Purification and Properties of a HeLa Cell Enzyme Able to Remove the 5'-Terminal Protein from Poliovirus RNA*

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Using a rapid phenol extraction assay, an enzyme was purified from uninfected HeLa cells that can cleave the 5'-terminal protein (VPg) from poliovirus RNA. Both cytoplasmic and nuclear extracts had enzymes with similar behavior. A polypeptide of molecular weight 27,000 was the major one present in the purified preparation. Assuming that this protein is the enzyme, a very low turnover number was calculated for it. The purified enzyme would cleave the tyrosine-phosphate bond linking VPg to poliovirus RNA with minimal degradation of the RNA or of VPg. If the RNA was first treated with proteinase K to degrade VPg, leaving a small peptide on the RNA, this peptide could also be removed by the enzyme. If the RNA was degraded with T1 RNase, leaving VPg attached to a nonanucleotide, the enzyme still would cleave off VPg, although incompletely. If the RNA was degraded completely, leaving either pUp or pU attached to VPg, the enzyme would not remove the nucleotides from the protein. Thus, for the enzyme to be active requires some length of polynucleotide attached to the protein but only a short peptide need be present for the enzyme to act.

The single-stranded RNA genome of poliovirus contains a protein (VPg)[1] covalently bound to its 5'-terminal phosphate (Flanegan et al., 1977; Nomoto et al., 1977a). VPg chromatographs as a molecule of molecular weight approximately 12,000 and contains 1 tyrosine residue that is linked by a phosphodiester bond to the RNA molecule (Ambros and Baltimore, 1978; Rothberg et al., 1978). VPg is found on negative strand viral RNA and was the only 5' end detectable on nascent poliovirus RNA molecules (Pettersson et al., 1978; Nomoto et al., 1977a). Poliovirus messenger RNA, however, lacks the 5'-terminal protein and instead terminates with a 5'-phosphate followed by the same nonanucleotide sequence that is 5' terminal in virion RNA (UUAAAACAG) (Nomoto et al., 1977b; Pettersson et al., 1977). Thus, poliovirion RNA and poliovirus mRNA differ by the presence of 5'-terminal VPg.

It has been suggested that poliovirus mRNA is formed by cleavage of the protein-RNA bond in newly made viral RNA, whereas the linkage remains intact in encapsidated RNA (Flanegan et al., 1977; Pettersson et al., 1977; Nomoto et al., 1977b). To investigate the nature of this cleavage reaction, we previously assayed cell-free extracts for an activity able to break the tyrosine-phosphate bond between VPg and poliovirus RNA (Ambros et al., 1978). We demonstrated an enzymatic activity in extracts of both poliovirus-infected and uninfected HeLa cells that was able to remove VPg from exogenously added poliovirus RNA and left the same 5' end structure (pUUAAAACAG) as found on mRNA. This activity, which we will refer to as unlinking enzyme or unlinking activity, had a requirement for divalent cations, was heat-labile, and sedimented at 3 S in a glycerol gradient.

To find the normal substrate for HeLa unlinking enzyme in uninfected cells, and to learn more about its possible role in poliovirus infection, it is first necessary to purify the enzyme and characterize the specificity of the cleavage reaction. Purification and partial characterization are described in this paper. Unlinking enzyme was purified from uninfected HeLa cells by DEAE-Sephadex and Cibacron blue-Sepharose chromatography. Enzyme recovered from the cytoplasmic fraction of HeLa cells and from a HeLa nuclear wash specifically cleaved the tyrosine-phosphate linkage between poliovirus RNA and VPg. Derivatives of poliovirus RNA·VPg complex were made by protease and ribonuclease digestion and those derivatives were tested for cleavage by the purified HeLa unlinking enzyme. Intact VPg was not necessary for cleavage of the tyrosine-RNA linkage, but a nucleic acid substituent longer than pUp was essential for cleavage of the linkage.

MATERIALS AND METHODS

Preparation of Substrates—[³H]Tyrosine-labeled poliovirion RNA was prepared as described elsewhere (Ambros and Baltimore, 1978) and stored at −70°C in 0.1 mM EDTA, pH 7.5, at a concentration of 0.2 pmol/μl (0.5 mg/ml). When 8 × 10⁸ poliovirus-infected HeLa cells were labeled with 5 mCi of [³H]tyrosine, specific activity of purified virion RNA was approximately 1000 cpm/μg.

Incubation Conditions for Removal of VPg from Poliovirus RNA—All incubations were performed in 1.5-ml polypropylene Eppendorf microcentrifuge vials. To each vial was added 15 μl of 10 mM Tris, pH 7.5, 1.5 mM MgCl₂, 5 mM β-mercaptoethanol, 10% glycerol (TMMG buffer), and 0.5 μl of poliovirus RNA substrate in 0.1 mM EDTA, pH 7.5 (approximately 250 cpm in 0.1 pmol of RNA). Five microliters of extract or TMMG buffer were then added and the mixture was mixed briefly and left to incubate at 30°C. At the end of the incubation time, the degree of removal of VPg from the RNA was immediately measured.

Phenol Extraction Assay for the Removal of VPg from [³H]-Tyrosine-labeled Poliovirus RNA—For routine analyses, removal of [³H]tyrosine-labeled VPg from poliovirus 35 S RNA was assayed by phenol extraction as described for the ³²P-labeled 5'-terminal nonanucleotide substrate (Ambros et al., 1978). When attached to 35 S RNA, VPg remains in the aqueous phase after phenol extraction;

[1] The abbreviations used are: VPg, 5'-terminal protein on poliovirion RNA; TMMG buffer, 10 mM Tris, pH 7.5, 1.5 mM MgCl₂, 5 mM β-mercaptoethanol, and 10% glycerol; SDS, sodium dodecyl sulfate; K-peptide, tyrosine-containing, proteinase-K-resistant peptide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

freed from RNA, it partitions to the phenol phase. The portion of VPg removed was expressed as percentage of ³H radioactivity which was phenol soluble.

When many samples or fractions were assayed at once, the following procedure was used. Identical 15-μl samples of TMMG buffer containing 0.1 pmol (approximately 250 cpm) of [³H]tyrosine-labeled poliovirus 35 S RNA substrate were transferred to individual 1.5-ml polypropylene Eppendorf vials. Five microliters of TMMG buffer was added to one tube (control) and to each of the rest was added 5 μl of a dilution of the fraction to be assayed. All tubes were incubated at 30°C for 30 min and then diluted and phenol extracted as described above. The entire aqueous phase of each sample was transferred to a scintillation vial and ³H radioactivity measured in Aquasol or Bray's solution. Unlinking activity was scored as decrease in radioactivity in the aqueous phase relative to the control sample aqueous phase. These data were converted to picomoles of ³H VPg released from RNA based on the measured specific activity of the [³H]tyrosine-labeled 35 S RNA substrate and 1 VPg molecule/RNA molecule.

K-Peptide Test for Cleavage of the Tyrosine-Phosphate Bond of Poliovirus RNA—The only tyrosine-containing proteinase K-resistant peptide of VPg, called K-peptide, is the one involved in the linkage to pUp. When the linkage is intact, treatment of [³H]tyrosine-labeled 35 S poliovirus RNA with a mixture of RNases T1, T2, and A, followed by proteinase-K, leaves ³H-labeled K-pUp. When the linkage is broken, free K-peptide is produced by protease-K. These two structures can be resolved by pH 3.5 3MM paper ionophoresis (see Fig. 8). Therefore, a definitive test for the cleavage of the tyrosine-phosphate bond employs the following protocol. [³H]Tyrosine-labeled poliovirus RNA is treated with an enzyme fraction under standard conditions. At the end of the incubation time, a mixture of ribonucleases T1, T2, and A is added and digestion is continued at 37°C for 1 h. Protease-K is then added to a final concentration of 1 mg/ml and the sample is further incubated at 37°C for 1 h. Samples are then fractionated by pH 3.5 3MM paper ionophoresis (Ambros and Baltimore, 1978). The paper is dried, the lanes are fractionated, and ³H radioactivity is measured.

S-100 Fractions of HeLa Cells—For purification of HeLa unlinking activity, the starting fractions were prepared differently than the extracts described previously (Ambros *et al.*, 1978). HeLa cells (4 × 10⁹ at 4 × 10⁵ cells/ml) were centrifuged at 1200 rpm in an IEC PR-J centrifuge for 5 min, washed twice with 10 mM Tris, 10 mM NaCl, and 1.5 mM MgCl₂, pH 7.5, by repeated centrifugation, and resuspended in 20 ml of hypotonic buffer (10 mM Hepes, pH 7.5, 15 mM magnesium acetate, and 6 mM 2-mercaptoethanol). The cells were allowed to swell at 0°C for 5 min and then were broken with 15 strokes of a Dounce homogenizer. Nuclei plus large cell debris were recovered by centrifugation at 5,000 rpm for 2 min in a Sorvall type SS-34 rotor at 4°C. The cytoplasmic supernatant was stored at 0°C. The nuclear pellet was washed by resuspension in TMMG buffer followed immediately by a 2-min centrifugation at 5,000 rpm in the Sorvall. The supernatant was discarded and the nuclei were resuspended in 25 ml of TMMG plus 200 mM KCl and were incubated at 0°C for 45 min with occasional gentle mixing. Both the nuclear suspension and cytoplasmic supernatant were then centrifuged at 10,000 rpm in a Sorvall type SS-34 rotor for 20 min at 4°C. The supernatants were then collected and centrifuged at 100,000 × *g* for 1 h in a Beckman SW 41 rotor at 4°C. The nuclear wash S-100 supernatant and cytoplasmic S-100 supernatant were then dialyzed against TMMG plus 50 mM KCl. Any precipitate which formed during dialysis was removed by centrifugation at 10,000 rpm for 10 min at 4°C. These supernatant fractions, designated nuclear S-100 and cytoplasmic S-100, were stored in a 4°C refrigerator in an ice bath.

DEAE-Sephadex Chromatography of HeLa Unlinking Activity— A column (1.5 × 15 cm) of DEAE-Sephadex A-25 was poured and equilibrated with TMMG + 50 mM KCl. Approximately 25 ml of sample (nuclear S-100 or cytoplasmic S-100) was applied to the column, and the column was washed with TMMG + 50 mM KCl until the per cent transmission at 254 nm (as monitored by an LKB Uvicord II spectrophotometer) returned to base-line value. The column was then eluted with 150 ml of a 50 mM to 200 mM KCl gradient in TMMG (flow rate = 10 to 20 ml/h). Fractions of 5 ml were collected and 5-μl samples were assayed for unlinking activity using [³H]tyrosine-labeled poliovirus RNA as substrate. Peak fractions were pooled and stored without dialysis (denoted Fractions IIN and IIC).

Cibacron Blue-Sepharose Chromatography of HeLa Unlinking Enzyme—Cibacron blue F3GA dye coupled directly to Sepharose 2B (Reyes and Sandquist, 1978) was a gift from Dr. Pim Zabel, Agricultural University, The Netherlands.

The pooled DEAE-Sephadex peak of unlinking activity was adjusted to 150 mM KCl and applied to a column (1 × 1.5 cm) of Cibacron blue-Sepharose equilibrated with 150 mM KCl in TMMG buffer. Per cent transmission was monitored as above and when the column was washed free of unbound protein, bound material was eluted with a 30-ml gradient of 150 mM to 1000 mM KCl in TMMG buffer. Fractions of 1 ml were collected. A 2-μl sample of each fraction was assayed for unlinking activity using the [³H]tyrosine-labeled 35 S RNA substrate. Removal of VPg from RNA was measured by phenol extraction. The peak fractions of activity, eluting at 400 to 600 mM KCl, were pooled and dialyzed for 12 h at 0°C against 10 mM Tris, 1.5 mM MgCl₂, 5 mM β-mercaptoethanol, and 50% glycerol, pH 7.5, to produce Fractions IIIN and IIIC. These were stored in 400-μl portions at −70°C. A working stock was removed from −70°C and kept at −20°C. The half-life of unlinking activity stored this way was about 2 months at −70°C in 50% glycerol and approximately 2 to 3 weeks at −20°C in 50% glycerol.

Agarose Gel Electrophoresis—Gel electrophoresis of poliovirus RNA through 1% agarose was performed as described elsewhere (Hewlett *et al.*, 1977).

RESULTS

Purification of HeLa Cell Unlinking Activity— To begin purification of the HeLa cell enzyme that unlinks VPg from poliovirus RNA, a nuclear wash S-100 and a cytoplasmic S-100 were prepared. These extracts were chromatographed on DEAE-Sephadex A-23 and fractions were assayed for their ability to unlink [³H]tyrosine-labeled VPg from poliovirus RNA using a phenol extraction assay. Both the cytoplasmic S-100 (Fig. 1*A*) and the nuclear S-100 (Fig. 1*B*) yielded peaks of unlinking activity eluting at approximately 120 mM KCl (Fractions IIC and IIN; Table I). In both cases, however, activity also appeared in the flow-through fractions. This activity was not characterized or purified further due to the large amount of contaminating ribonuclease and phosphodiesterase activities in the flow through (data now shown). When Fractions IIC and IIN were chromatographed on Cibacron blue-Sepharose, both the cytoplasmic (Fig. 2*A*) and nuclear (Fig. 2*B*) activities eluted at 400 to 500 mM KCl (Fractions IIIC and IIIN), well separated from the bulk of the
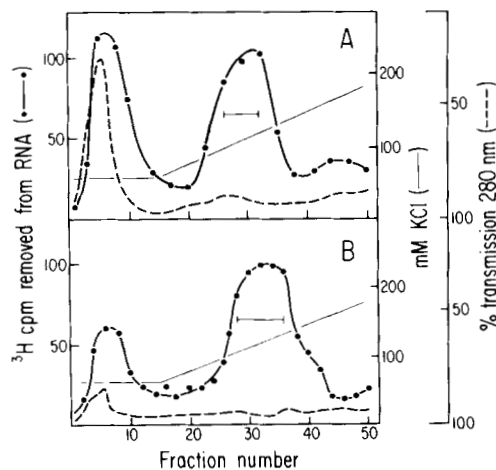

FIG. 1. **DEAE-Sephadex chromatography of HeLa unlinking enzyme.** Nuclear and cytoplasmic S-100 fractions were prepared as described under "Materials and Methods." DEAE-Sephadex columns were prepared and equilibrated as described under "Materials and Methods." Samples were applied in 50 mM KCl and TMMG buffer and per cent transmission at 254 nm was monitored during elution. Columns were eluted with a 50 to 200 mM KCl concentration gradient. Salt concentration (———) was measured by conductance. Fractions of 5 ml were collected and samples of alternate fractions were assayed for removal of ³H VPg from [³H]tyrosine-labeled poliovirus RNA (●——●). VPg removal was measured by phenol extraction. *A*, cytoplasmic S-100; *B*, nuclear S-100.

TABLE I

Purification of HeLa cell unlinking activity

Fraction	Enzyme fraction	Protein[a]	Units of enzyme[b]	Yield of enzyme	Specific activity	Net purification
		mg		*%*	*units/mg*	
IC	Cytoplasmic S-100	500	4,800	100	9.5	1
IIC	Cytoplasmic DEAE-Sephadex	18	2,200	45	120	13
IIIC	Cytoplasmic Cibacron blue pool	0.32	760	16	2,400	250
IN	Nuclear S-100	84	12,000	100	140	1
IIN	Nuclear DEAE-Sephadex pool	1.6	5,400	45	3,300	24
IIIN	Nuclear Cibacron blue pool	0.07	3,000	25	42,000	280

[a] Protein was measured by the assay of Lowry *et al.* (1951).

[b] One unit is defined as the amount of enzyme that will remove 0.04 pmol of VPg from poliovirion RNA in 30 min at 30°C at a substrate concentration of 0.1 pmol of poliovirus RNA/20-μl reaction.
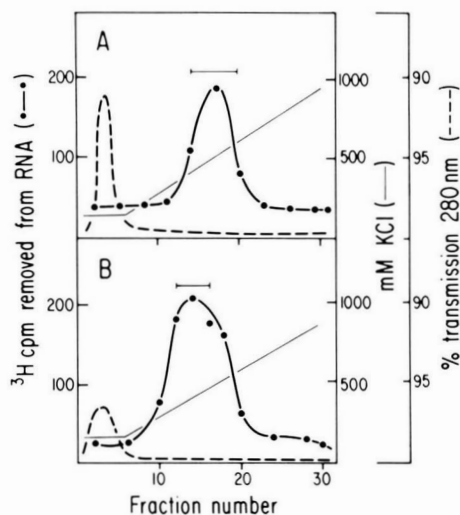

FIG. 2. **Cibacron blue-Sepharose chromatography of HeLa unlinking activity.** The pooled fractions from the peak of unlinking activity that eluted from DEAE-Sephadex at 120 mM KCl (Fig. 1) were applied to Cibacron-Sepharose columns, and the columns were eluted and fractionated as described under "Materials and Methods." Salt concentration (——), per cent transmission at 254 nm (- - -), and unlinking activity (●——●) were measured as for Fig. 1. *A*, cytoplasmic DEAE-Sephadex pool of unlinking activity; *B*, nuclear DEAE-Sephadex pool of unlinking activity.

FIG. 3. **Electrophoretic analysis of HeLa cell unlinking enzyme fractions.** Cell fractionation and purification of unlinking activity were performed as described under "Materials and Methods." Fraction IIIN enzyme was concentrated by binding and eluting from DEAE-Sephadex. SDS-polyacrylamide slab gel electrophoresis containing 12.5% acrylamide was carried out as described previously (Laemmli, 1970) and the gels were stained with Coomassie brilliant blue. *Lanes 1* and *4*, molecular weight markers of phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). *Lane 2*, approximately 45 μg of Fraction IN. *Lane 3*, approximately 2 μg of Fraction IIIN.

protein. These steps gave an overall purification of about 250-fold for each fraction with about 20% yields (Table I).

Analysis of Fractions IN (the nuclear starting material) and IIIN by SDS-polyacrylamide gel electrophoresis showed that a minor polypeptide in the starting material of molecular weight 27,000 was purified by the procedure (Fig. 3). Two independent preparations contained only this polypeptide. Fraction IIIC of unlinking activity, obtained from the cytoplasm, contained the molecular weight 27,000 polypeptide as well as larger quantities of other polypeptides of various sizes (data not shown). The relative impurity of Fraction IIIC is consistent with its lower specific acitvity (Table I).

It was shown previously (Ambros *et al.*, 1978) that the unlinking activity sediments in a glycerol gradient at 3 S, indicating a molecular weight of about 20,000 to 40,000. Thus, the 27,000 polypeptide in Fraction IIIN may represent the unlinking enzyme itself which sediments as a monomer in glycerol gradients.

With Fraction IIIN enzyme, activity measured under standard assay conditions was linearly related to added protein up to about 2.5 μg/ml (Fig. 4A). The amount of VPg freed by 1 unit (0.025 μg) of Fraction IIIN enzyme in 30 min at 30°C was proportional to poliovirus RNA substrate concentration up to about 10 nM or 0.2 pmol (0.5 μg) of 35 S RNA/20 μl of reaction
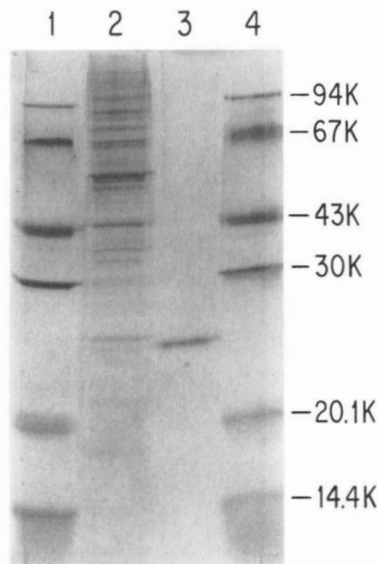
volume (Fig. 4B). A rough estimate of the K_m for the removal of VPg from poliovirus RNA at half-maximal velocity is approximately 7.5 nM. It should be noted that standard assays were performed at an RNA concentration of 5 nM, which is below the K_m. Assuming that the value of 0.2 pmol of VPg released in 30 min reflects the maximum velocity of the reaction, then the maximum specific activity of the enzyme under these conditions is 0.27 pmol of VPg removed/min/μg of enzyme. If the molecular weight 27,000 protein represents the unlinking enzyme and the Fraction IIIN enzyme is assumed to be 100% pure, then the turnover number of the enzyme is about 7×10^{-3} min^{-1}. This very low value could either indicate that the assumptions used in the calculation are wrong or suggest that the unlinking activity is a minor activity of the enzyme.

Gel Filtration Assay of Released VPg—For the purification, a rapid phenol extraction assay was used. To confirm that the purified enzyme had the properties originally described for cruder preparations (Ambros and Baltimore, 1978),
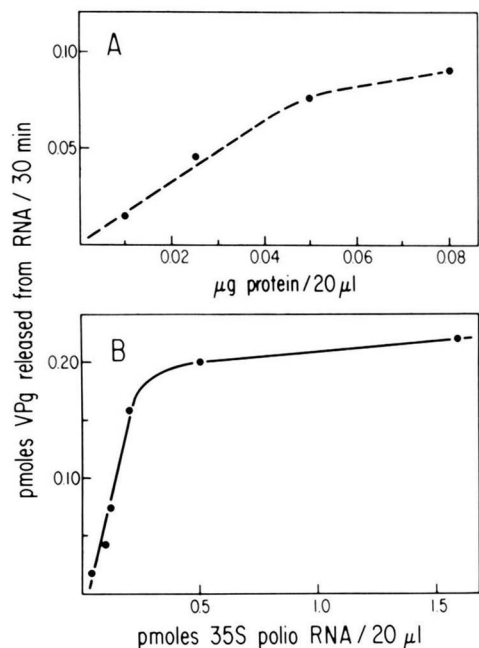
FIG. 4. **Concentration dependence for removal of VPg from 35 S poliovirus RNA.** Incubations were performed under standard conditions for 30 min at 30°C. *A*, reactions of 20 μl contained 0.1 pmol of [³H]tyrosine-labeled poliovirus RNA as substrate and various amounts of Fraction IIIN unlinking activity. *B*, reactions of 20 μl contained 1 unit (0.025 μg) of Fraction IIIN unlinking enzyme and various concentrations of [³H]tyrosine-labeled poliovirus RNA. Removal of VPg from RNA was measured by phenol extraction as described under "Materials and Methods." 35 S poliovirus RNA concentration is expressed in terms of molarity of 5′ ends.

the VPg released from viral RNA by the enzyme was characterized by Bio-Gel A-1.5m exclusion chromatography. Three samples containing 0.2 pmol (0.5 μg) of [³H]tyrosine-labeled poliovirus RNA plus 1200 cpm of [³²P]poliovirus RNA were incubated under standard assay conditions at 30°C for 1 h either with or without Fraction III enzyme. The samples were then processed and chromatographed through Bio-Gel A-1.5m in the presence of SDS (Fig. 5). Virtually all of the ³H in the sample receiving no enzyme eluted in the excluded volume, coincident with the [³²P]35 S RNA marker (Fig. 5A). This behavior is a consequence of the covalent attachment of VPg to RNA. The samples incubated with Fraction IIIN (Fig. 5B) or Fraction IIIC (Fig. 5C) had a large portion of their ³H eluting like free VPg, coincident with a cytochrome *c* marker. In neither case was the [³²P]35 S poliovirus RNA marker significantly degraded. Therefore, these purified fractions contain activity able to release VPg from poliovirus RNA. The protease activity detected in the crude cytoplasmic extract (Ambros *et al.*, 1978) was apparently absent from Fraction IIIC.

Agarose Gel Electrophoresis of Treated RNA—To examine the intactness of poliovirus RNA after incubation with the enzyme, agarose gel electrophoresis was employed. Samples of poliovirus RNA were incubated with either buffer, Fraction IIIN, or Fraction IIIC for 1 h. These conditions were sufficient to remove approximately 60 to 80% of VPg. At the end of the incubation period, each sample was heated to 70°C for 1 min to break up potential RNA aggregates, electrophoresed through agarose, and visualized by staining with ethidium bromide. In all three samples, full length poliovirus RNA was evident, although the control sample (Fig. 6, *lane 1*) contained about twice as much full length RNA as either the sample treated with Fraction IIIN (Fig. 6, *lane 2*) or with Fraction IIIC (Fig. 6, *lane 3*). Both enzyme preparations, therefore, had

small amounts of ribonuclease but were able to cleave VPg from RNA without extensive degradation of the RNA.

Removal of a Peptide from Viral RNA—To examine the substrate specificity of the unlinking enzyme, a variety of poliovirus RNA preparations and fragments were studied as substrates. For the first assay, [³H]tyrosine-labeled 35 S polio-
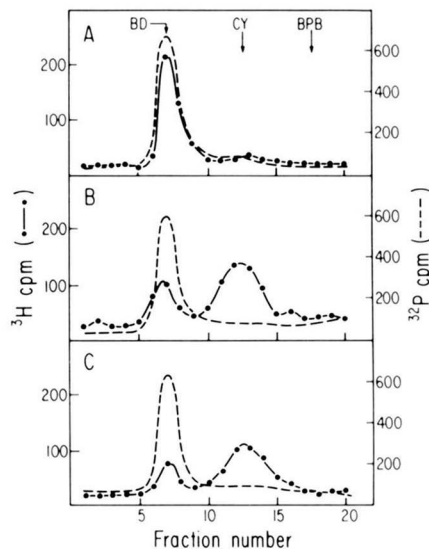

FIG. 5. **Gel filtration assay of Cibacron blue-Sepharose-purified unlinking enzyme.** Reactions of 20 μl containing 0.2 pmol of [³H]tyrosine-labeled poliovirion RNA and 1200 cpm of [³²P]poliovirion RNA were incubated with 4 μl of Cibacron blue-Sepharose-purified unlinking activity for 1 h at 30°C or with no enzyme under identical conditions. Samples were chromatographed through Bio-Gel A-1.5m as described previously (Ambros *et al.*, 1978). ³H Radioactivity in VPg (●——●) and ³²P radioactivity in poliovirus RNA (- - -) were measured by liquid scintillation counting. The column was calibrated with blue dextran 2000 (*BD*), cytochrome *c* (*CY*), and bromphenol blue (*BPB*) in a parallel run. *A*, control sample, with no enzyme treatment; *B*, sample treated with Fraction IIIN (nuclear) enzyme; *C*, sample treated with Fraction IIIC (cytoplasmic) enzyme.
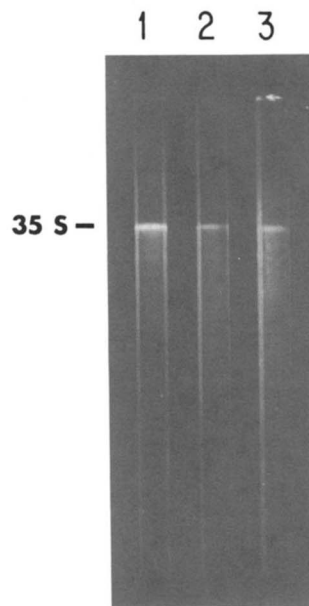

FIG. 6. **Agarose gel electrophoresis of poliovirus RNA treated with unlinking enzyme.** Samples of 0.5 μg of poliovirus virion RNA were electrophoresed through 1% agarose as described under "Materials and Methods" after treatment for 1 h at 30°C with buffer (*1*), 4 μl of Fraction IIIN unlinking enzyme (*2*), or 4 μl of Fraction IIIC unlinking enzyme (*3*).
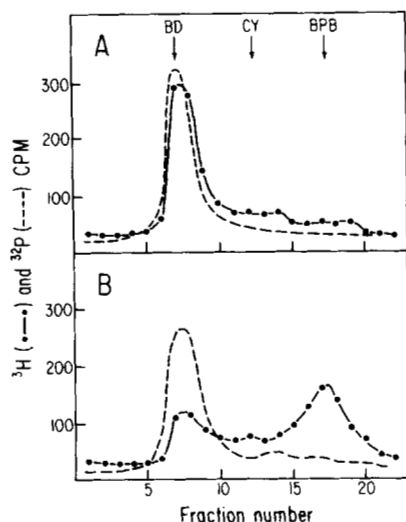
FIG. 7. **Gel filtration assay for removal of K-peptide from poliovirus 35 S RNA by Fraction IIIN enzyme.** Reaction conditions and chromatographic procedures were exactly as described in the legend to Fig. 5. Protease-K-treated poliovirus RNA was prepared as described in the text and chromatographed on Bio-Gel A-1.5 before (*A*) and after treatment with Fraction IIIN unlinking enzyme (*B*).

virus RNA was treated with proteinase-K to digest away all of VPg but the "K-peptide" that remains linked to the RNA via [³H]tyrosine (Ambros and Baltimore, 1978). This material was phenol extracted, ethanol precipitated, and used as substrate in the standard unlinking enzyme assay with ³²P-labeled 35 S poliovirus RNA included as marker. Removal of K-peptide was measured by Bio-Gel A-1.5m exclusion chromatography. Without enzyme treatment, [³H]tyrosine-labeled, proteinase-K-treated RNA and [³²P]RNA co-eluted at the void volume of the column (Fig. 7A). After incubation with 2.5 units of Fraction IIIN enzyme, elution of the [³²P]RNA was virtually unaffected, but low molecular weight ³H-labeled material was released (Fig. 7B). This ³H-labeled material eluted near the inclusion volume of the column, consistent with the expected small size of the K-peptide. Therefore, full size VPg was apparently not required for cleavage by the unlinking enzyme of the 5'-terminal tyrosine-phosphate bond in poliovirus 35 S RNA.

Removal of VPg from an Oligonucleotide or from Mononucleotides—Crude extracts of HeLa cells have previously been shown to remove VPg from the 5'-terminal T1 RNase-resistant oligonucleotide of poliovirus (Ambros *et al.*, 1978). To confirm that the purified enzyme retained activity on this substrate, [³H]tyrosine-labeled 5'-terminal oligonucleotide was purified from a T1 RNase digest of 35 S poliovirus RNA and incubated with 2.5 units of Fraction IIIN unlinking enzyme. Both this sample and one incubated in the absence of the enzyme were analyzed by paper ionophoresis after proteinase-K digestion. The sample incubated without enzyme contained ³H migrating near the position of the XC dye marker (Fig. 8A). This material represented the [³H]tyrosine-containing K-peptide linked to the 5'-terminal T1 RNase-resistant oligonucleotide (Flanegan *et al.*, 1977; Nomoto *et al.*, 1977b; Ambros *et al.*, 1978). Much of the ³H in the sample incubated with enzyme migrated back from the origin (toward the cathode), consistent with the behavior of the free K-peptide (Ambros and Baltimore, 1978) (Fig. 8B). Some of the ³H remained in K-peptide attached to the oligonucleotide. Thus, the purified unlinking enzyme could cleave the protein-nucleic acid linkage between VPg and the 5'-terminal T1 oligonucleotide of poliovirus RNA although the reaction was incomplete even after extensive treatment.

To test whether the enzyme could cleave VPg from mononucleotide linkage, [³H]tyrosine-labeled VPg-pUp and VPg-pU were generated and tested as substrates. Treated and untreated samples were analyzed by paper ionophoresis after digestion with proteinase-K. As a control, [³H]tyrosine-labeled intact RNA was digested and shown to liberate free K-peptide (Fig. 8C). In the samples with VPg-pUp, treated and untreated material migrated coincidentally (Fig. 8, *D* and *E*) at the position shown previously to correspond to K-pUp, thus, VPg-pUp was not a substrate for the unlinking enzyme. Similarly, no free K-peptide was released from VPg-pU after digestion because the digestion product had the characteristic migration rate for K-pU (Ambros and Baltimore, 1978) (Fig. 8, *F* and *G*).

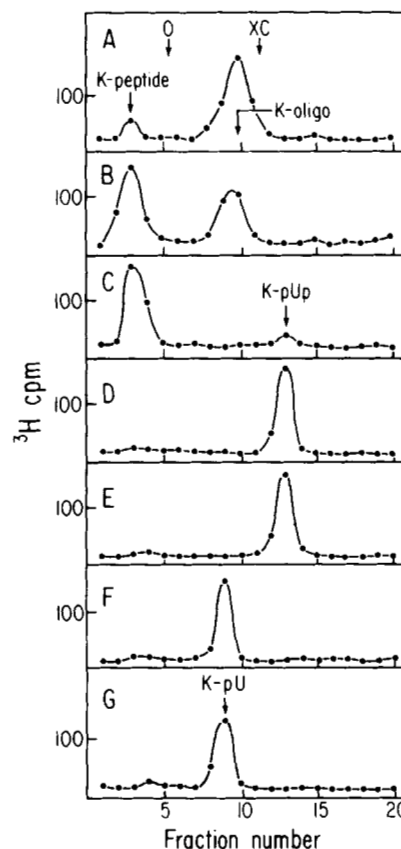For the previous experiments, the substrates were generated

FIG. 8. **Paper ionophoresis of enzymatic digestion products of [³H]tyrosine-labeled poliovirus RNA.** [³H]Tyrosine-labeled VPg attached to the 5'-terminal T1 RNase-resistant oligonucleotide of poliovirus RNA (VPg-oligo), VPg-pUp, and VPg-pU were generated by ribonuclease digestion of poliovirus RNA as follows. VPg-oligo was purified by cellulose acetate electrophoresis as described previously (Flanegan *et al.*, 1977). VPg-pUp was produced by treating 0.4 pmol (1 µg) of 35 S poliovirus RNA in TMMG with 0.1 µg each of ribonucleases T1, T2, and A for 1 h at 37°C. VPg-pU was generated from 0.4 pmol of RNA with 0.2 µg of P1 nuclease for 1 h at 37°C. Without further treatment, these samples were assayed for unlinking activity by adding 2.5 units of Fraction IIIN or an equivalent volume of buffer and incubating 18 h at 30°C. VPg concentration in all reactions was 40 nM. All samples were then digested with 10 µg of proteinase-K for 1 h at 37°C and analyzed by 3MM pH 3.5 paper ionophoresis as described under "Materials and Methods." *A*, VPg-oligo control; *B*, VPg-oligo treated with unlinking enzyme; *C*, 35 S poliovirus RNA treated with unlinking activity; *D*, VPg-pUp control; *E*, VPg-pUp treated with unlinking enzyme; *F*, VPg-pU control; *G*, VPg-pU treated with unlinking enzyme. *O*, origin; *XC*, xylene cyanol dye marker. Cathode was on the left. K-peptide, K-oligo, K-pUp, and K-pU mark the positions of the protease-K digestion products of VPg, VPg-oligo, VPg-pUp, and VPg-pU, respectively (Ambros *et al.*, 1978).

by appropriate enzyme digestions and then tested without further purification because of the difficulty of handling VPg. To test whether the residual enzymes or nucleotides might have been inhibitory, electrophoretically purified K-pUp was prepared and tested as a substrate for the unlinking enzyme. No digestion of this substrate could be shown (data not presented).

The fact that the electrophoretic mobility of VPg-pUp was not altered by unlinking enzyme also indicates that the enzyme does not contain appreciable phosphomonoesterase activity because the 3'-phosphate of VPg-pUp should be sensitive to such an activity. Phosphate was also not released from *p*-nitrophenyl phosphate by unlinking enzyme under standard assay conditions (data not shown).

DISCUSSION

We have described here the purification from HeLa cells of an enzyme that cleaves the tyrosine-phosphate bond linking VPg to poliovirus RNA. Both cytoplasmic and nuclear extracts yielded enzymes with identical chromatographic behavior but 3- to 4-fold more activity was found in the nuclear extract than in the cytoplasm. During the first chromatography step, using DEAE-Sephadex, a large amount of apparent unlinking activity was discarded, but most of that represented nucleases and proteases that can be confused with unlinking activity when a simple phenol extraction assay is used.

We cannot be at all sure, however, that the enzyme we have purified carries out the unlinking reaction in the cell. Until specific inhibitors of the enzyme are developed or mutants can be isolated, this will be a difficult question to answer. Two characteristics of the enzyme raise doubts about whether it interacts with poliovirus RNA in the cell. First, poliovirus replicates in the cytoplasm, but the bulk of the unlinking activity is recovered from a nuclear fraction of HeLa cells. Second, the very low specific activity of the purified enzyme, which is not a consequence of large losses of activity during purification, suggests that our assay may only be measuring a minor activity of the enzyme. Neither of these characteristics, however, argues compellingly that the enzyme we have isolated is not the one that unlinks VPg from poliovirus RNA in the cell. First, cell fractionation studies cannot give definitive evidence about intracellular location of enzymes. The minor fraction of unlinking activity in cytoplasmic extracts is sufficient to explain the removal of VPg from poliovirus RNA as shown by our previous work with crude extracts (Ambros *et al.*, 1978). Second, the low specific activity could well mean that in the uninfected cell the enzyme has a role quite different from the activity we measure on poliovirus RNA. Nonetheless, this may be the enzyme responsible for unlinking VPg from poliovirus RNA. Although the calculated turnover number of the enzyme was low, the K_m indicated a high affinity for poliovirion RNA. Hopefully, further study of its activity on a variety of substrates will define the preferred activity of the enzyme and thus give some insight into its role in the uninfected cell. Even if the enzyme does not cleave VPg from poliovirus RNA in cells, it is a unique enzyme with very interesting and unusual specificity.

Because the unlinking activity purified from the nuclear wash contained a single major polypeptide of molecular weight 27,000, we assumed that this represents the unlinking enzyme itself. The enzyme does sediment in a glycerol gradient at a rate consistent with such a small size and because our purifi-

cation did not use a sizing step, fortuitous purification of a low molecular weight polypeptide would not be expected. The low specific activity could be explained, however, if the molecular weight 27,000 polypeptide were a contaminant and, therefore, further study of this possibility is warranted.

Whatever the role of this enzyme in the cell, its specificity as revealed by the few substrates we have tested suggests that it interacts with nucleic acids. Because mononucleotides linked to VPg were not released, the enzyme is not a simple phosphodiesterase. Even its activity on a nonanucleotide linked to VPg was less complete than its activity on viral RNA, suggesting that it requires a long polynucleotide for maximal activity. The enzyme cleaved a small peptide from viral RNA as easily as it removed intact VPg from RNA; thus, it shows less interaction with the protein side of the bond it hydrolyzes than it does with the polynucleotide side of the bond.

Because the unlinking activity is found in uninfected cells, it is possible that protein-nucleic acid linkages via phosphodiester bonds to tyrosine may be found in normal cells, and that poliovirus uses for maturation of its mRNA a cellular enzyme normally involved in the cleavage of such linkages. Covalent linkages of nucleic acid to tyrosine are known to exist in bacteria. *Escherichia coli* glutamine synthetase and its regulatory protein pII are adenylated and uridylated, respectively, via a 5'-phosphodiester linkage to tyrosine (Adler *et al.*, 1975). *E. coli* ω protein binds to the 5'-phosphoryl end of a nick in duplex DNA via a similar bond.[2] Thus far, the picornavirus VPg-RNA bond is the only known example of a eukaryotic tyrosine-nucleic acid bond. Viral transforming proteins, however, are phosphorylated at tyrosine residues (Witte *et al.*, 1980; Hunter and Sefton, 1980) and the isolation of tyrosine-*O*-phosphate from *Drosophila* larvae has been reported (Mitchell and Lunan, 1964).

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[2] J. Wang, personal communication.