

# Two Nucleic Acid-dependent Nucleoside Triphosphate Phosphohydrolases from Vaccinia Virus

## NUCLEOTIDE SUBSTRATE AND POLYNUCLEOTIDE COFACTOR SPECIFICITIES

(Received for publication, September 14, 1973)

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### SUMMARY

The two purified nucleoside triphosphate phosphohydrolases from vaccinia virus are distinct enzymes as judged by their nucleotide substrate and polynucleotide cofactor specificities. Phosphohydrolase I hydrolyzes only ATP and dATP, whereas phosphohydrolase II hydrolyzes the four common ribonucleoside triphosphates as well as dATP and TTP. The range of  $K_m$  values for ATP and dATP hydrolysis by phosphohydrolase I ( $1.5$  to  $1.6 \times 10^{-4}$  M) was lower than the range of  $K_m$  values for ATP, dATP, TTP, GTP, CTP, and UTP hydrolysis by phosphohydrolase II ( $6.4$  to  $11.2 \times 10^{-4}$  M). Phosphohydrolase I had little or no detectable activity in the absence of added nucleic acids. Phosphohydrolase II frequently had some endogenous activity, probably due to trace amounts of nucleic acid present in the eluate from the DNA-cellulose column. This endogenous activity could be reduced by passage through a DEAE-cellulose column. Neither phosphohydrolase I nor II was stimulated by completely double-stranded DNA unless the DNA was first denatured. Only phosphohydrolase II was stimulated by RNA but again only single-stranded forms were usable. Phosphohydrolase II was also stimulated by all tested homopolynucleotides of either the ribose or deoxyribose series and by all possible hybrid forms. Phosphohydrolase I exhibited a greater specificity and was incapable of using single-stranded homopolynucleotides. However, homopolydeoxyribonucleotide duplexes and some homopolyribonucleotide:homopolydeoxyribonucleotide duplexes were effective. These results suggest that phosphohydrolase I requires some secondary structure such as adjacent single-stranded and hydrogen-bonded regions. This interpretation was supported by the preferential inhibition of phosphohydrolase I with actinomycin D and low concentrations of proflavine. The ability of both enzymes to form stable complexes with nucleic acids was shown by glycerol gradient sedimentation. It was concluded that both nucleoside triphosphate phosphohydrolases have unique properties which make them distinct from any previously described enzymes. Their biological role is unknown, although it is tempting to speculate an involvement in replication and packing of DNA or transcription and extrusion of nascent RNA from viral cores.

The preceding paper (1) described the purification and properties of two nucleic acid-dependent enzymes that hydrolyze ATP to ADP and  $P_i$ . In this report the nucleotide substrate and nucleic acid cofactor specificities of the two nucleic acid-dependent nucleoside triphosphate phosphohydrolases (phosphohydrolase I and II) are considered.

### MATERIALS AND METHODS

**Nucleic Acids**—Salmon sperm DNA, calf thymus DNA, and *Escherichia coli*-soluble RNA were purchased from Worthington Biochemical Corp. Yeast ribosomal RNA and  $\phi$ X174 DNA were from Calbiochem and Miles Laboratories, respectively. Vaccinia virus DNA was isolated from purified virus using SDS and phenol. HeLa cell and  $\lambda$ DNA were provided by Dr. H. Rosemond-Hornbeak, adenovirus 2 DNA by Dr. J. A. Rose, SV40 DNA Component I by Dr. M. A. Martin (all at National Institutes of Health) and reovirus RNA by Dr. A. J. Shatkin (Roche Institute of Molecular Biology). [ $^3$ H]Thymidine-labeled T7 DNA was purified according to published procedures (2) with the help of Dr. N. Nossal (National Institutes of Health).

**Shearing of Nucleic Acids**—SV40 DNA Component I and  $\phi$ X174 DNA were sheared by sonication with the Branson Sonifier at half-maximal setting using the microprobe attachment. Total sonication time was 2 min at 15-s bursts with cooling of the sample throughout.

**Nucleic Acid Denaturation**—Where indicated, nucleic acids were denatured at  $100^\circ$  for 10 min followed by rapid quenching of the sample in ice.

**Synthetic Polynucleotides**—Poly(dA), poly(dT), poly(C), poly(U), and poly[d(AT)] were obtained from Miles Laboratories, and poly(dG), poly(dC), poly(dI), and poly(I) were from P-L Biochemicals. Synthetic duplexes were made by mixing equal amounts of the respective homopolynucleotides (125  $\mu$ g each per ml in 0.01 M Tris-HCl, pH 7.4, and 0.2 M NaCl) and allowing them to stand at room temperature for 15 min as described by Spiegelman *et al.* (3).

**Enzymes**—Phosphohydrolase I and phosphohydrolase II were solubilized from vaccinia virus and purified and assayed as described in the previous communication (1).

**Glycerol Gradient Sedimentation of DNA-Enzyme Complex**—Phosphohydrolase I and phosphohydrolase II were incubated in a 300- $\mu$ l reaction mix with or without heat-denatured [ $^3$ H]thymidine-labeled T7 DNA at  $37^\circ$  for 15 min. Of these reaction mixtures, 200  $\mu$ l were then applied to separate 10 to 30% glycerol gradients in 0.01 M Tris-HCl, pH 7.4, 0.05 M NaCl, 0.1% bovine serum albumin, and 0.05% Triton X-100. Following centrifugation for 2.5 hours at 45,000 rpm in the SW 50.1 rotor at  $4^\circ$ , the gradients were collected from the bottom of the tube. Portions

of each fraction were counted for [ $^3\text{H}$ ]thymidine radioactivity or assayed for phosphohydrolase activity under standard conditions with salmon sperm DNA as cofactor.

**Nucleoside Triphosphate**—[ $G$ - $^3\text{H}$ ]adenosine 5'-triphosphate (13.4 Ci per mm), [ $G$ - $^3\text{H}$ ]guanosine 5'-triphosphate (48 Ci per mm), [ $C$ - $^3\text{H}$ ]cytidine 5'-triphosphate (31.4 Ci per mm), [ $U$ - $^3\text{H}$ ]uridine 5'-triphosphate (26.9 Ci per mm), [ $G$ - $^3\text{H}$ ]deoxyadenosine 5'-triphosphate (18.4 Ci per mm), and [ $methyl$ - $^3\text{H}$ ]thymidine 5'-triphosphate (21.4 Ci per mm) were purchased from New England Nuclear. Unlabeled nucleoside triphosphates were obtained from P-L Biochemicals.

**Drugs**—Proflavine was purchased from Allied Chemicals and rifampicin was a gift of the Dow Chemical Co. Actinomycin D was provided by Dr. N. P. Salzman.

## RESULTS

**Specificity of Phosphohydrolase I and Phosphohydrolase II for Nucleoside Triphosphates**—Purified phosphohydrolase I and phosphohydrolase II both catalyze the DNA-dependent hydrolysis of ATP (1). Whole vaccinia virions, however, are capable of hydrolyzing all four common ribonucleoside triphosphates (4, 5). Further experiments established that the soluble extracts of vaccinia cores contain guanosine, cytidine, and uridine triphosphate phosphohydrolase as well as adenosine triphosphate phosphohydrolase activity. After removal of endogenous viral DNA by DEAE-cellulose chromatography, hydrolysis of each nucleoside triphosphate was stimulated about 10-fold by added DNA (Table I). Since phosphohydrolase I and phosphohydrolase II are separable by DNA-cellulose chromatography (1), the column fractions were assayed with each of the four radioactively labeled ribonucleoside triphosphates. The results indicated that phosphohydrolase I hydrolyzed only ATP while phosphohydrolase II hydrolyzed all four ribonucleoside triphosphates tested (Fig. 1). Further evidence that phosphohydrolase II contained all four hydrolytic activities was provided by gel filtration. A single peak of activity capable of hydrolyzing ATP, GTP, UTP, and CTP was eluted from a Sephadex G-200 column after application of a sample of phosphohydrolase II (data not presented).

The hydrolysis of deoxyribonucleoside triphosphates by purified phosphohydrolase I and phosphohydrolase II was tested using dATP and TTP as representative purine and pyrimidine deoxyribonucleoside triphosphates. The data in Table II indicated that the purified phosphohydrolase I used dATP as efficiently as ATP but did not hydrolyze TTP to any appreciable extent. Purified phosphohydrolase II, on the other hand, hydrolyzed both dATP and TTP as efficiently as ATP. The  $K_m$  values for each of the ribo- and deoxyribonucleoside triphosphate substrates tested were determined from Lineweaver-Burk plots of the saturation data and are presented in Table III. Several important points may be derived. First, the  $K_m$  of phospho-

hydrolase I for ATP and dATP are virtually identical. Second, the  $K_m$  of phosphohydrolase I for either substrate is lower than the corresponding values for phosphohydrolase II. Third, the  $K_m$  of phosphohydrolase II for each substrate lies within a narrow range ( $6.4$  to  $11.2 \times 10^{-4}$ ).

**Effect of Native and Denatured DNA on Activity of Phosphohydrolase I and Phosphohydrolase II**—The effects of native and denatured DNA on the hydrolysis of ATP by phosphohydrolase I and phosphohydrolase II are shown in Fig. 2. In this experiment adenovirus DNA was used. Both phosphohydrolase I and phosphohydrolase II were stimulated by denatured DNA but not to any appreciable extent by native DNA. An appreciable rate of ATP hydrolysis in the absence of added DNA was found with some purified phosphohydrolase II preparations,

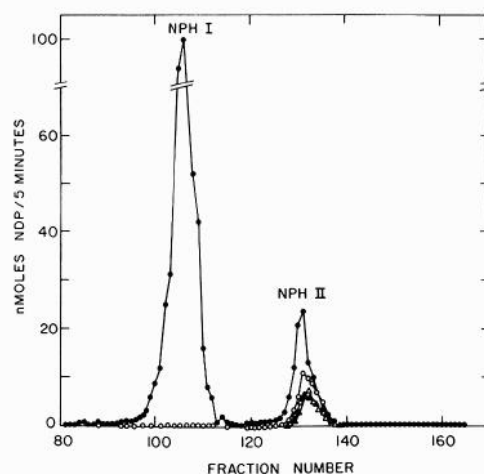


FIG. 1. Hydrolysis of ribonucleoside triphosphate by phosphohydrolase I and phosphohydrolase II. Phosphohydrolase I (NPH I) and II (NPH II) were separated by DNA-cellulose chromatography as described (1). Aliquots from each fraction were assayed with the following substrates: ATP, ●—●; GTP, ○—○; CTP, ▲—▲; UTP, △—△; using the standard assay conditions described under "Materials and Methods."

TABLE II  
Hydrolysis of dATP and dTTP by phosphohydrolase I and II

Substrate	Phosphohydrolase I	Phosphohydrolase II
	<i>nmoles NDP/5 min</i>	
ATP.....	5.8	5.7
dATP.....	5.1	5.3
dTTP.....	0.1	5.4

TABLE I  
Stimulation of ATP, GTP, CTP, and UTP hydrolysis by DNA

Purification fraction	Substrate							
	ATP		GTP		CTP		UTP	
	-DNA	+DNA	-DNA	+DNA	-DNA	+DNA	-DNA	+DNA
	enzyme units $\times 10^5$							
I. Total Nonidet P-40-treated virus.....	0.76	1.92	0.45	1.55	0.13	1.13	0.06	0.97
II. Cores.....	0.58	1.48	0.22	1.07	0.12	0.77	0.08	0.64
III. Disrupted cores.....	1.24	3.48	0.25	0.97	0.16	0.29	0.06	0.33
IV. 136,000 $\times g$ material.....	0.68	1.97	0.13	0.39	0.16	0.33	0	0.14
V. High salt DEAE-cellulose.....	0.26	2.80	0.03	0.37	0.03	0.33	0	0.12
VI. Low salt DEAE-cellulose.....	0.20	3.29	0.05	0.48	0.03	0.47	0	0.31

TABLE III  
*K<sub>m</sub>* values for nucleoside triphosphate substrates of  
phosphohydrolase I and II

Nucleoside triphosphate	<i>K<sub>m</sub></i> values	
	Phosphohydrolase I	Phosphohydrolase II
	<i>M</i> × 10 <sup>-4</sup>	
ATP.....	1.4	6.4
CTP.....		6.4
UTP.....		9.1
GTP.....		11.2
TTP.....		7.1
dATP.....	1.6	6.5

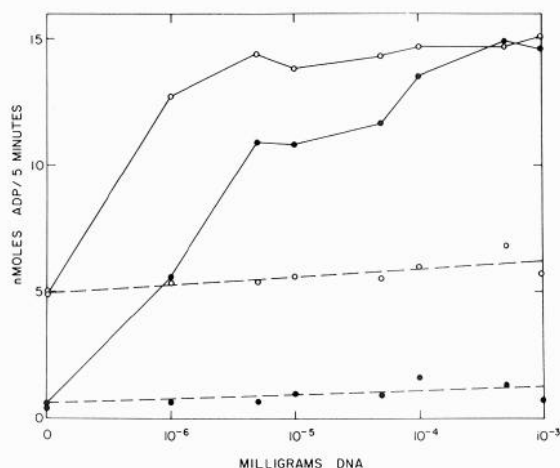


FIG. 2. Native and denatured DNA as cofactor for phosphohydrolase I and phosphohydrolase II. Activity was measured in a standard phosphohydrolase assay as described under "Materials and Methods" using increasing amounts of native or denatured adenovirus DNA. ●—●, phosphohydrolase I + native DNA; ●—●, phosphohydrolase I + denatured DNA; ○—○, phosphohydrolase II + native DNA; ○—○, phosphohydrolase II + denatured DNA.

while in other preparations endogenous activity was quite low. Filtration of the DNA-cellulose-purified phosphohydrolase II enzyme through a DEAE-cellulose column served to reduce the endogenous activity and suggested that residual nucleic acid may sometimes be present in the enzyme preparations after DNA-cellulose chromatography. It is also evident from Fig. 2 that phosphohydrolase II was saturated at lower concentrations of added DNA than was phosphohydrolase I, indicating that phosphohydrolase II would be stimulated to a greater extent by trace amounts of nucleic acid. Saturation at low concentrations of DNA also occurred with preparations of phosphohydrolase II which had little endogenous activity.

**Comparison of Natural Nucleic Acids as Cofactors for Phosphohydrolase I and Phosphohydrolase II**—A number of nucleic acids from various sources were examined for their ability to stimulate the hydrolysis of ATP. The results of such a survey are presented in Table IV. With all nucleic acids that were well defined as to size and structure such as adenovirus, T7, and SV40 DNA, it was evident that both phosphohydrolase I and phosphohydrolase II had a strong or absolute preference for denatured DNA. SV40 DNA Component I, the closed double-stranded circular superhelical form, failed to serve as cofactor for either phosphohydrolase I or phosphohydrolase II. This was true even when the DNA was sheared to produce diplotonic

TABLE IV  
Natural nucleic acids as cofactors for phosphohydrolase I and II

Nucleic Acid <sup>a</sup>	nmoles ADP/5 min.			
	Phosphohydrolase I		Phosphohydrolase II	
	Native	Denatured	Native	Denatured
None	0.2	—	1.6	—
Adenovirus 2 DNA	0.7	13.0	2.6	6.4
T7 DNA	0.4	11.1	1.7	7.1
SV40 DNA I	0.6	—	1.7	—
SV40 Sheared DNA	0.6	10.6	2.6	6.4
φX174 DNA	11.0	—	7.2	—
φX174 Sheared DNA	11.0	—	6.3	—
Vaccinia DNA	0.6	6.5	3.3	4.4
λ DNA	2.3	11.9	4.4	6.3
Salmon Sperm DNA	6.0	11.2	6.1	7.7
Calf thymus DNA	2.5	10.3	5.2	6.2
Hela DNA	2.3	11.6	5.6	5.6
E. Coli s-RNA	0.3	0.3	2.8	2.6
Yeast Ribosomal RNA	0.3	—	4.8	—
Reovirus RNA	0.1	0.2	0.9	5.6
Dextran Sulfate	0.2	—	1.4	—

<sup>a</sup> Each was present at 0.1 μg/enzyme assay.

breaks. Only when the DNA was sheared and then denatured did it function as a cofactor for both phosphohydrolase I and phosphohydrolase II, again indicating the requirement by both enzymes for single-stranded DNA.

The ability of both phosphohydrolase I and phosphohydrolase II to use the closed circular single-stranded form of φX174 DNA as efficiently as when it had been sheared suggested that ends are not required for the nucleic acid-enzyme interreaction resulting in ATP hydrolysis. This point must be explored further since some linear molecules were present in the unsheread preparation.

Native vaccinia DNA exhibited a limited capacity to serve as cofactor for phosphohydrolase II but not for phosphohydrolase I. Since vaccinia DNA is rather large, with a molecular weight of  $160 \times 10^6$  (6), and difficult to isolate intact, this limited capacity of vaccinia DNA to serve as cofactor for phosphohydrolase II may be due to some single-stranded DNA present in the preparation. The fact that phosphohydrolase II is saturated at much lower concentrations of denatured DNA than is phosphohydrolase I may account for the preferential utilization of the DNA by the former enzyme. Alternatively, a specific association of phosphohydrolase II with a specific region on the vaccinia DNA is possible.

The ability of native λDNA to serve to some extent as cofactor for both phosphohydrolase I and phosphohydrolase II may be due to the single-stranded regions at the ends of the λDNA molecule (7).

Commercial preparations of native salmon sperm DNA and calf thymus DNA as well as native HeLa cell DNA all served to varying degrees as cofactors for both phosphohydrolase I and phosphohydrolase II. This was probably due to single-stranded DNA present in these preparations.

Yeast ribosomal RNA and reovirus RNA and to a lesser extent *E. coli*-soluble RNA functioned as cofactors for phosphohydrolase II but not for phosphohydrolase I. As seen with native and denatured reovirus RNA, phosphohydrolase II could use only the denatured form as cofactor. This result was similar to the requirement for denatured DNA. The deoxysugar polynucleotide specificity of phosphohydrolase I provided another means of distinguishing the two enzymes in addition to the nucleotide substrate specificity. Non-nucleotide polyanions such



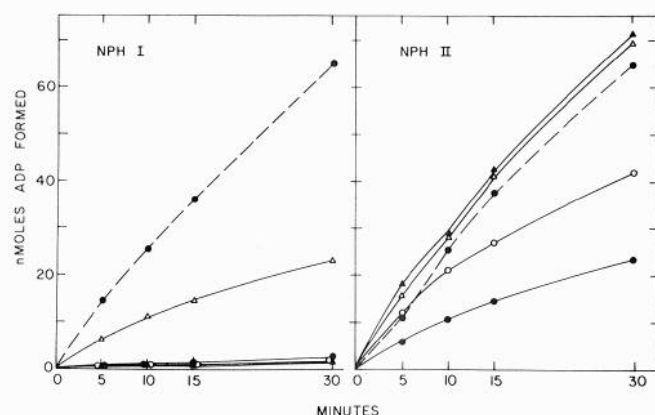


FIG. 3. Synthetic polynucleotides as cofactors for phosphohydrolase I and phosphohydrolase II. Phosphohydrolase I (NPH I) and II (NPH II) were assayed in the presence of various polynucleotides as described under "Materials and Methods." Aliquots of the enzyme assay mixture were withdrawn at intervals and the amount of ADP produced determined as described under "Materials and Methods." No DNA, ●—●, denatured salmon sperm DNA, ●—●—●, poly(dA·dT), △—△, poly(dA), ○—○, poly(dT), ▲—▲.

TABLE V  
Single-stranded homopolymers<sup>a</sup> as cofactors for phosphohydrolase I and II

Polynucleotide	nmoles NDP <sup>b</sup> formed							
	ATP		UTP		CTP		GTP	
	NPH <sup>c</sup> I	NPH <sup>c</sup> II	NPH I	NPH II	NPH I	NPH II	NPH I	NPH II
poly rA	1.4	21.5	<1	21.8	<1	18.4	<1	23.7
poly rC	<1	31.6	<1	27.2	<1	25.5	<1	36.3
poly rU	<1	29.8	<1	26.3	<1	24.3	<1	37.4
poly rI	<1	24.0	<1	19.3	<1	21.6	<1	27.5
poly dA	<1	20.2	<1	19.7	<1	19.6	<1	28.2
poly dC	4.9	29.7	<1	25.2	<1	23.5	<1	30.3
poly dT	1.4	26.3	<1	19.6	<1	19.5	<1	32.0
poly dI	<1	25.4	<1	20.9	<1	20.3	<1	26.4
poly dG	<1	10.1	<1	10.0	<1	10.9	<1	14.6
DNA	25.7	24.1	1	21.8	1.8	21.5	1	29.2

<sup>a</sup> All synthetic homopolymers as well as commercial salmon sperm DNA were present at 0.6 μg per reaction.

<sup>b</sup> NDP, nucleoside diphosphate.

<sup>c</sup> NPH I and II, phosphohydrolase I and II, respectively.

as dextran sulfate did not stimulate either phosphohydrolase I or phosphohydrolase II.

**Synthetic Polynucleotides as Cofactors for Phosphohydrolase I and Phosphohydrolase II**—Preliminary studies carried out with poly(dA) and poly(dT) indicated that phosphohydrolase I and phosphohydrolase II responded quite differently to synthetic polynucleotides (Fig. 3). Phosphohydrolase II was stimulated by the single-stranded homopolymers or by the synthetic duplex poly(dA):poly(dT). In contrast, phosphohydrolase I was stimulated only by the duplex (Fig. 3). This was a totally unexpected result in view of the previous demonstration that phosphohydrolase I had shown a complete dependence on thermally denatured DNA and was not stimulated by double-stranded DNA. These findings led us to investigate the ability of a number of synthetic ribo- and deoxyribohomopolymers as well as their respective hybrids to serve as cofactors for phosphohydrolase I and phosphohydrolase II.

**Single-Stranded Synthetic Homopolymers as Cofactors for Phosphohydrolase I and Phosphohydrolase II**—The effects of various synthetic homopolymers on the hydrolysis of ATP, UTP, CTP, and GTP by phosphohydrolase I and phosphohydrolase II are presented in Table V. Phosphohydrolase II could use any of the synthetic homopolymers tested as cofactor for the hydrolysis

TABLE VI  
Synthetic double-stranded DNA and RNA polymers and copolymer d(AT) as cofactors<sup>a</sup> for phosphohydrolase I and II

	nmoles NDP <sup>b</sup> formed							
	ATP		UTP		CTP		GTP	
	NPH <sup>c</sup> I	NPH <sup>c</sup> II	NPH I	NPH II	NPH I	NPH II	NPH I	NPH II
dA · dT	6.3	21.8	<1	19.3	<1	23.7	<1	32.7
dG · dC	12.0	23.2	<1	17.8	<1	21.4	<1	29.5
dC · dI	19.3	22.7	<1	15.7	<1	20.3	<1	31.1
rA · rU	<1	17.9	<1	18.4	<1	20.2	<1	23.1
rI · rC	<1	23.6	<1	22.7	<1	23.9	<1	34.5
d(AT)	11.5	19.6	<1	18.3	<1	19.6	<1	28.1
DNA	22.4	19.6	<1	15.5	1.3	16.4	<1	29.2

<sup>a</sup> Each synthetic polynucleotide as well as commercial salmon sperm DNA were present at 0.6 μg per assay.

<sup>b</sup> NDP, nucleoside diphosphate.

<sup>c</sup> NPH I and NPH II, phosphohydrolase I and II, respectively.

of any of the four ribonucleoside triphosphates. There was no clear preference by phosphohydrolase II for any synthetic homopolymer as cofactor, nor did phosphohydrolase II indicate any preference for a ribonucleoside triphosphate substrate.

Phosphohydrolase I, on the other hand, did not use to an appreciable extent any of the tested synthetic homopolymers as cofactor for the hydrolysis of ATP with exception of poly(dC). The latter was used by phosphohydrolase I only about one-fifth as well as commercial salmon sperm DNA. Except for a small amount of CTP hydrolyzed in the presence of DNA, only ATP served as a substrate for phosphohydrolase I.

**Synthetic Double-Stranded DNA and RNA Hybrids as Cofactors for Phosphohydrolase I and Phosphohydrolase II**—The ability of synthetic double-stranded DNA and RNA hybrids to function as cofactors for phosphohydrolase I and phosphohydrolase II is demonstrated in Table VI. Phosphohydrolase II hydrolyzed each of the ribonucleoside triphosphate substrates using any of the tested synthetic double-stranded DNA and RNA polymers, including the copolymer d(AT), as efficiently as with commercial salmon sperm DNA. Phosphohydrolase I utilized the synthetic double-stranded DNA polymers and copolymer d(AT) quite efficiently, while it failed to utilize the double-stranded RNA hybrids. Again, phosphohydrolase I hydrolyzed only ATP to any appreciable extent.

**Synthetic DNA-RNA Hybrids as Cofactors for Phosphohydrolase I and Phosphohydrolase II**—The ability of synthetic DNA-RNA hybrids to function as cofactors for phosphohydrolase I and phosphohydrolase II is shown in Table VII. Phosphohydrolase II was stimulated by all tested synthetic DNA-RNA hybrids and all four ribonucleoside triphosphates were hydrolyzed. Only the hybrids dT·rA and dC·rI were effective cofactors for phosphohydrolase I and, as expected, phosphohydrolase I was able to extensively hydrolyze only ATP.

**Effect of Actinomycin D, Proflavine, and Rifampicin on Phosphohydrolase I and Phosphohydrolase II Activity**—When commercial salmon sperm DNA was used as a cofactor, actinomycin D preferentially inhibited phosphohydrolase I activity (Table VIII). Actinomycin D had little or no effect on phosphohydrolase II. Similarly, proflavine, which binds more readily to duplex than to single-stranded polynucleotide structures (8), inhibited phosphohydrolase I more strongly than it inhibited phosphohydrolase II activity (Table VIII). This specificity was especially evident at lower concentrations of the drug. Rifampi-

TABLE VII  
Synthetic DNA-RNA hybrids<sup>a</sup> as cofactors for  
phosphohydrolase I and II

	nmoles NDP <sup>b</sup> formed							
	ATP		UTP		CTP		GTP	
	NPH <sup>c</sup> I	NPH <sup>c</sup> II	NPH I	NPH II	NPH I	NPH II	NPH I	NPH II
dA · rU	<1	30.4	<1	18.7	<1	16.2	<1	28.5
dT · rA	5.3	28.1	<1	19.7	<1	18.0	<1	27.8
dC · rI	8.6	24.9	<1	12.7	<1	14.9	<1	25.8
dI · rC	<1	30.0	<1	17.4	<1	19.8	<1	33.3
dG · rC	<1	30.0	<1	17.8	<1	23.2	<1	39.2
DNA	27.6	30.4	<1	14.7	1.3	16.0	<1	33.9

<sup>a</sup> Each synthetic hybrid as well as commercial salmon sperm DNA were present at 0.6  $\mu$ g per reaction.

<sup>b</sup> NDP, nucleoside diphosphate.

<sup>c</sup> NPH I and NPH II, phosphohydrolase I and II, respectively.

TABLE VIII  
Effect of actinomycin D, proflavine, and rifampicin on  
phosphohydrolase I and II<sup>a</sup>

Reagent added, $\mu$ g/ml	Phosphohydrolase I		Phosphohydrolase II	
	nmoles of ADP/5 min	Per cent control	nmoles of ADP/5 min	Per cent control
None	4.1	100	4.2	100
Actinomycin D, 6.25	2.6	63	4.7	112
Actinomycin D, 12.5	1.8	44	4.9	117
Actinomycin D, 25	1.1	27	4.8	114
Proflavine, 10	0.8	20	3.2	76
Proflavine, 25	0.3	7	2.1	50
Proflavine, 50	0.1	2	0.6	14
Rifampicin, 5	3.9	95	4.8	100
Rifampicin, 25	4.1	100	4.8	114
Rifampicin, 50	4.3	105	4.8	114

<sup>a</sup> Concentration of commercial salmon sperm DNA in these assays was 0.1  $\mu$ g/0.1 ml of reaction mixture.

cin was not found to inhibit either phosphohydrolase I or phosphohydrolase II activity (Table VIII).

**DNA Binding of Phosphohydrolase I and Phosphohydrolase II**—We took advantage of the ability of phosphohydrolase I and phosphohydrolase II to bind to DNA-cellulose for their purification (1). Additional studies showed that under standard assay conditions both enzymes bind strongly to DNA in solution and form stable DNA-enzyme complexes that can be isolated by glycerol gradient sedimentation (Fig. 4). Control experiments indicated that phosphohydrolase I and phosphohydrolase II incubated in a standard enzyme reaction without nucleic acid remained on top of glycerol gradients (Fig. 4). The conditions needed to form the DNA-enzyme complex, such as requirements for divalent cation, ATP, single- or double-stranded nucleic acid, will be the subject of future studies.

#### DISCUSSION

Phosphohydrolase I and phosphohydrolase II appear to be distinct enzymes as judged by their elution from DNA-cellulose, divalent cation requirements, nucleotide substrate specificities, polynucleotide cofactor requirements, and inhibition by intercalating agents. A summary of the major differences is presented in Table IX. The most striking difference between the two enzymes is their specificity for hydrolysis of nucleoside triphosphates. Phosphohydrolase II hydrolyzed any of the four common ribonucleoside triphosphates as well as dATP and

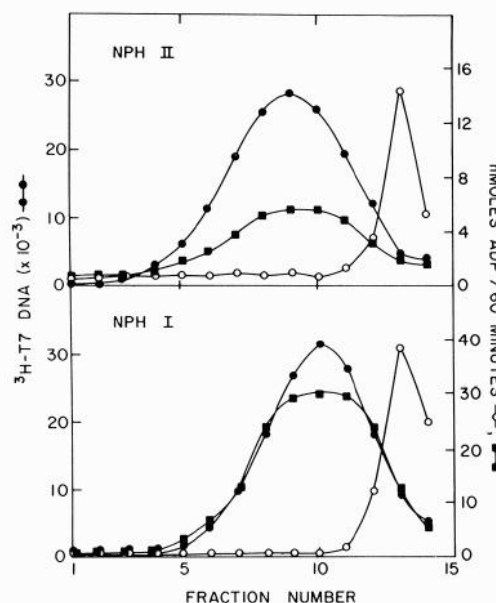


FIG. 4. Stable phosphohydrolase-DNA complex formation. The stable enzyme-nucleic acid complex formed between phosphohydrolase II (NPH II) and denatured T7 DNA and phosphohydrolase I (NPH I) and denatured T7 DNA are shown. Enzymes preincubated in the absence of T7 DNA (○—○), or in the presence of T7 DNA (■—■) and <sup>3</sup>H-labeled, denatured T7 DNA preincubated without enzyme (●—●) were run on separate 10 to 30% glycerol gradients and the fractions assayed as described under "Materials and Methods."

TABLE IX  
Summary of major differences between phosphohydrolase I and II

	Phosphohydrolase I	Phosphohydrolase II
1. NaCl concentration eluted from DNA-cellulose	0.1 M	0.17 M
2. Divalent cation requirement	Mg <sup>2+</sup> , Mn <sup>2+</sup> , Ca <sup>2+</sup>	Mg <sup>2+</sup> , Mn <sup>2+</sup>
3. Inhibition by Cu <sup>2+</sup>	+	—
4. Ribonucleoside triphosphates hydrolyzed	ATP	ATP, GTP, CTP, or UTP
5. Deoxyribonucleoside triphosphates hydrolyzed	dATP	dATP, TTP
6. Polynucleotide cofactors	Denatured DNA Synthetic DNA-DNA hybrids Synthetic DNA-RNA hybrids	Denatured DNA Denatured RNA Synthetic homopolynucleotides Synthetic RNA-RNA hybrids Synthetic DNA-DNA hybrids Synthetic DNA-RNA hybrids
7. Inhibition by actinomycin D	+	—

TTP, while phosphohydrolase I hydrolyzed only ATP and dATP to any appreciable extent. The second major difference is the polynucleotide cofactor requirement. Phosphohydrolase II utilized denatured DNA or RNA, while phosphohydrolase I utilized only denatured DNA. Furthermore, phosphohydrolase

II was active with single-stranded homoribo- or deoxyribonucleotide polymers and all of the hybrid duplexes tested, while phosphohydrolase I utilized only hybrids of deoxyribonucleotide polymers and some deoxyribonucleotide:ribonucleotide polymer hybrids.

The requirement for denatured nucleic acids indicated that both phosphohydrolase I and II required single-stranded base sequences. Thus it was surprising to find that synthetic homopolynucleotide hybrids rather than single-stranded molecules activated phosphohydrolase I. Evidently phosphohydrolase I requires regions of DNA containing some secondary structure such as single-strands adjacent to hydrogen-bonded regions. These structures would be present in denatured or naturally occurring single-stranded nucleic acids such as  $\phi$ X174 (9, 10), but not in synthetic homopolynucleotides. The preferential inhibition of phosphohydrolase I by actinomycin D and proflavine, which interact more readily with hydrogen-bonded structures than with single-stranded polynucleotides, supports this interpretation. Additional studies with model oligo- and polynucleotides will be needed to define the precise structure needed for cofactor function. In contrast to the complex requirement of phosphohydrolase I, phosphohydrolase II utilized any tested single-stranded polynucleotide.

Vaccinia virus cores exhibit phosphohydrolase activity without the addition of exogenous nucleic acids. Some possible explanations are that the packaged double-stranded viral DNA contains suitable single-stranded regions, that the enzymes act in concert with an unwinding protein (11), or that the enzymes are associated with separate small polynucleotides.

Efforts were made to determine whether nucleoside triphosphate hydrolysis is coupled to a modification of DNA. We could not detect either adenylation or phosphorylation of DNA isolated by gel filtration after addition of high specific activity [ $^3$ H]ATP and [ $\gamma$ - $^{32}$ P]ATP to the standard enzyme assay. No ligase activity was detected using SV40 DNA Component II as substrate. Native T7 DNA was not nicked as judged by alkaline sucrose gradient sedimentation after incubation with either phosphohydrolase I or II under a variety of conditions and in the presence of ATP or *S*-adenosylmethionine. Some endonuclease activity was detected by examining the sedimentation of  $^3$ H-labeled denatured T7 DNA on alkaline sucrose gradients after incubation with high concentrations of phosphohydrolase I. This endonuclease was neither stimulated nor dependent on the presence of ATP. Rechromatography of the enzyme on a DNA-cellulose column reduced but did not eliminate the nuclease activity, while no loss of phosphohydrolase I activity occurred. Nuclease activity was more pronounced at pH 4.4 than at pH 7 and was specific for single-stranded DNA. It is likely that phosphohydrolase I is contaminated with trace amounts of a very active deoxyribonuclease with the latter properties which has been purified from vaccinia virus as a separate enzyme (12). Although there is no evidence of an ATP-dependent DNase activity, further studies are needed to completely rule out the possibility that phosphohydrolase I has nuclease activity. Another point of difference between the enzymes described here and ATP-dependent DNases which also hydrolyze ATP (13-22) is that the latter prefer, or at least utilize, duplex DNA as cofactor for ATP hydrolysis.

The biological roles of these enzymes are unknown. The similar  $K_m$  values of phosphohydrolase I for ATP and dATP and the similar  $K_m$  values of phosphohydrolase II for all tested nucleoside triphosphates provide no clue as to whether one or all are physiological substrates. Vaccinia virus has an active DNA-

dependent RNA polymerase packaged within the core (23, 24). Kates and Beeson have presented data suggesting that extrusion of messenger RNA from viral cores is an ATP-dependent process (25). Either of these enzymes is a good candidate, since both enzymes require a polynucleotide cofactor and hydrolyze nucleoside triphosphates. If the enzymes worked in tandem, phosphohydrolase I might act at the site of transcription, since it requires DNA, and phosphohydrolase II might act at a later point since it can be stimulated by RNA. Alternatively, these enzymes may function during *in vivo* DNA replication or packaging. It is interesting to note that histochemical studies by Gold and Dales (5) indicated that ATPase activity can be demonstrated in immature vaccinia virus particles. Whether this finding is significant in relation to the time of action of the enzyme is unknown. It is likely that further work with these enzymes will provide more insight into their biological roles. It is evident that the properties of the two enzymes make them unlike any previously described.

**Acknowledgments**—We would like to extend our appreciation to the following people for gifts of various nucleic acids: H. Rosemond-Hornbeak, J. A. Rose, M. A. Martin, A. J. Shatkin, and N. Nossal.

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