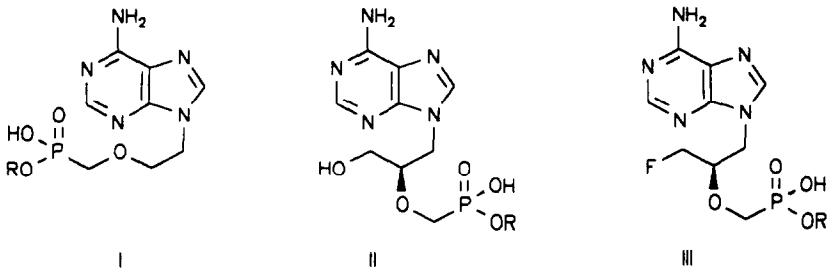
† Abbreviations: CP/CPK, creatine phosphate/creatine kinase; FPMPA, 9-(3-fluoro-2-phosphonomethoxypropyl)-adenine; FPMPAp, phosphoryl derivative of FPMPA; FPMPApp, diphosphoryl derivative of FPMA; HPMPA, 9-(3-hydroxy-2-phosphonomethoxypropyl)adenine; HPMPAp, phosphoryl derivative of HPMPA; HPMPApp, diphosphoryl derivative of HPMPA; HPMPC, 9-(3-hydroxy-2-phosphonomethoxypropyl)cytosine; HPMG, 9-(3-hydroxy-2-phosphonomethoxypropyl)guanine; PMEA, 9-(2-phosphonomethoxyethyl)adenine; PMEAp, phosphoryl derivative of PMEA; PMEApp, diphosphoryl derivative of PMEA; PRPP, 5-phosphoribose-1-diphosphate; PRPP synthetase, 5-phosphoribose-1-diphosphate synthetase (EC 2.7.6.1); and TBAHS, tetrabutylammonium hydrogen sulfate.



I II III

PMEA (I), HPMPA (II), FPMPA (III): R = H

PMEAp (I), HPMPAp (II), FPMPAp (III): R = $P(O)(OH)_2$

PMEApp (I), HPMPApp (II), FPMPApp (III): R = $P(O)(OH)-O-P(O)(OH)_2$

Fig. 1. Structures of PMEA, HPMPA, FPMPA and their mono- and diphosphoryl derivatives.

MATERIALS AND METHODS

Chemicals. (*S*)-HPMPA, PMEA and their mono- and diphosphoryl derivatives, (*R*)-HPMPA and (*S*)- and (*R*)-FPMPA were prepared according to published procedures [13, 15–17]. [Adenine-U-^{14}C]-(*S*)-HPMPA (4.96 MBq/μmol) and [adenine-U-^{14}C]-PMEA (4.96 MBq/μmol) were synthesized as described by Votruba *et al.* [7]. [^{14}C]AMP (9.25 MBq/μmol), [^{14}C]ADP (9.26 MBq/μmol) and [U-^{14}C]-dAMP (17.2 MBq/μmol) were obtained from the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia). Creatine kinase (CPK) (EC 2.7.3.2) was purchased from Boehringer (Germany); creatine phosphate (CP), PRPP and PRPP synthetase (P-0287, Lot 69F8130) were from Sigma (U.S.A.). Phosphocellulose P-11 and DEAE-cellulose DE-52 were purchased from Whatman (Great Britain); molecular weight calibration kits, Columns PD-10, Sephacryl S-300, DEAE-Sephadex A-50, Polybuffer Exchanger PBE 94 and Polybuffers PB 74 and PB 96 for chromatofocusing were products of Pharmacia (Sweden).

Cells. Mouse L1210 leukemia cells were grown in inbred DBA/2 male mice (25 g) after i.p. transfer of 10^5 cells and harvested 8 days after inoculation. The cells were released from the peritoneum in phosphate-buffered saline (PBS), washed twice in 10 mM potassium phosphate buffer with 0.15 M KCl and 5 mM 2-mercaptoethanol, and stored at −78°.

Purification of PMEA phosphorylating enzyme from L1210 cells. All procedures were conducted at 4°, and mixtures were centrifuged for 20 min at 30,000 *g*; all buffers contained 2 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. Protein concentration was estimated by the method of Bradford [18].

Cells obtained from ten mice were thawed, resuspended in 10 mM Tris–HCl buffer (pH 7.4) to a final volume of 24 mL and, after sonication (60 sec, amplitude 12 at 22 kc) the cell-free extract was centrifuged at 30,000 *g* for 20 min and at 100,000 *g* for an additional 100 min. Nucleic acids were precipitated with streptomycin sulfate (0.7%) and removed by centrifugation. Proteins were concentrated by salting out in 80% ammonium sulfate and dissolving in 50 mM Tris–HCl buffer (pH 8.0, buffer A). The crude extract was then dialyzed overnight against the same buffer.

Chromatofocusing was performed on a polybuffer exchanger PBE 94 column (0.8 × 48 cm) equilibrated in 25 mM Tris-acetate buffer (pH 8.8). After 5 mL of elution buffer (polybuffer PB 74 and PB 96 mixed in a 1:1 ratio, diluted ten times with water, adjusted to pH 5.0 by acetic acid), 6 mL of crude extract (81 mg protein) was applied onto the column and the pH gradient was created in the column by elution buffer (12 mL/hr, fractions 6 mL). Active fractions were pooled and components of polybuffer were removed by ammonium sulfate precipitation (100%). Precipitated proteins were dissolved in buffer A and desalted on PD-10 columns equilibrated in the same buffer.

Samples of 4.5 mL (8.7 mg protein) obtained by chromatofocussing were applied onto the DEAE-cellulose DE-52 column (1.6 × 5.5 cm) equilibrated with buffer A. After a 40-mL wash with buffer A the elution continued with an 80 mL gradient of 0 to 0.5 M KCl in the same buffer (flow rate 24 mL/hr, fractions 4 mL). Active fractions were pooled, concentrated in an Amicon concentration cell equipped with a YM-10 membrane, and dialyzed against 50 mM Tris–HCl buffer (pH 7.5).

A column of phosphocellulose P-11 (0.8 × 20 cm) was equilibrated in 50 mM Tris–HCl buffer (pH 7.5). After the dialyzed DE-52 fraction (3.9 mL, 1.7 mg) was applied onto the column (7 mL/hr), the elution continued with 50 mL of equilibration buffer and a 100-mL gradient of 0 to 1.0 M KCl in the same buffer. The active fractions were pooled, concentrated on YM-10 membrane, supplemented with bovine serum albumin to a concentration of 50 μg/mL, dialyzed against 20 mM Tris–HCl buffer (pH 7.5), and stored at −75°.

Preparation of DE-fraction. Onto the column of DEAE-Sephadex A-50 (1.4 × 2.2 cm), equilibrated in 50 mM Tris–HCl buffer (pH 7.4), was applied 10 mL of crude extract previously dialyzed against the equilibration buffer. The PMEA phosphorylating enzyme did not bind and was eluted with the same

buffer. This preparation (6.2 mg protein/mL), termed DE-fraction, was used for some kinetic studies.

Electrophoresis. Discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 4.5% stacking gel and 12.5% separating gel was run on a 2050 MIDGET-Electrophoresis Unit (LKB) by the method of Laemmli [19] and stained with Coomassie Brilliant Blue. Gradient polyacrylamide gel electrophoresis of native enzyme was performed overnight in 64 × 62 × 1.5 mm non-denaturing gel with acrylamide gradient 4–30% in the same apparatus. Voltage during the preelectrophoresis and sample application was 70 V, during the run 110 V, total 2000 Vhr. The gel was cut into 2-mm slices and assayed for (*S*)-HPMPA and AMP phosphorylation activities. LMW and HMW calibration kits from Pharmacia were used as molecular weight standards.

Enzyme activity measurements. All activity estimations were performed at 37°. Reactions with radioactive substrates were terminated by the spotting of appropriate aliquots on PEI-cellulose TLC plates and fast drying, and samples for HPLC measurements were prepared by adding 1.5 vol. of methanol. Unless otherwise stated, the sum of mono- and diphosphoryl derivatives is taken as product concentration.

The enzyme assays during purification and most kinetic studies were performed in a reaction mixture (20 or 40 μL) containing 10 μM [^{14}C]PMEA or [^{14}C]-AMP, 0.2 mM ATP, 1 mM $MgCl_2$, 5 mM creatine phosphate, 0.05 mg/mL creatine kinase, 40 mM HEPES (pH 8.0) and an appropriate amount of enzyme. Reaction was terminated after 10 min (for AMP phosphorylation) or 120 min (for PMEA phosphorylation).

Stereospecificity of HPMPA and FPMPA phosphorylation with non-radioactive substrates (0.1 mM) was measured in the presence of 1 mM ATP, 1 mM $MgCl_2$, 40 mM HEPES (pH 8.0), 10 mM creatine phosphate, 0.1 mg/mL creatine kinase and an appropriate amount of DE-fraction. Phosphorylation of PMEAp and (*S*)-HPMPAp by purified PMEA phosphorylating enzyme was measured in the same mixture, except that CP and CPK were omitted. The reaction was stopped after 120 min by adding methanol and products were separated by HPLC.

Phosphorylation of 5 mM [^{14}C]ADP, (*S*)-HPMPAp and PMEAp by creatine kinase was performed in a 20-μL reaction mixture consisting of 40 mM HEPES (pH 8.0), 20 mM $MgCl_2$, 20 mM creatine phosphate and 0.001 mg/mL (for ADP phosphorylation) or 0.05 mg/mL [for (*S*)-HPMPAp and PMEAp phosphorylation] creatine kinase and evaluated by TLC and HPLC, respectively.

Analysis of reaction products. Reaction products were separated on PEI-cellulose TLC plates (Macherey–Nagel, Germany) in 0.8 M LiCl/0.8 M acetic acid. Non-radioactive PMEA, PMEAp and PMEApp (R_f: 0.80, 0.59 and 0.24, respectively) were used as carrier standards in experiments with both PMEA and HPMPA, and adequate carrier mono-, di-, and triphosphates were used when other radioactive substrates were used. Either product spots or 0.4-mm slices were evaluated for radioactivity in toluene-based scintillation fluid.

Analysis of products of radioactive PMEA and (*S*)-HPMPA phosphorylation was performed on a column (4.6 × 250 mm) of Silasorb C18, 5 μm (Lachema, Czechoslovakia), in 3 mM tetrabutylammonium hydrogen sulfate (TBAHS) and 50 mM KH_2PO_4 (pH 6.8) with acetonitrile gradient (4–12%, v/v) (flow rate 1 mL/min, 25 min). Fractions per 25 sec were collected and evaluated for radioactivity in Brej scintillation mixture. Non-radioactive standards were used to verify the identity of the reaction products.

Analysis of samples with non-radioactive substrates was performed on 4.6 × 150 mm Separon SGX RPS (7 μm) columns (Tessek, Czechoslovakia). Products of PMEA and HPMPA phosphorylation in the presence of ATP were separated in 3 mM TBAHS and 50 mM KH_2PO_4 (pH 3.2) with acetonitrile gradient (2–4% in 5 min, 4–20% in 30 min). The separation of FPMPA and its phosphorylation products was accomplished in the same mobile phase with pH 5.5 and acetonitrile gradient from 6 to 20% in 50 min. Separation of products of PMEAp and (*S*)-HPMPAp phosphorylation by creatine kinase (no ATP present) was accomplished in 20 min by 10–50% methanol gradient in the above buffer with pH 3.2. Positions of products were identified by the use of non-radioactive standards.

RESULTS

Purification of PMEA phosphorylating enzyme. Purification from extracts of L1210 cells by chromatofocusing and chromatography on DEAE-cellulose and phosphocellulose is documented in Fig. 2. A pH gradient between 8.5 and 5.0 was used for the separation of crude extract proteins on polybuffer exchanger column, where 83% of PMEA phosphorylating activity eluted at pH range 7.4 to 6.9 and 13% at pH 6.3 to 6.2. The PMEA phosphorylating activity co-purified with AMP kinase activity during all purification steps; the final recovery was 1.5%. To prevent the loss of activity the active fractions after the phosphocellulose step were supplemented with bovine serum albumin prior to dialysis. The final enzyme preparation was stable for at least 3 months at −70° when 20% glycerol was added before freezing, and exhibited a specific activity of 24 nmol/min/mg and 33 μmol/min/mg when measured with 10 μM PMEA and AMP, respectively.

Molecular weight estimation and isoelectric point. (*S*)-HPMPA and AMP phosphorylating activities were detected in the same positions on a gradient polyacrylamide gel and corresponded to a molecular weight of 68,000 (Fig. 3). Similar results were obtained when PMEA was used as substrate. Elution patterns of PMEA and AMP phosphorylating activities were also identical when partially purified enzyme preparations were subjected to chromatography on Sephacryl S-300 (data not shown). SDS electrophoresis of purified enzyme revealed two dominant bands with molecular weights of 40,000 and 29,000 (Fig. 4). The isoelectric point was

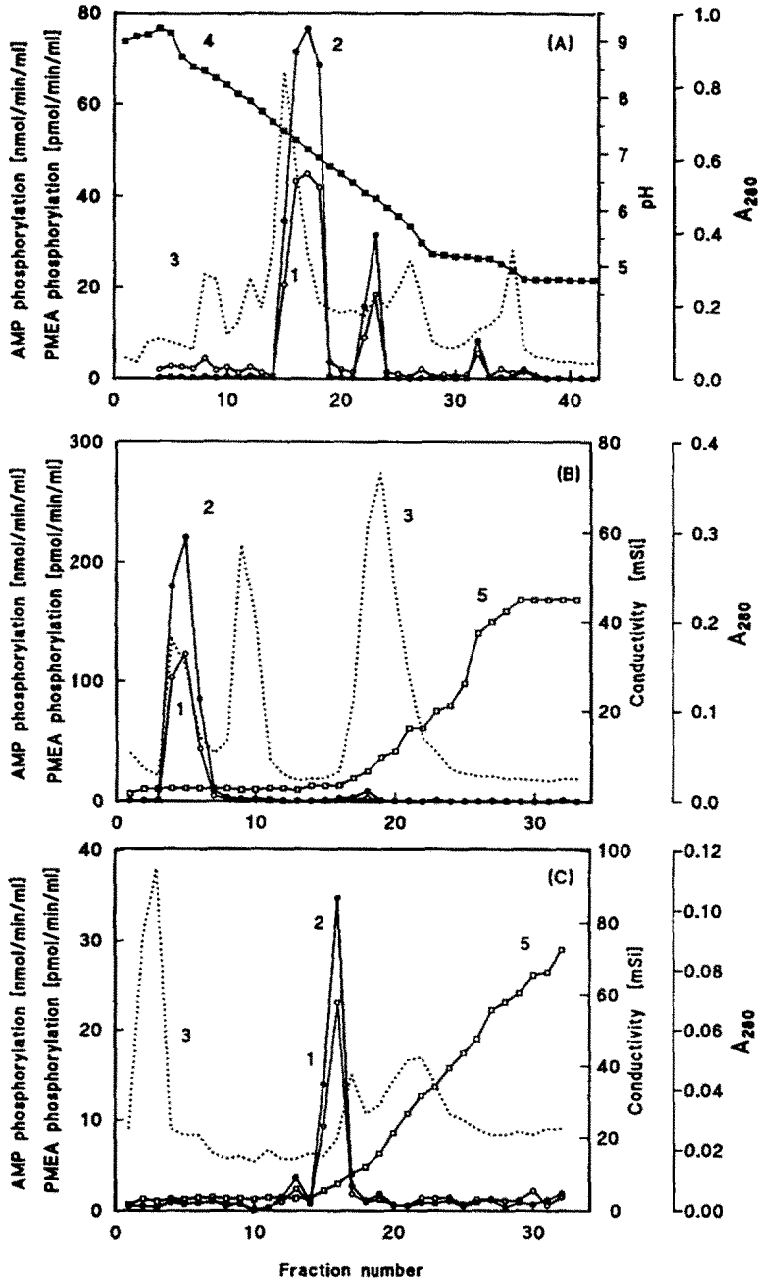


Fig. 2. Purification of PMEA phosphorylating enzyme. Key: (A) chromatofocusing, (B) chromatography on DEAE-cellulose, and (C) chromatography on phosphocellulose; (1) PMEA phosphorylating activity, (2) AMP kinase activity, (3) absorbance at 280 nm, (4) pH gradient and (5) KCl gradient.

estimated by chromatofocusing, and its value was 7.1.

Substrate specificity. Among potential substrates of purified PMEA phosphorylating enzyme, only AMP, dAMP and ADP were efficiently phosphorylated (Table 1); their conversion to ATP (dATP) with excess ATP as phosphate donor proceeded with a velocity of 33, 22 and 20 μmol/min/mg, respectively. The phosphorylation of AMP is a reversible reaction, because in the absence of ATP, ADP was transformed to AMP and ATP. Other natural nucleoside 5′-mono- and 5′-diphosphates (GMP, CMP, UMP, GDP, CDP, UDP) were not substrates of the purified enzyme (data not shown). Phosphorylation of (*S*)-HPMPA and PMEA proceeded with 210 and 1400 times lower velocity than the reaction with AMP. No phosphorylation of (*S*)-HPMPC was detected.

Kinetic constants were estimated for AMP and dAMP: K values were 87 ± 9 and 346 ± 45 μM, respectively, and the reaction proceeded with a V_{max} of 180 ± 12 and 120 ± 10 μmol/min/mg, respectively.

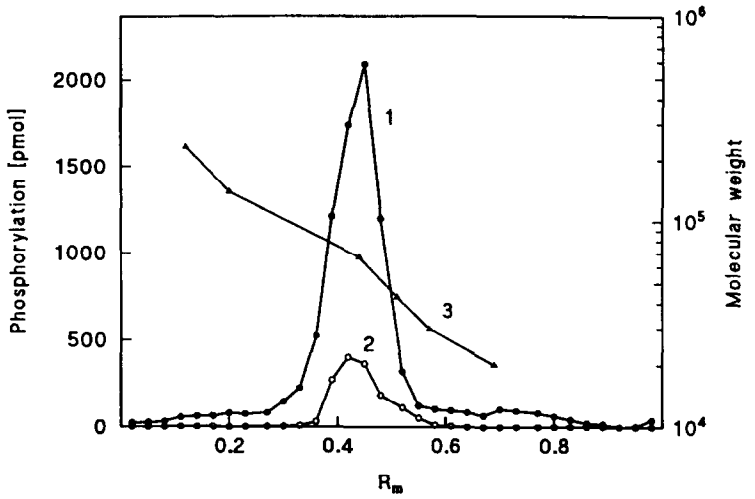


Fig. 3. Gradient PAGE of PMEA phosphorylating enzyme. Enzyme protein (5 or 0.5 μg) was applied onto the gel; the phosphorylation activity was measured in 2-mm slices incubated in reaction mixture (40 μL) containing 15 μM (*S*)-HPMPA or 100 μM AMP for 120 or 20 min, respectively. Key: (1) AMP phosphorylation, (2) HPMPA phosphorylation and (3) calibration curve. Molecular weight standards: catalase (232,000), lactate dehydrogenase (140,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,100). R_m = relative mobility.

Phosphate donors for PMEA phosphorylation and the influence of an ATP-regenerating system. The ability of ribonucleoside 5′-triphosphates to provide a phosphate group for PMEA phosphorylation is documented in Table 2. Of the four natural nucleoside 5′-triphosphates tested only ATP was effective; PMEA was phosphorylated to PMEAp and PMEApp regardless of the presence or absence of an ATP-regenerating system consisting of creatine phosphate and creatine kinase. No phosphorylation of PMEA was found when PRPP (0.2, 1.0 or 10.0 mM) replaced ATP in the reaction without the ATP-regenerating system; also AMP was not transformed to ATP under these conditions (data not shown).

Time course of PMEA and (S)*-HPMPA phosphorylation.* PMEA and (*S*)-HPMPA were converted to their respective mono- and diphosphoryl derivatives in both the presence and the absence of a CP/CPK ATP-regenerating system (Fig. 5). In the absence of the regenerating system (panels A and C), the monophosphoryl derivative appeared as the first phosphorylation product; its concentration reached a plateau after 40–60 min of the incubation period. Synthesis of the diphosphoryl derivative continued after the initial lag phase until the end of

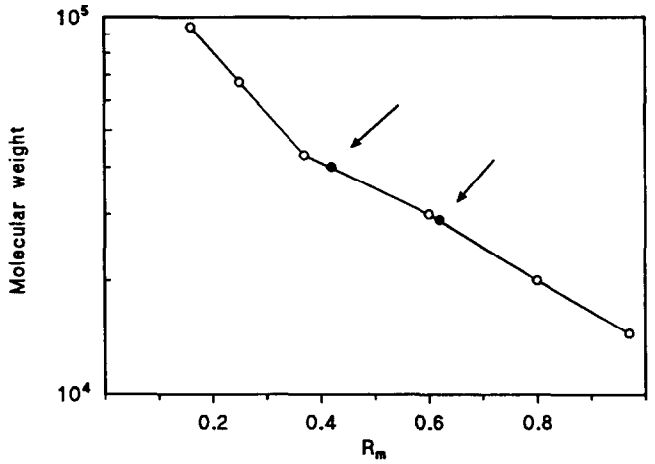


Fig. 4. SDS electrophoresis of purified PMEA phosphorylating enzyme. Arrows indicate positions of enzyme subunits obtained by the separation of 5 μg of enzyme preparation. Molecular weight standards: phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). R_m = relative mobility.

Table 1. Substrate specificity of PMEA phosphorylating enzyme

Substrate	ATP added	Reaction velocity (μmol/min/mg)	Reaction velocity (%)
AMP	+	33.0	100
dAMP	+	22.0	67
ADP	−	4.1	12.6
ADP	+	20.0	61
PMEA	+	0.024	0.07
HPMPA	+	0.157	0.48

Substrate specificity of purified PMEA phosphorylating enzyme was measured in reaction mixtures (20 μL) containing 10 μM phosphate acceptor, 40 mM HEPES (pH 8.0) and 1 mM $MgCl_2$ in the presence (+) or absence (−) of 0.2 mM ATP. Phosphorylation of ^{14}C-labeled nucleotides was initiated by adding an appropriate amount of enzyme and evaluated by TLC as described in Materials and Methods.

Table 2. Phosphate donors for PMEA phosphorylation by the DE-fraction of PMEA phosphorylating enzyme

Phosphate donor	CP/CPK added	Phosphorylation (pmol)
ATP	−	55
ATP	+	78
GTP	−	2.1
GTP	+	3.3
UTP	−	2.1
UTP	+	2.0
CTP	−	1.1
CTP	+	2.0
PRPP	−	Not detected

The phosphorylation of [^{14}C]PMEA (10 μM) to PMEApp by the DE-fraction (2 μL) of PMEA phosphorylating enzyme with 0.2 mM phosphate donors (1 or 10 mM PRPP) was measured in a reaction mixture (40 μL) containing 40 mM HEPES (pH 8.0) and 1 mM $MgCl_2$ in the presence (+) or absence (−) of creatine phosphate (5 mM) and creatine kinase (0.05 mg/mL) and evaluated by TLC. $MgCl_2$ (10 mM) was used in the reaction with PRPP.

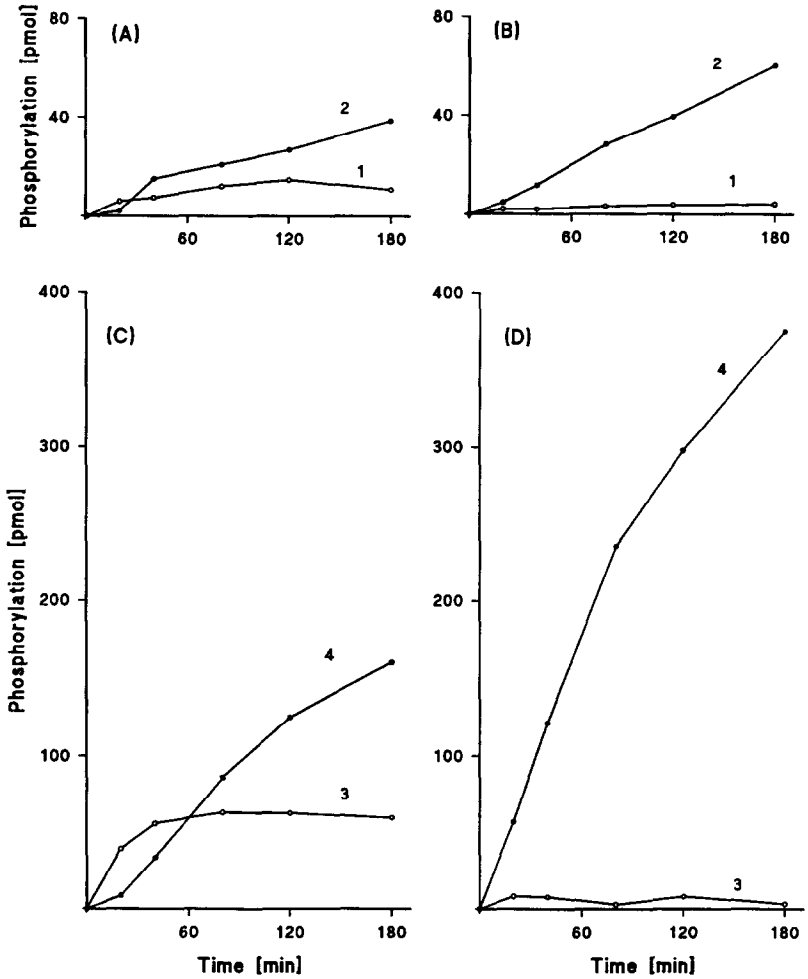


Fig. 5. Time course of PMEA and (*S*)-HPMPA phosphorylation by purified PMEA phosphorylating enzyme. Phosphorylation of [^{14}C]PMEA (10 μM, A and B) and [^{14}C](*S*)-HPMPA (15 μM, C and D) was measured as described in Materials and Methods in the absence (A, C) or the presence (B, D) of creatine phosphate and creatine kinase. Key: (1) PMEAp, (2) PMEApp, (3) HPMPAp, and (4) HPMPApp.

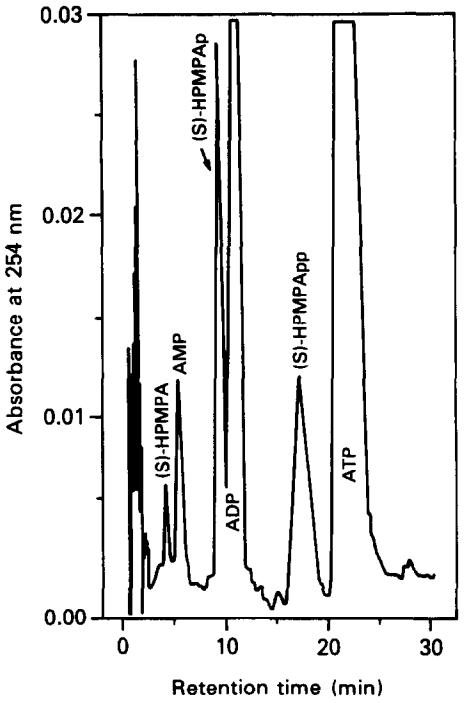


Fig. 6. Phosphorylation of (*S*)-HPMPAp by purified PMEA phosphorylating enzyme. The reaction was carried out and products were separated by HPLC as described in Materials and Methods.

the incubation period. In the presence of the CP/CPK ATP-regenerating system (panels B and D), PMEApp and (*S*)-HPMPApp appeared in the reaction mixture from the beginning of the incubation while the concentration of PMEAp and (*S*)-HPMPAp remained below 1.5% of the initial substrate concentration until the end of the incubation. The synthesis of the diphosphoryl derivatives was significantly higher with the ATP-regenerating system than without it; in the case of (*S*)-HPMPA 63% of the substrate was converted to the diphosphoryl derivative in 180 min. It was evident that the phosphorylation proceeded in two steps, with PMEAp or (*S*)-HPMPAp as intermediate products, the phosphorylation of which was increased significantly by the presence of creatine phosphate and creatine kinase.

Phosphorylation of PMEAp and (S)-*HPMPAp by PMEA phosphorylating enzyme.* To prove that PMEAp and (*S*)-HPMPAp are intermediates during the phosphorylation reaction, the phosphorylation of non-radioactive PMEAp and (*S*)-HPMPAp was studied. We found that purified PMEA phosphorylating enzyme could catalyze the synthesis of both diphosphoryl derivatives when 0.1 mM substrates and 1.0 mM ATP were used (Fig. 6).

Phosphorylation of PMEAp and (S)-*HPMPAp by creatine kinase.* Creatine kinase from rabbit muscle (specific activity of 98 μmol/min/mg for the synthesis of ATP from ADP and creatine phosphate) readily converted 5 mM PMEAp and (*S*)-HPMPAp to their

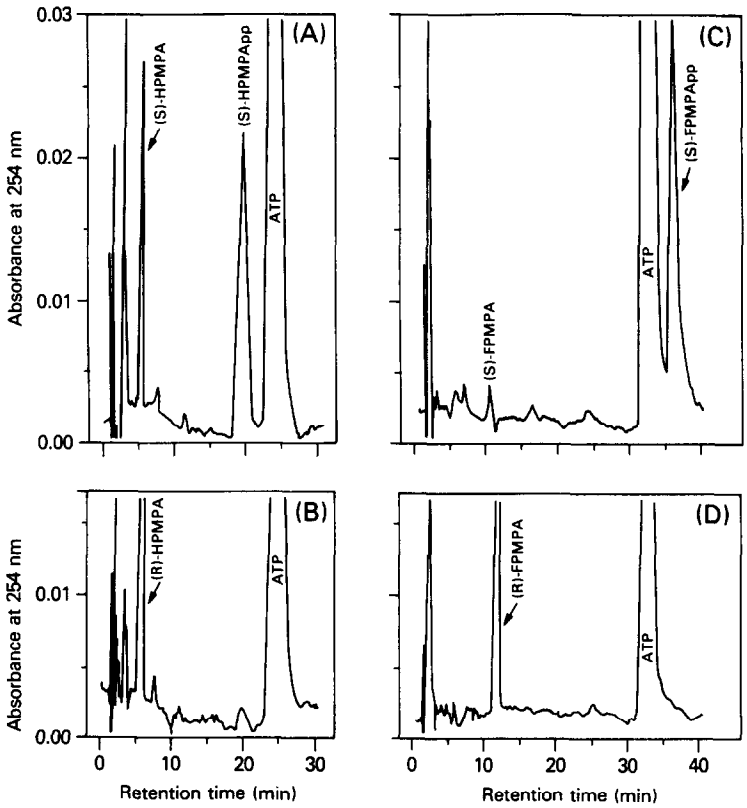


Fig. 7. Phosphorylation of (*S*)-HPMPA (A), (*R*)-HPMPA (B), (*S*)-FPMPA (C) and (*R*)-FPMPA (D) by DE-fraction of PMEA phosphorylating enzyme. The reaction was carried out and products were separated by HPLC as described in Materials and Methods.

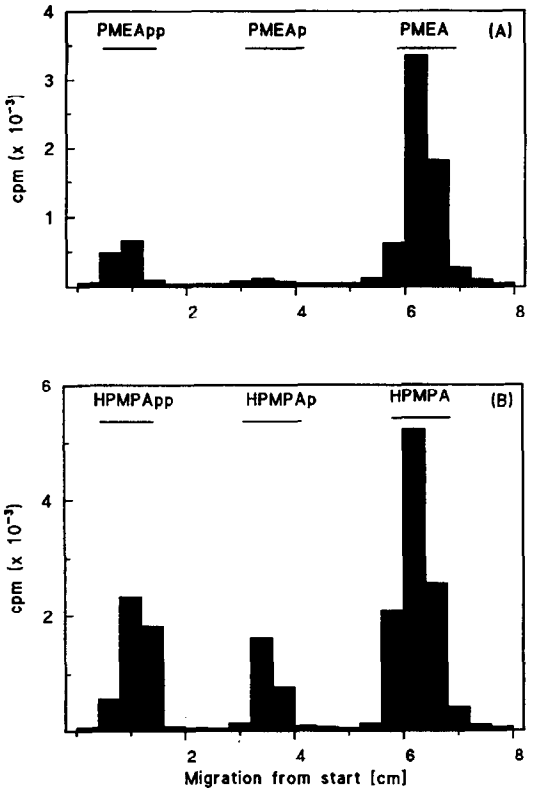


Fig. 8. Identification of products of PMEA (A) and HPMPA (B) phosphorylation by TLC on PEI-cellulose. Lines depict the positions of reference compounds.

respective diphosphoryl derivatives; the phosphorylation rates with 20 mM creatine phosphate as phosphate donor were 5.1 and 4.6 μmol/min/mg, respectively. No phosphorylation of PMEA or (*S*)-HPMPA was detected under these conditions.

Phosphorylation of (S)- *and* (R)-*isomers of HPMPA and FPMPA.* Phosphorylation of HPMPA and FPMPA was limited to the (*S*)-enantiomers only (Fig. 7). Under conditions where (*S*)-FPMPA was converted completely to (*S*)-FPMPApp and 74% of (*S*)-HPMPA to (*S*)-HPMPApp (panels A and C), only 3.6% of (*R*)-HPMPApp appeared and no phosphorylation of (*R*)-FPMPA was observed (panels B and D). The stereospecificity of HPMPA and FPMPA phosphorylation was confirmed with two enzyme preparations at several substrate concentrations. The minor phosphorylation observed with (*R*)-HPMPA was most probably due to the known presence of the (*S*)-enantiomer (<3%).

Identification of reaction products. The identity of the reaction products was proven by TLC (Fig. 8) and HPLC (Fig. 9). In both cases radioactivity comigrated with non-radioactive standards PMEA, PMEAp and PMEApp or (*S*)-HPMPA, (*S*)-HPMPAp and (*S*)-HPMPApp. No other reaction products were detected with either crude extract or partly purified enzyme preparations. These results indicate that during the course of reaction only two phosphorylated products are created.

* See Addendum.

DISCUSSION

Phosphorylation of (*S*)-HPMPA and PMEA, antivirals from the group of acyclic phosphonomethoxyalkyl analogues of nucleoside 5′-monophosphates, has been shown to proceed in Vero, HEL and MT-4 cells [5–7] and in cell-free extracts of mouse leukemia L1210 cells [11].

The enzymes capable of PMEA and (*S*)-HPMPA phosphorylation have not been identified properly yet. It has been only mentioned that AMP kinase from rabbit muscle and several other kinases (GMP kinase, nucleoside 5′-monophosphate kinase, nucleoside 5′-diphosphate kinase) cannot catalyze this reaction [11]. Also the mechanism of phosphorylation of (*S*)-HPMPC, another potent antiviral compound of this group, that is converted in cells to phosphoryl derivatives (and also to their conjugates with choline), is not known [20]. On the other hand, the phosphorylation of (*R,S*)-HPMPG can be catalyzed to the first step by GMP kinase and to the second step by nucleoside 5′-diphosphate kinase [21]. However, whether these steps are general and enantiospecific remains obscure.*

In this study we investigated in detail the phosphorylation reaction and purified the enzyme that is capable of phosphorylating (*S*)-HPMPA and PMEA to their respective diphosphoryl derivatives. We have purified the PMEA phosphorylating enzyme to near homogeneity by chromatofocusing and by chromatography on DEAE-cellulose and phosphocellulose. During the purification, the elution of PMEA and AMP phosphorylating activities followed the same pattern. The final enzyme preparation used AMP, dAMP and ADP as preferred substrates and ATP could not be substituted as the phosphate donor (the mechanism, by which UTP, CTP and GTP can participate in PMEA and (*S*)-HPMPA phosphorylation in crude extracts of L1210 cells [11], remains obscure). From these data, and also because the AMP and PMEA phosphorylating activities were detected in the same positions on gradient gels, it is evident that the enzyme, which catalyzes PMEA and (*S*)-HPMPA phosphorylation, is AMP(dAMP) kinase; it is not contaminated by nucleoside mono- or diphosphate kinase because of its inability to phosphorylate other than adenine containing nucleotides. The purified enzyme has a molecular weight of 68,000; it is most likely composed of two subunits of 40,000 and 29,000.

PMEA phosphorylating enzyme can catalyze both phosphorylation steps occurring during the PMEA or (*S*)-HPMPA phosphorylation as demonstrated by the conversion of non-phosphorylated substrates to both mono- and diphosphoryl derivatives and of the monophosphoryl derivatives to their respective diphosphates. The latter observation demonstrates that the reaction proceeds in two steps.

The reaction catalyzed by PMEA phosphorylating enzyme is specific for phosphonomethoxyalkyl analogues containing adenine; it can phosphorylate PMEA, (*S*)-HPMPA and (*S*)-FPMPA, but not (*S*)-HPMPC. The enzyme seems to be responsible for the antiviral action of adenine phosphonomethoxyalkyl derivatives, but not of HPMPC. Hence, if the

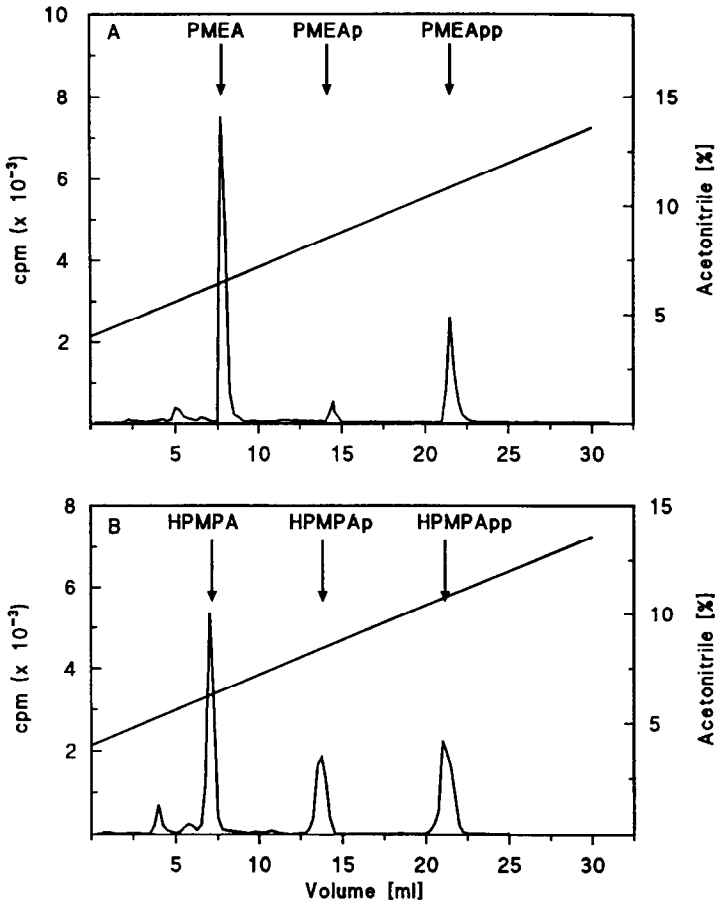


Fig. 9. Identification of products of PMEA (A) and HPMPA (B) phosphorylation by HPLC. Arrows indicate the positions of reference unlabeled compounds. Separation on Silasorb C18 column was performed as described in Materials and Methods.

phosphorylation of HPMPC is required for its activation, then another enzyme must exist that can catalyze this reaction.*

Due to the high ATPase activity in the crude extracts of L1210 cells, a creatine phosphate/creatine kinase ATP-regenerating system is necessary for ATP regeneration during the phosphorylation of PMEA and (*S*)-HPMPA by this preparation [11]. However, the overall phosphorylation of PMEA and (*S*)-HPMPA by purified PMEA phosphorylating enzyme, which is devoid of ATPase activity, is also significantly higher in the presence of the ATP-regenerating system than in its absence. This can be explained either by the competition of PMEA with AMP, which can appear in the reaction mixture after ATP is used as the phosphate donor and resulting ADP is transformed to ATP and AMP via the PMEA phosphorylating enzyme, or by the participation of creatine kinase in PMEAp phosphorylation. Even if the first explanation is possible (AMP was found to interfere with PMEA phosphorylation, data not shown), the second is more likely: our findings confirmed that PMEAp and HPMPAp are substrates of creatine kinase.

It is well known that in the case of HPMPA only the (*S*)-enantiomer is an effective antiviral compound [1] but no explanation for this phenomenon has been proposed yet. Our data show that the phosphorylation of HPMPA and FPMPA is specific for the (*S*)-enantiomers and suggest that the enantiospecificity of this reaction may be one of the reasons why (*S*)-HPMPA is efficient while the (*R*)-enantiomer is inefficient against viral multiplication. This observation further supports the presumption that the phosphorylation is necessary for the antiviral action of the studied compounds, and that one (or more) of the enzymes, that were shown to be inhibited by mono- and diphosphates of 9-(phosphonomethoxyalkyl)adenine [5, 8–10], is the target for their antiviral action. The antiviral activity of FPMPA has been measured with the racemates only [13]. It would be interesting to find out whether it is also limited solely to the (*S*)-enantiomer as in the case of (*S*)-HPMPA.

One-step phosphorylation of PMEA, (*S*)-HPMPA and FPMPA catalyzed by PRPP synthetase from *E. coli* which leads directly to the respective diphosphate derivatives was reported recently by Balzarini *et al.* [5, 12, 13]. The authors suggest that this reaction may be the source of diphosphoryl derivatives of

* See Addendum.

PMEA, (*S*)-HPMPA and FPMPA also in eukaryotic cells. However, no data were presented thus far to prove that such a reaction can be catalyzed also by eukaryotic PRPP synthetases. Moreover, our attemps to repeat the experiments with bacterial PRPP synthetase were unsuccessful (data not shown); negative results were also obtained by Černý and Cheng.*

PRPP is an important intermediate in cellular metabolism and its synthesis is viewed as the first step of several divergent biosynthetic pathways leading not only to *de novo* synthesis of purine and pyrimidine nucleotides, coenzymes NAD^+ and $NADP^+$ and of histidine and tryptophan, but also to reutilization of nucleobases via the phosphoribosyltransferase reactions (for review see Becker *et al.* [22]). The cellular function of PRPP synthetase is the formation of PRPP from ATP and ribose-5-phosphate. This reaction, the sole source of PRPP both in bacteria and in eukaryotic cells, is subjected to complex metabolic regulation by the end products of respective metabolic pathways. Its direct products, PRPP and AMP, are considered to be inhibitors of PRPP synthetase rather than potential substrates for the reverse reaction, the physiological significance of which is doubtful. (The only known example of such a reverse reaction which presumably occurs in cells is the synthesis of 5-amino-4-imidazolecarboxamide riboside triphosphate (AICAR 5′-triphosphate) catalyzed by human erythrocyte PRPP synthetase [23].)

Our attempts to use PRPP as the pyrophosphate donor in extracts from L1210 cells were equally unsuccessful and none of the partially purified AMP(dAMP) kinase preparations utilized PRPP as substrate (data not shown). For this reason, and also because of the above-mentioned strict regulation of PRPP synthetase activity *in vivo* as well as the very low affinity of the enzyme for PRPP and AMP [24], we believe that the synthesis of PMEApp, (*S*)-HPMPApp and FPMPApp in these cells is not catalyzed via PRPP synthetase.

In conclusion in this paper we present several pieces of evidence showing that AMP(dAMP) kinase is capable of the two-step phosphorylation of PMEA and (*S*)-HPMPA to their diphosphoryl derivatives. We also showed that other nucleoside 5′-triphosphates or PRPP cannot be substituted for ATP as the phosphate donor. We have also demonstrated that at least one additional enzyme (creatine kinase) is capable of transforming monophosphoryl derivatives to their respective diphosphates.

Addendum: The mechanism of HPMPC phosphorylation has been recently explained by Cihlář *et al.* (Cihlář T, Votruba I, Horská K, Liboska R, Rosenberg I and Holý A, Metabolism of 1-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl) cytosine (HPMPC) in human embryonic lung cells. *Collect Czech Chem Commun* **57**: 661–672, 1992).

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* Černý J and Cheng Y-C, personal communication. Cited with permission.

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