Two Nucleic Acid-dependent Nucleoside Triphosphate Phosphohydrolases from Vaccinia Virus

**PURIFICATION AND CHARACTERIZATION***

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**SUMMARY**

Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases have been found in vaccinia virus cores. Both enzymes hydrolyze ATP to ADP and Pi in the presence of nucleic acid. The enzymes were solubilized from purified viral cores by treatment with sodium deoxycholate and a Tris buffer at alkaline pH. DNA-free viral extracts were prepared by adsorption of viral nucleic acid to DEAE-cellulose columns in the presence of high salt, and the two enzymes were purified by DNA-cellulose chromatography. Phosphohydrolase I was purified 300- to 400-fold with yields of 80 to 90% and is approximately 95% pure. The smaller amount of phosphohydrolase II prevented accurate determinations of specific activity or purity. The molecular weight of the native enzyme was calculated to be 68,000 and it may also consist of a single polypeptide.

Nucleic acids were required by phosphohydrolase I and II for the hydrolysis of ATP, and optimal activities were obtained at neutral pH in the presence of divalent cations. The hydrolysis of ATP resulted in the production of stoichiometric amounts of ADP and Pi. The $K_m$ values of phosphohydrolases I and II were $1.4 \times 10^{-4}$ M and $6.4 \times 10^{-5}$ M, respectively.

A number of enzymatic activities are associated with purified preparations of vaccinia virus. These include a DNA-dependent RNA polymerase (1, 2) a deoxyribonuclease (3, 4), a nucleoside triphosphate phosphohydrolase (5, 6), a protein kinase (7, 8), and a polyadenylate polymerase (9, 10). None of the enzymes are released upon treatment of the virion with nonionic detergents, indicating that they are contained within the core. Recently, we noted that the phosphohydrolase was solubilized during disruption of the vaccinia core and that the released enzyme was dependent for activity upon the presence of nucleic acids (11). The latter finding was novel since similar enzymes have not been described in either animal cells or other viruses. DNA-dependent ATPase activities of at least two varieties have been found in prokaryotic cells. The ATP-dependent deoxyribonucleases from uninfected or phage-infected bacteria exhibit DNA-dependent ATPase activities (12-21). Certain other briefly described DNA-dependent ATPases lack demonstrable nuclease activities and are of unknown function (22, 23).

In this report we describe the purification, some physical properties, and a preliminary characterization of the nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus. In the following communication (24) we will describe the nucleotide substrate and nucleic acid cofactor specificities of the purified enzymes.

**MATERIALS AND METHODS**

**Cell and Virus Strains**—Vaccinia virus (strain WR) was purified from HeLa cells by the procedure of Joklik (25) which included sedimentation through a sucrose cushion and two sucrose gradient centrifugations as previously described (26). Virus grown in the presence of [3H]leucine was purified in a similar manner. Phosphohydrolase Assay—Phosphohydrolase activity was assayed by following the formation of [3H]ADP from [3H]ATP. The reaction mixture (100 μl) contained 10 μmoles of morpholino-propanesulfonic acid (pH 7.0), 0.1 μmole of MgCl₂, 0.1 μmole of [3H]ATP, 0.5 μmole of DTT, 1% nonionic detergent, NP-40, at a final concentration of 0.05%, and 5 μg of commercial salmon sperm DNA. After incubation with the enzyme at 37º, 10 μl were applied to PEI-cellulose sheets which had been prespotted with the appropriate carrier nucleotide standards. After drying, the chromatography plates were washed in beakers with deionized water, dried, and developed with 1 M CH₃COOH-4 M LiCl (8:2, v/v) as described by Randerath and Randerath (27). After chromatography the plates were dried and the ADP and ATP spots visualized under ultraviolet light, cut out, and placed in scintillation vials. LiCl (0.5 ml of a 2 M solution) was added to elute the nucleotides which were counted after addition of 5 ml of a toluene-based scintillation fluid containing Triton X-100 (28). By definition, 1 enzyme unit will form 1 nmole of ADP from ATP in 1 min.

*The abbreviations used are: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PEI-cellulose, polyethyleneimine-substituted cellulose; NP-40, Nonidet P-40.*
SDS Polycrylamide Gels—Purification of the enzymes was monitored by SDS polycrylamide gel electrophoresis according to published procedures (29). Dilute solutions of protein were concentrated by precipitation with 50% trichloroacetic acid. The pellet was neutralized with NaOH prior to solubilization of the material with SDS and 2-mercaptoethanol at 100° (29). Coomassie brilliant blue stain (0.1% in 25% trichloroacetic acid) was used for detection of polypeptides (30). Gels containing radioactively labeled proteins were sliced into 1-mm sections, dissolved with hydrogen peroxide, and counted as described (31).

Separation of Enzymes—Chromatography—Purified [3H]leucine-labeled phosphohydrolase I or phosphohydrolase II was applied in a 0.5 ml volume to a column (1.5 x 83.5 cm) of Sephadex G-200 equilibrated with a buffer consisting of 0.1 M Tris-HCl, pH 8.4; 0.25 M NaCl; 1 mM EDTA; 1 mM DTT; 0.2%; NP-40; and 0.68% bovine serum albumin. The columns were eluted with 5% above buffer and fractions of 2.4 ml were collected. After assaying the fractions for phosphohydrolase activity the remainder of the sample was used to determine [3H]leucine-labeled protein. Bovine serum albumin (10 mg) and ovalbumin (10 mg) standards were applied and eluted from the column in the same fashion, and their elution volumes was determined by turbidity measurements (32). The elution volumes of myoglobin (1 mg) and cytochrome c (1 mg) were determined by absorbance at 410 and 405 nm, respectively. The void volume and total volume of the column were measured with blue dextran and [14C]glucosamine, respectively.

Sucrose Gradient Sedimentation—A 100-μl sample of phosphohydrolase I or phosphohydrolase II containing myoglobin (1 mg per ml), E. coli alkaline phosphatase (0.6 enzyme units), catalase (50,000 enzyme units), and bovine serum albumin (1 mg per ml) as standards was layered onto a 5 to 20% sucrose gradient in the same buffer system as was used for Sephadex filtration except that bovine serum albumin was omitted. The gradients were centrifuged in the SW 50.1 rotor at 38,000 rpm for 17.5 hours. Phosphohydrolase activity was assayed as described. Alkaline phosphatase was assayed according to Goren and Levitt (33). Catalase was assayed as described by Beers and Sizer (34). Myoglobin was detected by absorbance at 410 nm and bovine serum albumin by turbidity measurements after addition of trichloroacetic acid (32).

The sedimentation values of the phosphohydrolase enzymes were calculated according to Martin and Ames (35) using the following sedimentation coefficients for the standards: myoglobin, 2.04; bovine serum albumin, 4.11; E. coli alkaline phosphatase, 6.3; and catalase 11.3.

Molecular Weight Determination by SDS Polycrylamide Gel Electrophoresis—Purified phosphohydrolase I and phosphohydrolase II were applied on 5% SDS polycrylamide gels. The apparent molecular weights of the enzymes were determined from the electrophoretic mobility relative to that of known molecular weight standards as described (36-38).

Enzymes and Molecular Weight Standards—Bovine liver catalase (50,000 units per mg), E. coli alkaline phosphatase (36 units per mg), glyceraldehyde phosphate dehydrogenase, and β-galactosidase were purchased from Worthington Biochemical Corp. Chymotrypsinogen, bovine serum albumin, and cytochrome c were obtained from Mann Research Laboratories. Myoglobin and ovalbumin were purchased from Sigma.

Reagents—DEAE-cellulose (Whatman DE52) was purchased from Reeve-Angel, Clifton, N.J. Deoxyribonuclease acid from salmon sperm and calf thymus were obtained from Worthington Biochemical Corp. PEF-cellulose particles were purchased from the J. T. Baker Chemical Co. [3H]ATP (35.4 Ci per mmole) and [3H]leucine (6 Ci per mmole) were from New England Nuclear. ATP was purchased from P-L Biochemicals. The non-ionic detergent NP-40 was a generous gift of the Shell Oil Co. Munktell 410 cellulose was purchased from Bio-Rad Laboratories.

RESULTS

Purification of Nucleic Acid-dependent Phosphohydrolase

Preparation of Viral Cores—The phosphohydrolase activity was known to be associated with viral cores (6, 11). Therefore, cores were prepared from purified vaccinia virus by procedures (39) modified from the original description of Easterbrook (40).

Purified vaccinia virus (70 to 80 mg) was incubated in a solution containing 50 mM Tris-HCl, pH 8.4, 50 mM DTT, and the non-ionic detergent NP-40 (0.5%) at 37° for 30 min with periodic shaking. This procedure effectively removes the outer lipoprotein coat of the virus (39, 40) and approximately 30 to 40% of the total viral protein. The cores were then purified by sedimentation through a 36% (w/v) cushion of sucrose in 10 mM Tris-HCl, pH 8.4, containing 1 mM DTT in the SW 50.1 rotor at 25,000 rpm for 30 min. The cores were resuspended by sonication in 10 mM Tris-HCl, pH 8.4.

Solubilization of Phosphohydrolase from Viral Cores—The phosphohydrolase activity was extracted from purified viral cores as follows. Cores at a concentration of 25 to 40 mg per ml were added to a solution composed of 0.3 M Tris-HCl, pH 8.4, 50 mM DTT, 0.1% sodium deoxycholate, and 0.25 M NaCl. This and all subsequent purification steps were performed at 4°. Upon the addition of cores to this solution, an immediate increase in viscosity was observed due to the release of viral DNA (11). After 30 min, the preparation was briefly sonicated with a Branson Sonifier to reduce the viscosity, and the preparation was centrifuged at 136,000 x g for 60 min. The supernatant, which contained approximately 30 to 35% of the total core protein, was recovered and processed as described below. An increase in total phosphohydrolase activity was always observed upon disruption of cores. This may be due to the increased accessibility of the phosphohydrolase enzymes to added nucleic acid or to substrate.

High Salt DEAE-cellulose Chromatography—Since we were dealing with nucleic acid-dependent enzymes, it was essential to remove all endogenous DNA from the extract in order to carry out the purification. DNase treatment was avoided since we also wished to purify the endogenous single-strand-specific DNase from vaccinia virus (41). Partition of the soluble extract in a polyethylene glyco-dextran, aqueous two-phase system (42-44) using a variety of conditions proved unsuccessful because of the presence of deoxycholate. We, therefore, tried the procedure of adsorbing the viral DNA to DEAE-cellulose in the presence of high salt concentrations (45). The supernatant was diluted to contain 0.2 M NaCl and made 10% with respect to glycerol, 0.1% with respect to Triton X-100, and 1 mM with respect to Na₂EDTA. This preparation was then applied to a column (0.9 x 8 cm) of DEAE-cellulose (Whatman DE52) which had been equilibrated with Buffer A (0.15 M Tris-HCl, pH 8.4, 0.1% Triton X-100, 2 mM DTT, 10% glycerol, and 1 mM Na₂EDTA) containing 0.9 M NaCl. The phosphohydrolase activity was recovered in the flow-through. Greater than 98% of the nucleic acid was retained on the column. Little or no protein was adsorbed to the exchanger under these conditions.

Low Salt DEAE-cellulose Chromatography—The flow-through from the high salt DEAE-cellulose column was collected and diluted with Buffer A to reduce the NaCl concentration of the sample to 0.05 M. This material was then applied to a column (0.9 x 8 cm) of DEAE-cellulose equilibrated with Buffer A containing 0.05 M NaCl. The phosphohydrolase activity was again recovered in the flow-through. This low salt DEAE-cellulose step serves to remove residual DNA from the sample as well as some high molecular weight viral polypeptides.

DNA-cellulose Chromatography—DNA-cellulose affinity chromatography has been used extensively for the detection and purification of DNA-specific proteins from a variety of organisms (46-54). Denatured calf thymus DNA-cellulose prepared as described by Alberts and Herrick (54) was packed into a column

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Fig. 1. DNA-cellulose chromatography of nucleic acid-dependent phosphohydrolase activity from vaccinia virus. Phosphohydrolase activity from a low salt DEAE-cellulose column was applied directly to a DNA-cellulose column (18 packed ml containing 280 μg of denatured calf thymus DNA per packed ml) and developed with a 350-ml gradient of 0.05 to 0.25 M NaCl in Buffer A (“Materials and Methods”). Fractions of 5.7 ml were collected at a flow rate of 12 ml per hr. Aliquots (0.1 ml) were removed from each fraction and counted for [3H]leucine (O—O). Aliquots (5 μl) were assayed for nucleic acid-dependent phosphohydrolase activity (•—•). NPH I and NPH II, phosphohydrolase I and II, respectively.

0.9-cm diameter, (18 packed ml, 280 μg of DNA per ml) and equilibrated with Buffer A containing 0.05 M NaCl. The flow-through from the low salt DEAE-cellulose column was applied directly to the DNA-cellulose column and after extensive washing with Buffer A containing 0.05 M NaCl, the column was developed with a gradient composed of Buffer A containing 0.05 and 0.25 M NaCl as the limiting salt concentrations. The results of such a DNA-cellulose column chromatography are shown in Fig. 1. About 40 to 50% of the protein did not adsorb to the column as monitored by following the distribution of [3H]leucine-labeled viral material. Several radioactive protein peaks were eluted between 0.1 and 0.25 M NaCl. In other experiments no additional protein was eluted with salt concentrations as high as 1 M. The column fractions were then assayed for phosphohydrolase activity and the results are presented in Fig. 1. Some phosphohydrolase activity appeared in the DNA-cellulose flow-through. The amount of this unadsorbed phosphohydrolase activity was greatly reduced in subsequent experiments by using longer columns. Two peaks of nucleic acid-dependent phosphohydrolase activity were eluted from the DNA-cellulose column on application of the salt gradient. The first peak, by far the major enzymatic component, was eluted at a salt concentration of approximately 0.1 M and was coincident with a [3H]leucine-labeled protein peak. A second, minor peak of phosphohydrolase activity was eluted at approximately 0.17 M. This peak of activity was not coincident with a [3H]leucine-labeled protein peak indicating the presence of multiple proteins in that region. Greater resolution of the minor phosphohydrolase activity has been obtained in subsequent purifications by increasing the total volume of the gradient used to develop the DNA-cellulose column. The two phosphohydrolase activities rechromatographed as distinct species at the expected salt concentrations and will be referred to as phosphohydrolase I and phosphohydrolase II.

Fig. 2. Purification of phosphohydrolase I and phosphohydrolase II as monitored by SDS polyacrylamide gel electrophoresis. Gels A to C show the polypeptide composition of fractions containing approximately 200 phosphohydrolase units derived from purified cores, high speed soluble material from deoxycholate-extracted cores, and low salt DEAE-cellulose flow-through material, respectively. Gels D and E represent approximately 4000 phosphohydrolase I and 2000 phosphohydrolase II units of enzyme purified from DNA-cellulose columns. These samples were concentrated by trichloroacetic acid precipitation as described under “Materials and Methods.” Similar but fainter bands were obtained with unconcentrated material. Gel F shows the polypeptide composition of material eluting from the DNA-cellulose column immediately after phosphohydrolase II. An equivalent volume of sample was concentrated for electrophoresis by trichloroacetic acid precipitation as that of phosphohydrolase I and phosphohydrolase II. Electrophoresis was from top to bottom.

Purification of Phosphohydrolase I and Phosphohydrolase II as Monitored by SDS Polyacrylamide Gel Electrophoresis—Standard SDS 7.5% polyacrylamide gels were used to monitor the degree of purification of the phosphohydrolase enzymes. Fig. 2 shows a typical set of results. Gel A in Fig. 2 indicates the polypeptide composition of purified viral cores. Gel B shows the polypeptide composition of soluble material extracted from cores by deoxycholate treatment. The major structural viral polypeptides 4a and 4b, which compose approximately 82% of the total viral protein, remain insoluble with this treatment. Gel C indicates the polypeptide composition of the flow-through material from the low salt DEAE-cellulose column. The effective removal of several high molecular weight polypeptides should be noted. Gels D and E show the rather extensive purification of phosphohydrolase I and phosphohydrolase II, respectively, which is achieved by DNA-cellulose affinity chromatography. Purified preparations of phosphohydrolase I contain a single stained
band, while phosphohydrolase II frequently contains two very closely spaced bands. Use of a more shallow salt gradient to develop the DNA-cellulose column of chromatography of phosphohydrolase II on a second DNA-cellulose column appeared to result in a diminution of the faster migrating polypeptide observed in Gel E without loss of phosphohydrolase II activity. Further work is needed, however, to clarify the relationship of the two bands. Gel F contains the polypeptides eluted immediately following phosphohydrolase II on DNA-cellulose. Subsequent studies have shown that this material contains polyadenylate polymerase activity (10). Gels A to C were loaded with approximately 200 enzyme units per gel, whereas more than 4000 and 200 enzyme units of purified phosphohydrolase I and phosphohydrolase II were applied on Gels D and E, respectively.

In order to determine the relative purity of phosphohydrolase I, approximately 4000 enzyme units of purified [3H]leucine-labeled phosphohydrolase I were concentrated by trichloroacetic acid precipitation and applied to 7.5% SDS polyacrylamide gels as described under "Materials and Methods." The gel was frozen, sliced into 1-mm sections, and dissolved with H2O2, and the fractions were counted for radioactivity.

The gel was frozen, sliced into 1-mm sections, and dissolved with polycrylamide gels as described under "Materials and Methods." Approximately 4000 enzyme units of purified [3H]leucine-labeled phosphohydrolase I were concentrated by trichloroacetic acid precipitation and prepared for electrophoresis on 7.5% SDS polyacrylamide gels (55). The enzymatic activity coincided with the [3H]leucine-labeled material was counted. The result of such a determination is shown in Fig. 3. The purity of phosphohydrolase I is in the order of 95% as estimated by SDS polycrylamide gel electrophoresis. A similar determination was not done for phosphohydrolase II since the relatively low amount of radioactivity found in this minor enzymatic component would have made a reliable estimate of purity difficult.

Purification of Phosphohydrolase I—The relative purification of the nucleic acid-dependent phosphohydrolase I during the various steps is outlined in Table I. Removal of protein during the formation of cores from whole virions resulted in a doubling of specific activity. An activation of phosphohydrolase activity occurred when the cores were disrupted by deoxycholate treatment. Since only 25 to 35% of the protein in the viral core was solubilized by deoxycholate treatment, a further increase in specific activity of the phosphohydrolase was obtained after high speed centrifugation. An additional 40-fold purification of phosphohydrolase I was achieved by DNA-cellulose chromatography. The total increase in specific activity achieved during the purification procedures was 300- to 400-fold and between 80 and 90% of the initial activity was recovered. Approximately 160 µg of purified phosphohydrolase I were recovered from 72 mg of purified virus. This suggests that about 0.2 to 0.6%, of the total viral protein is phosphohydrolase I. The range of this estimate is dependent upon whether one uses the values of NP-40-treated total virus or deoxycholate-disrupted cores as the starting point for phosphohydrolase activity in calculating the recovery of enzyme. The latter is perhaps a more reliable starting point.

No precise estimates regarding the increase in specific activity of phosphohydrolase II were obtained because of the small physical amounts of this enzymatic component. However, examination of the polycrylamide gels indicated a similar order of purification.

Determination of Molecular Weights

Sephadex Gel Filtration—Purified preparations of [3H]leucine-labeled phosphohydrolase I were chromatographed on columns of Sephadex G-200 as described under "Materials and Methods." Protein standards were also analyzed on the same column under comparable conditions. The results are shown in Fig. 4. Phosphohydrolase I eluted at the same volume as bovine serum albumin which was compatible with a Stokes radius of 3.5 nm (35). The enzymatic activity coincided with the [3H]leucine radioactivity indicating the absence of any detectable impurities that could be separated by gel filtration. Phosphohydrolase II also eluted from Sephadex G-200 at the same volume as bovine serum albumin and is not shown.

Sucrose Gradient Centrifugation—The technique described by Martin and Ames (35) was used to determine the sedimentation behavior of phosphohydrolase I and phosphohydrolase II. The enzymes were sedimented on 5 to 20% gradients with standards of known sedimentation coefficients. The results of such analyses are shown in Fig. 5 for phosphohydrolase I and Fig. 6 for phosphohydrolase II. In other experiments it was demonstrated that the included protein standards did not affect the sedimentation of phosphohydrolase I or phosphohydrolase II. A partial separation of phosphohydrolase I and phosphohydrolase II was observed when the two enzymes were run on the same gradient with bovine serum albumin and assayed with divalent cations and cofactors specific for each enzyme (24). Sedimentation coefficients of 4.17 for phosphohydrolase I and 4.72 for

| Fraction | Volume | Total protein | Total activity | Specific activity |
|----------|--------|---------------|----------------|------------------|
| I. NP-40-treated virus | 10 | 27.1 | 160,000 | 2,219 |
| II. Cores | 2 | 38.4 | 150,000 | 3,896 |
| III. Deoxycholate-disrupted cores | 5 | 38.0 | 390,000 | 10,283 |
| IV. 150,000 × g supernatant | 5 | 9.0 | 270,000 | 30,000 |
| V. High salt DEAE-cellulose | 10 | 9.0 | 150,000 | 16,666 |
| VI. Low salt DEAE-cellulose | 53 | 9.0 | 212,000 | 23,556 |
| VII. DNA-cellulose | 27 | 0.162 | 135,000 | 833,333 |

* Calculated as 64 µg of viral protein per absorbance unit at 260 nm. Specific activity of [3H]leucine-labeled virus used here was 7.3 × 10⁶ cpm per mg.
FIG. 4. Sephadex G-200 gel filtration of purified phosphohydrolase I. [3H]Leucine-labeled phosphohydrolase I was chromatographed on a Sephadex G-200 column as described under "Materials and Methods." Aliquots of fractions were counted for [3H]leucine (O-O) and assayed for phosphohydrolase activity (•). The elution volumes of blue dextran (V₀), bovine serum albumin ovalbumin, myoglobin, cytochrome c, and [¹C] glucosamine (V₁) are also indicated.

FIG. 5 (left). Sucrose gradient sedimentation of phosphohydrolase I. Purified phosphohydrolase I was analyzed on 5 to 20% sucrose gradients along with known standards as described under "Materials and Methods." The sedimentation profiles of catalase, alkaline phosphatase, bovine serum albumin, and myoglobin are indicated by dashed lines and that of phosphohydrolase I (NPH I) with a solid line. Recovery of phosphohydrolase I activity on the gradient was 100%. Absorbance measurements are as follows: catalase activity was measured by the decrease in absorption at 240 nm, alkaline phosphatase activity was measured by the increase in absorption at 410 nm, bovine serum albumin was measured by turbidity at 450 nm, and myoglobin by absorption at 410 nm as described under "Materials and Methods."

FIG. 6 (right). Sucrose gradient sedimentation of phosphohydrolase II. Purified phosphohydrolase II was analyzed on 5 to 30% sucrose gradients along with known standards as described under "Materials and Methods." The sedimentation profiles of catalase, alkaline phosphatase, bovine serum albumin, and myoglobin are indicated by dashed lines and that of phosphohydrolase II (NPH II) by a solid line. Recovery of phosphohydrolase II activity on the gradient was greater than 100%. Absorbance measurements are as follows: Catalase activity was measured by the decrease in absorption at 240 nm, alkaline phosphatase activity was measured by the increase in absorption at 410 nm, bovine serum albumin was measured by turbidity at 450 nm, and myoglobin by absorption at 410 nm as described under "Materials and Methods."

Fig. 7. Molecular weight determination of phosphohydrolase I on SDS polyacrylamide gels. Purified phosphohydrolase I (NPH I) was subjected to electrophoresis on 5% SDS polyacrylamide gels and its electrophoretic mobility compared with that of known molecular weight standards run on separate gels.

phosphohydrolase II were obtained from a number of such gradient analyses.

The sedimentation coefficients derived above and the Stokes radii derived from Sephadex G-200 gel chromatography (56, 57) were used as described by Siegel and Monty (58) to calculate the molecular weights. Values of approximately 61,000 and 69,000 were obtained for phosphohydrolase I and phosphohydrolase II, respectively, assuming partial specific volumes of 0.73 ml g⁻¹.

SDS Polyacrylamide Gel Electrophoresis—Purified preparations of phosphohydrolase I and phosphohydrolase II were analyzed on SDS polyacrylamide gels in order to estimate molecular weights (36-38). A value of approximately 68,000 was calculated for phosphohydrolase I by comparison to standards of known molecular weight (Fig. 7). The similar molecular weights obtained for phosphohydrolase I under native and denaturing conditions indicate that the enzyme is a monomer. Phosphohydrolase II has not been obtained in a homogeneous form. The band which is predominant in our most highly purified preparations has an electrophoretic mobility, however, almost identical to that of phosphohydrolase I. Thus, phosphohydrolase II probably also exists as a monomeric enzyme.

Conditions for Optimal Enzyme Activities

Nucleic Acid—The stimulatory effects of nucleic acids on the activities of phosphohydrolase I and phosphohydrolase II will be described in detail in the accompanying communication (24). The amount of commercial salmon sperm DNA contained in the standard reaction mixture described under "Materials and Methods" provided maximum activity.

pH—Using a variety of buffer systems both phosphohydrolase I and phosphohydrolase II demonstrated a very broad pH optimum around neutrality. A pH 7.0 morpholinopropane sulfonic acid buffer (pKᵢ = 7.2) was selected. No difference in activity of either enzyme was observed at several concentrations of buffer tested.

Requirements of Nonionic Detergent and Reducing Reagents—Neither purified phosphohydrolase I nor phosphohydrolase II showed any requirement for added nonionic detergents (NP-40, Triton X-100) or sulphydryl-reducing reagents (DTT). These reagents, however, were retained in the phosphohydrolase assay.
for comparative purposes since the early fractions, particularly whole virions and cores, required these reagents for maximal activity. The requirement for these reagents during the early purification steps was probably due to the disruptive effect of these reagents on the virus.

Requirement for Divalent Cation—Both phosphohydrolase I and phosphohydrolase II required a divalent cation for activity. This is shown in Fig. 8. No activity with either enzyme was observed in the absence of added Mg$^{2+}$. Both enzymes showed optimal activity when Mg$^{2+}$ was present in the range of 1 to 5 mM.

The effect of other divalent cations either alone or with Mg$^{2+}$ was investigated. The data in Table II indicate that in the absence of Mg$^{2+}$, Mn$^{2+}$ was utilized quite efficiently by both enzymes. Ca$^{2+}$ could support the activity of phosphohydrolase I but not that of phosphohydrolase II. Neither Cu$^{2+}$ nor Zn$^{2+}$ could substitute for Mg$^{2+}$ as the divalent cation. When these reagents were added together, it was observed that Zn$^{2+}$ was inhibitory to both enzymes. Addition of Cu$^{2+}$ inhibited phosphohydrolase I activity but allowed considerable phosphohydrolase II activity but allowed considerable phosphohydrolase II activity. Ca$^{2+}$ did not inhibit phosphohydrolase I appreciably while it inhibited phosphohydrolase II by 90%. The addition of Mn$^{2+}$ depressed neither phosphohydrolase I nor phosphohydrolase II appreciably.

Effect of ATP Concentration on Phosphohydrolase Activity—Fig. 9 shows the rate of ATP hydrolysis by phosphohydrolase I and phosphohydrolase II as a function of ATP concentration. Double reciprocal plots of the data are shown in the inset. $K_m$ values of 1.4 x 10$^{-4}$ M and 6.4 x 10$^{-4}$ M were calculated for phosphohydrolase I and phosphohydrolase II, respectively. It should be noted that both enzymes were inhibited by high concentrations of ATP. This experiment was carried out using a constant amount of Mg$^{2+}$ while varying the ATP concentration.

Effect of Time and Enzyme Concentration on Phosphohydrolase I and Phosphohydrolase II Activity—Both phosphohydrolase I (Fig. 10) and phosphohydrolase II (Fig. 11) hydrolyze ATP without a lag period. Both enzymes show an initial linear response with respect to time and the hydrolysis of ATP, within certain limits, was proportional to the amount of enzyme added (insets, Figs. 10 and 11). These figures also show the dependence of both phosphohydrolase I and phosphohydrolase II activity on the presence of added DNA as illustrated by the low levels of endogenous activity in the absence of added DNA.

Stoichiometry of Phosphohydrolase I and Phosphohydrolase II Reactions—The stoichiometry of ATP hydrolysis to ADP and P$_i$ was studied using both [32P]ATP and [γ-32P]ATP. As can be seen in Table III the amount of labeled substrate hydrolyzed was equal to the amount of ADP and P$_i$ that was formed. This indicates that under standard assay conditions the products of ATP hydrolysis were not consumed by further reactions.

Side Reactions—A number of side reactions that might occur with ATP have been looked for. As expected from the stoichiometry data, hydrolysis of ADP does not occur. The enzymes also fail to hydrolyze AMP. The enzymes fail to produce detectable ATP from ADP and inorganic phosphate and no exchange reactions were observed. Under standard conditions of assay, no formation of polynucleotides by either enzyme was observed.

Effects of Various Substances on Enzyme Activity—The data in Table IV indicate that the end products of ATP hydrolysis, P$_i$ and ADP, at equimolar concentrations with ATP are not very inhibitory to either phosphohydrolase I or phosphohydrolase II. Likewise PP$_i$ and AMP did not extensively inhibit either phosphohydrolase I or phosphohydrolase II. The phosphonic acid analogs of ATP with a methylene bridge between either the β-γ- or α-β-phosphates were not very inhibitory to either phosphohydrolase I or phosphohydrolase II. Phosphohydrolase I was more sensitive to the inclusion of monovalent ions in the assay mixture than was phosphohydrolase II. In fact, phosphohydrolase II showed a small stimulation of activity at low monovalent ion concentration. Neither cyclic adenosine 3':5'-monophosphate nor 3'-adenosylmethionine had any effect on either phosphohydrolase I nor phosphohydrolase II at various concentrations tested (data not presented).

Stability of Purified Enzymes—Both enzymes have been stored at -20°C for several months without loss of activity even when frozen as dilute solutions directly from the DNA-cellulose (data not presented).

![Fig. 8. Effect of Mg$^{2+}$ on phosphohydrolase I and phosphohydrolase II activity. Purified phosphohydrolase I, ○—○, and phosphohydrolase II, ●—●, were assayed under standard conditions as described under “Materials and Methods” with the concentration of Mg$^{2+}$ as indicated on the abscissa.](image-url)
Two enzymes which split ATP to ADP and P1 were purified from vaccinia virus cores. Both enzymes are stimulated by nucleic acid and extensive purification was achieved by DNA-cellulose chromatography. Phosphohydrolase I, the more abundant enzyme, was purified to near homogeneity and represented approximately 0.2 to 0.6% of the total viral protein. The correspondence of molecular weights determined by sucrose gradient sedimentation, gel filtration, and SDS polyacrylamide gel electrophoresis indicates that the enzyme exists as a monomer with a molecular weight of about 88,000. From the recovery and molecular weight values we estimate that each virion contains between 100 and 300 molecules of phosphohydrolase I. A turnover number of approximately $1 \times 10^4$ molecules of ATP per min per enzyme molecule was estimated. Similar estimates were not made for phosphohydrolase II which has a similar molecular weight because the low amounts did not allow reliable estimates of enzyme concentration.

As described by Siegel and Monty (58) frictional ratios of the enzymes could be determined by making further calculations using the molecular weights derived from sucrose gradient sedimentation and the Stokes radii derived from Sephadex gel filtration. Frictional ratios of approximately 1.35 and 1.29 for phosphohydrolase I and phosphohydrolase II, respectively, were calculated, suggesting axial ratios of about 7:1 and 6:1 for these enzymes if considered as prolate ellipsoids (59). One must bear in mind that deviations of the partial specific volumes of these enzymes from the assumed value of 0.73 ml g$^{-1}$ or unusual hydrations could introduce rather large errors.

With the exception of reverse transcriptase (60), purification

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**TABLE III**

Stoichiometry of ATP hydrolysis by phosphohydrolase I and II

| Enzyme              | [P$\text{I}^\text{32P}$]ATP hydrolyzed/5 min | [P$\text{II}^\text{32P}$]ATP hydrolyzed/5 min | [P$\text{I}^\text{32P}$]ADP formed/5 min | [P$\text{II}^\text{32P}$]ADP formed/5 min |
|---------------------|---------------------------------------------|---------------------------------------------|----------------------------------------|----------------------------------------|
| Phosphohydrolase I  | 7.2                                         | 7.1                                         | 4.5                                    | 4.6                                    |
| Phosphohydrolase II | 5.5                                         | 5.5                                         | 5.1                                    | 4.8                                    |

$^*$ $^{32}$P$_1$ was separated from $[\gamma^{32}$P]ATP using the standard chromatographic procedure described under "Materials and Methods" and was located by radioautography.

**TABLE IV**

Effect of various reagents on phosphohydrolase I and II

| Reagent added to reaction assay, mg | Phosphohydrolase I | Phosphohydrolase II |
|-------------------------------------|--------------------|---------------------|
| Control activity                    | 1.00               | 1.00                |
| P$_1$, 1                            | 0.96               | 0.96                |
| PP$_{1}$, 1                         | 0.89               | 0.69                |
| AMP, 1                              | 0.98               | 1.02                |
| ADP, 1                              | 0.72               | 0.90                |
| APPCP, 1                            | 0.71               | 0.85                |
| APCPP, 1                            | 0.66               | 0.77                |
| NaCl                                | 0.92               | 1.22                |
| 10                                  | 0.55               | 0.92                |
| 50                                  | 0.22               | 0.51                |
| KCl                                 | 0.78               | 1.18                |
| 10                                  | 0.68               | 1.03                |
| 50                                  | 0.28               | 0.62                |

$^*$ Control activity for phosphohydrolase I and phosphohydrolase II was 6.4 and 4.7 nmoles of ADP/5 min, respectively.
of other enzymes associated with animal viruses has not been reported. This has undoubtedly been due to the relatively small physical amounts of purified virus available as starting material and the insoluble particulate nature of viruses. The procedure that we have developed has been found sufficient for the solubilization and purification of the two nucleic acid-dependent phosphohydrolases reported here from relatively small amounts of purified virus (70 to 80 mg). This procedure also has been used to solubilize the deoxyribonuclease (41), polyadenylate polymerase (10), and protein kinase (61) from vaccinia virions.

The question of whether phosphohydrolase I and phosphohydrolase II are virus-induced enzymes must be approached in several ways. Our preliminary unpublished experiments indicate that DNA dependent phosphohydrolase activity first appears in the cytoplasm of infected cells several hours after infection. A similar activity was not detected in the cytoplasm of uninfected cells. In addition, further characterization of the two enzymes indicates that they are unlike any previously described (12-23). Isolation of specific temperature-sensitive mutants of the two enzymes indicate that they are unlike any previously described. Synthesis of the enzymes in a DNA-directed, cell-free protein synthesizing system is a possibility that remains to be explored.

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