JOURNAL OF VIROLOGY, Oct. 1973, p. 827-835
Vol. 12, No. 4

Printed in U.S.A.

Polypeptide Composition of Influenza B Viruses and Enzymes Associated with the Purified Virus Particles

J. S. OXFORD[1]

Department of Microbiology, The John Curtin School of Medical Research, The Australian National University, Canberra, Australia

Received for publication 25 April 1973

Influenza B/LEE/40, B/Rome/1/67, B/Hong Kong/8/73, and B/Victoria/98926/70 viruses have a similar polypeptide composition as analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. These viruses are composed of six or seven polypeptides, depending on whether one or two high-molecular-weight polypeptides are resolved, ranging in molecular weights from 27,000 to 90,400. Three of these polypeptides, namely the heavy and light hemagglutinin chains and the neuraminidase, have attached carbohydrate. Highly purified influenza B/LEE/40 and B/Rome/1/67 virus preparations have RNA-dependent RNA polymerase activity equivalent to the incorporation of 100 and 30 pmol, respectively, of ^{3}H-UMP per mg of virus protein per h at 37 C, which is demonstrated only in detergent-treated virus suspensions. However, no RNA-dependent DNA polymerase enzyme activity was detected in the two viruses although virus suspensions were "activated" by heat, α-chymotrypsin, and detergents. Other enzymatic activities were associated with purified preparations of influenza B virus and were attributed to minor contamination of virus with host cell enzymes. Thus, nucleoside and deoxynucleoside phosphohydrolase enzymes were active in the absence of detergents and catalyzed the release of 1,200 and 1,800 nmol of P_i per mg of virus protein in 30 min at 37 C from ATP and dATP substrates. Thin-layer chromatography indicated that the products of the phosphohydrolase enzymes of influenza B/LEE/40 were mainly nucleoside diphosphate and monophosphate. The latter enzymes were tightly bound to influenza B/LEE/40 virus and could not be removed completely by repeated centrifugation, including centrifugation of the virus to equilibrium in density gradients of 25 to 40% (wt/vol) cesium chloride. A low degree of RNase (approximately 0.01 μg% contamination) and phosphatase (10-30 nmol of P_i released per mg of virus protein per 30 min) activity was detected in some, but not all, influenza B/LEE/40 virus preparations.

Several recent studies have described the polypeptide composition of influenza A strains (5, 13, 18, 24, 26, and summarized in 12) and the molecular weights of the six to eight polypeptides constituting the virus range from 21,000 to 94,000 daltons. Much less is known about the protein composition of influenza B virus (8, 16, 18). Influenza A and B virus strains have a similar morphology but are not related serologically and differ markedly in their epidemiology, including the absence of influenza B viruses in animal hosts other than man (10, 30).

The present study describes the polypeptide composition of two strains of influenza B virus, B/LEE/40 and B/Rome/1/67, which are distantly related by serological tests (2) and attempts to correlate the polypeptides with biologically active proteins of the virus. Also, the association of certain enzymes with the purified preparations of the two influenza B viruses is investigated. Two enzymatic activities have been described as closely associated with influenza A and B virus particles, namely neuraminidase (30) and RNA-dependent RNA polymerase enzyme (4, 22, 25). The presence of other enzymes such as RNase, nucleotide phosphohydrolases, and nucleic acid polymerases as detected in certain myxoviruses and paramyx-

[1] Present address: Division of Virology, National Institute for Medical Research Mill Hill, London NW7 1AA, England.

oviruses (20, 23, 27), reovirus (11), vaccinia (7), and Rous sarcoma virus (19), either as adsorbed cellular contaminants or as virus-coded enzymes, could markedly affect studies of product RNA synthesized in vitro and also enzyme kinetics of the influenza B virus particle-associated, RNA-dependent RNA polymerase. Such studies are being undertaken in this laboratory to determine the mode of action of a series of inhibitors of influenza RNA-dependent RNA polymerase (22).

MATERIALS AND METHODS

Viruses. Influenza strains B/LEE/40 and B/Rome/1/67 were supplied by W. G. Laver, Department of Microbiology, John Curtin School of Medical Research, Canberra, Australia. Influenza B/Hong Kong/8/73 and B/Victoria/98926/70 were supplied by G. C. Schild, National Institute for Medical Research, Mill Hill, London NW7.

Virus purification. Influenza viruses were grown in 10-day-old embryonated hen eggs and purified as described previously (15) with the addition of an equilibrium sedimentation of the virus in potassium tartrate (26). Such standard preparations of influenza B/LEE/40 virus were purified from a specific activity in crude allantoic fluid of 0.87×10^{-3} hemagglutination (HA) units/mg of protein to 357.1×10^{-3} HA units/mg of protein with a virus hemagglutinin yield of approximately 60% of the starting material.

RNA polymerase test. The reaction mixture (100 μliters) contained 0.05 mM GTP, ATP, CTP, 0.005 mM ^{3}H-UTP, (approximately 7.5 μCi), 0.2% (wt/vol) Nonidet P-40, and 50 μg of purified influenza virus protein in 50 mM Tris-hydrochloride buffer (pH 8.0), and the test was carried out as described previously (22).

Chemicals. Unlabeled deoxy- and ribonucleoside triphosphates and TTP-*methyl*-^3H (16.8 Ci/mmol) were purchased from Schwartz-Mann, Orangeburg, N. Y., and ^{3}H-UTP (15.9 Ci/mmol) from New England Nuclear, Boston, Mass. RNA polymerase from *Escherichia coli* K-12 and DNA polymerase from *Micrococcus lysodeikticus* were purchased from Miles Laboratories Inc., Elkhart, Indiana.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out as described by Skehel and Schild (26). In some experiments the high-pH Tris-borate buffer system of Laver, Wrigley, and Pereira was used (17). For analysis of biological functions of virus proteins, virus was disrupted with 2% sodium dodecyl sulfate (SDS) and virus proteins were separated on cellulose acetate strips in Tris-boric acid-EDTA buffer at pH 9 containing 0.4% SDS and then precipitated with ethanol (14) before electrophoresis in polyacrylamide gels. Standard proteins used in co-migration experiments were ovalbumin crystallized two times, lactate dehydrogenase, and chymotrypsinogen A (Worthington Biochemical Corp.), and pyruvate kinase type 11 from rabbit skeletal muscle (Sigma Chemical Co., St. Louis).

Nucleotide phosphohydrolase activity. The reaction mixture (100 μliters) contained 6 mM Mg^{2+}, 50 mM Tris-hydrochloride buffer (pH 8.0), and 0.5 mM nucleotide tri-, di-, or monophosphate. Approximately 100 μg of virus protein was added, and the mixture was incubated at 37 C for 30 min. Increase in the concentration of free phosphate compared to unincubated control preparations was estimated in duplicate tubes by use of standard techniques (3). In some experiments ^{3}H-UTP (15.9 Ci/mmol) was used as a substrate; and conversion to ^{3}H-UDP or ^{3}H-UMP was determined by thin-layer chromatography.

Thin-layer chromatography of nucleotide triphosphates. The solvent was *n*-butanol (35 vol), acetone (25 vol), acetic acid (15 vol), 5% ammonium hydroxide (15 vol), and water (10 vol). Precoated silica gel F_{254} plates (5 by 20 cm) with fluorescent background (E. Merck AG, Darmstadt, Germany) were spotted with approximately 10 μg (in 2 μliters) of nucleotide, and the plates were developed vertically for 4 h at room temperature.

RNase activity. Half-confluent Vero cells were incubated for 4 h in medium containing 5 μCi of uridine-5-^3H per ml (sp act 22.8 Ci/mmol). The cells were lysed with 0.1% (wt/vol) SDS, and the tritium-labeled RNA was immediately extracted using the hot phenol-SDS method, reprecipitated two times with cold ethanol, and stored at −20 C. To detect RNase activity, 100 μg of virus protein was added to 100 μliters of 50 mM Tris-hydrochloride buffer (pH 8.0) containing 10,000 counts/min of ^{3}H-RNA, samples were removed at time 0, and the reaction mixtures were then incubated for 30 min at 37 C. Portions (20 μliters) were pipetted onto paper disks and washed in trichloroacetic acid, and acid-precipitable counts were determined by liquid scintillation spectrometry (22). Parallel tubes contained concentrations of RNase from 0.0001 to 10 μg, and from the calibration curve the comparative amount of RNase activity present in the sample was calculated.

RESULTS

Polypeptide composition of influenza B viruses. The influenza B viruses were purified, disrupted by SDS and β-mercaptoethanol, and subjected to electrophoresis under reducing conditions in SDS polyacrylamide gels as described (26). Similar gel patterns, both in the number of staining bands, the percentage of bound stain, and the relative rates of polypeptide band migrations, were obtained for influenza B/LEE/40, B/Hong Kong/8/73, B/Victoria/98926/70 and influenza B/Rome/1/67. Six or seven bands were detected in these gels (Fig. 1), and the approximate molecular weights (Table 1) were estimated by co-migration of standard proteins in parallel gels. Identical results were obtained when virus polypeptides were carboxymethylated (6) to reduce any possibility of reassociation during electrophoresis by disulfide bond formation. Also, no difference in polypeptide composition was detected between freshly harvested, purified influenza virus and virus which had been stored at 4 C in

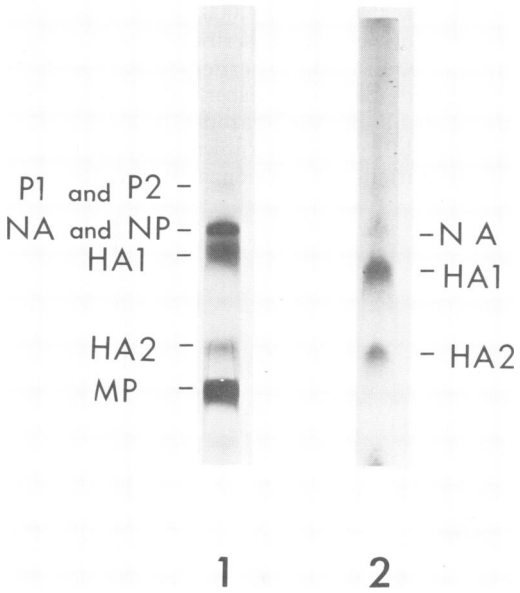

FIG. 1. *Polyacrylamide gel electrophoresis of influenza B/LEE/40 virus polypeptides. 1, Virus polypeptides stained by Coomassie blue. 2, Virus polypeptides stained by the periodic acid-Schiff test for glycoproteins. Virus proteins were solubilized in SDS and β-mercaptoethanol and subjected to electrophoresis as described (26). Samples were added at the top of the gels in the figure. Note that in gel 1 the neuraminidase cannot be distinguished from the NP band. In most experiments the neuraminidase electrophoresed as a minor band ahead of the NP. P1 and P2 were present as two closely spaced bands as seen by eye, although in some experiments only a single polypeptide band was discernible.*

TABLE 1. *Polypeptide composition of influenza B/LEE/40 virus by SDS polyacrylamide gel electrophoresis*

Mol wt of polypeptides[a] ($\times 10^{-3}$)	% of bound stain	Presence of carbohydrate	Identification
90.4 ± 1.1	2.7	0	P
69.1 ± 0.9	26.9	0	NP
63.3 ± 1.2	5.1	+	Neuraminidase
56.2 ± 0.9	21.7	+	HA1
29.8 ± 1.0	11.7	+	HA2
27.1 ± 1.0	32.4	0	MP

[a] Average molecular weights and standard errors from 10 experiments. Molecular weight estimates of the glycoproteins in particular should be regarded as approximate only, since the effects of glycosylation on the migration of polypeptides in these gels is not known.

0.08% (wt/vol) sodium azide for 3 weeks.

To correlate the polypeptide bands detected in SDS polyacrylamide gels with the biological activities of different structural components of the virus, influenza B/LEE/40 virus was disrupted first with 2% SDS and subjected to electrophoresis on cellulose acetate strips at room temperature for 5 h. When the strips were stained for protein by use of Coomassie blue, three separate bands were detected migrating towards the anode (Fig. 2), whereas a single band migrated towards the cathode as described previously (14). The latter protein band (Fig. 2, band 4) was identified as the virus neuraminidase by testing the eluates from the strips by the method of Warren (29). The protein eluates from the separated bands were precipitated with 3 vol of ethanol and subjected to electrophoresis in SDS polyacrylamide gels under reducing conditions. The neuraminidase thus eluted from the cellulose acetate strips gave a single band in polyacrylamide gels migrating at a rate corresponding to a polypeptide of molecular weight 63,300.

Two bands were obtained in polyacrylamide gels from the cellulose acetate fraction 3 (Fig. 2) migrating slowly towards the anode, with molecular weights of 56,200 and 29,800 (Fig. 3B). As detected by staining with Coomassie blue and subsequent densitometry tracing (Fig. 4), the latter two polypeptides were present in a ratio of approximately 2:1 and were further identified, together with the neuraminidase band at 63,300 daltons, as glycoproteins by the periodic acid-Schiff test. The two bands thus had very similar characteristics to the heavy and light chains of influenza hemagglutinin as shown by others for influenza A strains (16, 26).

The polypeptide at 69,100 daltons was identified as ribonucleoprotein (NP), since NP prepared from influenza virus-infected egg allan-

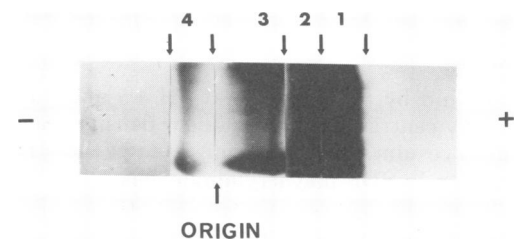

FIG. 2. *Electrophoretic separation of influenza B/LEE/40 virus proteins on cellulose acetate. Virus was disrupted with 2% SDS and virus proteins separated on cellulose acetate strips in Tris-boric acid-EDTA buffer (pH 9) containing 0.4% SDS at 10 V/cm for 5 h at room temperature. Strips were stained in a solution of Procion brilliant blue dye (0.5%, wt/vol) and hydrochloric acid (1%, vol/vol) in methanol. For preparative studies the strips were cut as illustrated by the arrows and the proteins were eluted into deionized water at 4 C for 12 h.*

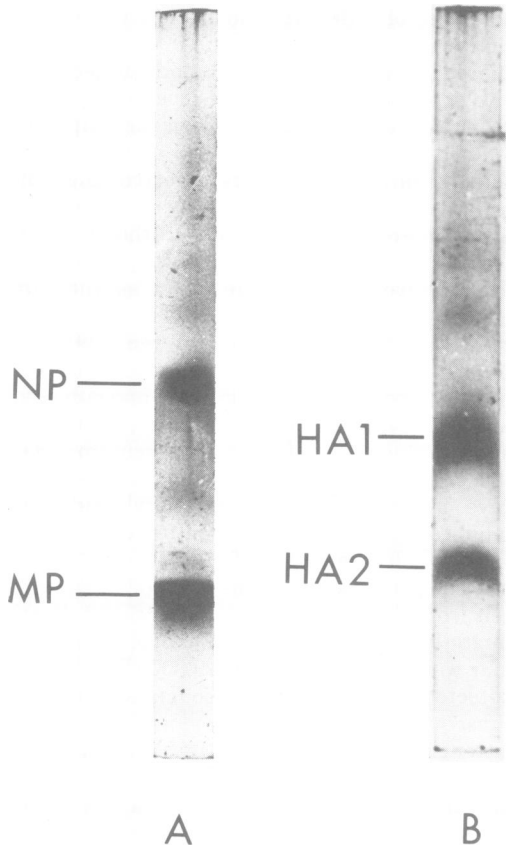

FIG. 3. *Polyacrylamide gel electrophoresis of influenza B/LEE/40 proteins eluted from cellulose acetate strips. A, Gel pattern of fractions 1 and 2 eluted from the cellulose acetate strip. B, Gel pattern of influenza B/LEE virus polypeptides eluted from fraction 3 of the cellulose acetate strip. Virus proteins were eluted from the cellulose acetate strip illustrated in Fig. 2 precipitated with 3 vol of ethanol to concentrate and then solubilized in SDS and β-mercaptoethanol as described (26). Samples were added to the top of the gels in the figure.*

toic fluid by precipitation at pH 4.5 and purified by centrifugation in a linear density gradient of cesium chloride (8) migrated at the same position in SDS polyacrylamide gels.

The band in closest proximity to the anode in the cellulose acetate strips was demonstrated by polyacrylamide gel electrophoresis to have a molecular weight of 27,100. As detected by densitometry (Fig. 4) of stained gels, it was also the most abundant polypeptide in the virus particle and appeared to be identical in molecular weight, absence of carbohydrate, and behavior on cellulose acetate to the matrix protein (MP) of influenza A viruses (12).

Two closely spaced polypeptides were detected at the top of the gel with molecular weights of 90,400 and 94,000, similar to the P1 and P2 polypeptides described for influenza A virus (12). In some experiments only a single polypeptide band was discernible in this region of the gel, and the reasons for this lack of reproducibility are not known at present.

Preparations of influenza B/LEE/40 virus

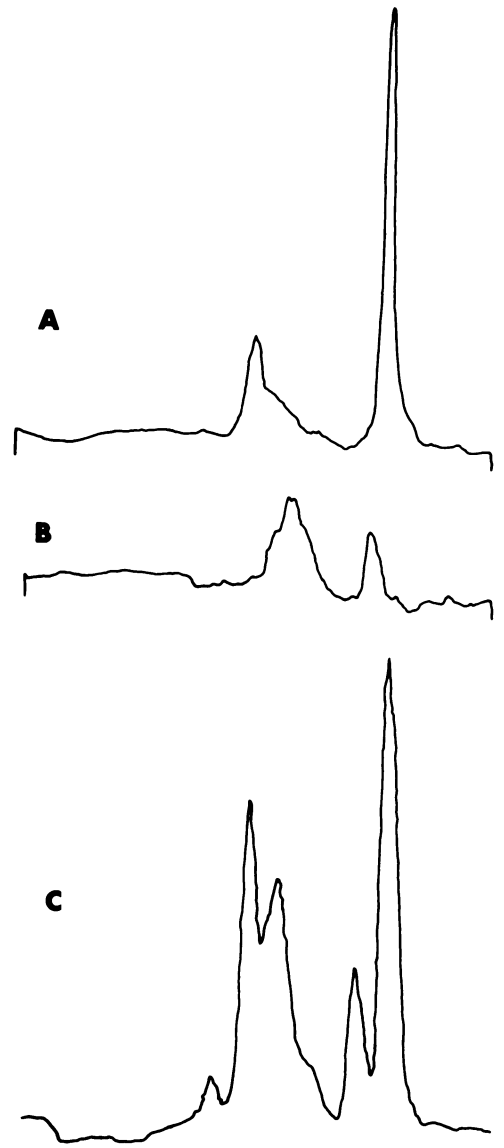

FIG. 4. *Densitometer tracings of Coomassie blue-stained polyacrylamide gels of influenza B/LEE/40 virus polypeptides. A, Tracing of gel A from Fig. 3 showing MP and NP polypeptides. B, Tracing of gel B from Fig. 3 showing HA1 and HA2 polypeptides. C, Tracing of gel 1 from Fig. 1 showing (from left to right) polypeptides P, NP, HA1, and neuraminidase, HA2, and MP. Note that neuraminidase and also P1 and P2 are incompletely resolved in the tracing.*

showing only six or seven polypeptide bands by electrophoresis in polyacrylamide gels, depending on the resolution of P1 and P2 polypeptides, and no other contaminating polypeptides detectable by this technique were used for the enzyme experiments described below.

Nucleoside phosphohydrolase activity associated with influenza B virus particles. Purified preparations of influenza B/LEE/40 and B/Rome/1/67 viruses had deoxynucleoside triphosphatase and ribonucleoside triphosphatase and diphosphatase activities (Table 2). Results in Table 2 were obtained with influenza B/LEE/40 virus, but influenza B/Rome/1/67 released approximately 1,000 nmol of inorganic phosphorus per mg of virus protein in 30 min at 37 C from uridine triphosphate substrate. The coincidence of UTPase activity with purified virus is shown in Fig. 5. In contrast, phosphatase activity using UMP as substrate was only detected at relatively low levels (10–30 nmol of P_i released per mg of protein per 30 min) in three out of five B/LEE/40 virus preparations.

Further purification of influenza B/LEE/40 virus by centrifugation to equilibrium in 25 to 40% (wt/vol) cesium chloride for 20 h at 22,000 rpm in the 27.1 rotor, removal of the virus band at a density of 1.25 g/cm^3, and overnight dialysis to remove cesium chloride resulted in a 50% decrease in ribonucleoside triphosphatase activity of one virus preparation, the release of 1,200 to 600 nmol of inorganic phosphorus per mg of virus protein in 30 min at 37 C from uridine triphosphate substrate, and a 10% decrease in a second virus preparation. However, the continued presence of nucleoside phosphohydrolase activity after centrifugation in high-molarity cesium chloride indicated that the enzyme(s) was tightly bound to the virus.

In further experiments the products of the nucleoside phosphohydrolase enzyme activity associated with a suspension of B/LEE/40 virus, additionally purified by equilibrium centrifugation in 25 to 40% (wt/vol) cesium chloride, were examined by thin-layer chromatography (Fig. 6). Uridine triphosphate was converted to uridine diphosphate and monophosphate by the virus-associated enzyme, and after 1 h of incubation at 37 C, 73.5% of the counts were detected as UDP or UMP.

TABLE 2. *Association of nucleoside and deoxynucleoside phosphohydrolase activity with purified preparations of influenza B/LEE/40 virus*

Nucleoside	P_i released from substrate[a]		
	Deoxynucleoside triphosphate	Ribonucleoside triphosphate	Ribonucleoside diphosphate
Guanosine	880	1,040	280
Cytidine	1,320	1,240	480
Uridine		1,400	560
Thymidine	1,400		
Adenosine	1,800	1,200	560

[a] Measured in nanomoles per milligram of virus protein per 30 min.

The addition of a twofold excess of unlabeled GTP, CTP, or ATP to the ^{3}H-UTP-containing reaction mixture (Table 3) partially inhibited enzymatic conversion of ^{3}H-UTP to UDP or UMP, suggesting that a single reaction site is present on the virus-associated enzyme(s) for these nucleotide triphosphates.

RNase activity associated with influenza B/LEE/40 virus particles. Variable degrees of RNase-activity were detected in four different purified influenza B/LEE/40 virus samples tested. One virus preparation had no detectable RNase activity at a virus concentration of 100 μg in the reaction mixture, whereas the highest virus-associated enzymatic activity was equivalent to 0.01 μg of added RNase per reaction mixture containing 100 μg of influenza B virus protein. Approximately 0.003 μg of RNase was detected in the other two virus preparations tested. Approximately a twofold increase in RNase activity was detected when the virus particles were partially disrupted by the addition of 0.1% (vol/vol) Nonidet P-40 detergent.

RNA-dependent RNA polymerase activity associated with influenza B virus particles. The strain of influenza B/LEE/40 tested incorporated approximately 100 pmol of ^{3}H-UMP per mg of virus protein per h at 37 C. Similar activity was obtained when virus was purified by differential centrifugation followed by centrifugation to equilibrium in linear gradients of 40 to 60% (wt/vol) sucrose, or purified by ammonium sulfate precipitation (8) followed by rate zonal centrifugation in linear gradients of 10 to 40% (wt/vol) sucrose. However, virus purified by adsorption and elution from red blood cells followed by rate zonal centrifugation in sucrose and finally equilibrium centrifugation in potassium tartrate showed less tendency to aggregate on storage at 4 C and was used for the remaining experiments. Influenza B/Rome/1/67 virus incorporated 30 nmol of ^{3}H-UMP per mg of virus protein per h at 37 C.

Suspensions of purified influenza B/LEE/40 virus could be stored at 4 C in the presence of sodium azide without significant decrease in RNA-dependent RNA polymerase activity for periods of 30 days or longer. However, 50% inhibition of RNA-dependent RNA polymerase activity was obtained when 10 mM sodium

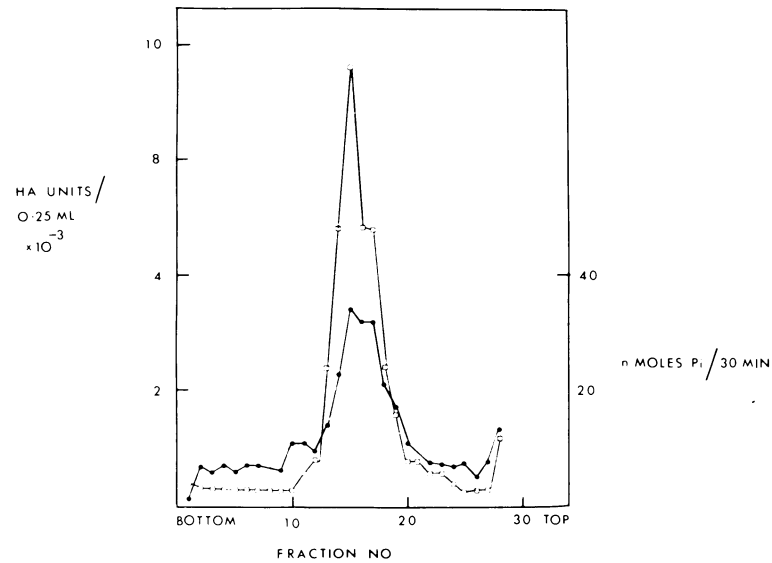

FIG. 5. *Association of UTPase activity with purified influenza B/LEE/40 virus. Symbols: ●, nanomoles of Pi released in 30 min; □, HA units. Influenza B/LEE/40 virus purified as described in Materials and Methods was further purified by rate zonal centrifugation in a linear 10 to 40% (wt/vol) sucrose gradient. A 0.3-ml amount of purified virus was layered on the gradient and centrifuged for 50 min at 19,000 rpm in the 25.1 rotor of the Spinco ultracentrifuge. Fractions (1 ml) were collected by bottom puncture and titrated for hemagglutinin and UTPase activity.*

azide or 3 mM potassium cyanide were added to the reaction mixture at zero time before incubation at 37 C.

A range of detergents was tested for ability to activate the virus particle polymerase enzyme since no polymerase activity was detected in the absence of detergents in the reaction mixture (Table 4). Optimum RNA polymerase activity was detected with the addition of 0.2% (wt/vol) of Nonidet P-40.

Absence of influenza B/LEE/40 and B/Rome/1/67 virus particle-associated DNA polymerases. No incorporation of ^{3}H-TMP into acid-precipitable product was detected with the addition to the reaction mixture of 200 μg of protein from three freshly purified preparations each of influenza B/LEE/40 virus and B/Rome/1/67/virus. Similar negative results were obtained from two preparations of influenza A/WSN/34 (HON1) virus. Treatment of virus particles in the reaction mixture with 0.1% Nonidet P-40, 4 μg of α-chymotrypsin, or heat shock (50 C for 2 min) failed to activate any DNA polymerase activity (Table 5). The experiments were controlled by use of DNA polymerase from *M. lysodeikticus* which incorporated 82.1×10^4 disintegrations per min of ^{3}H-TMP per mg of protein per h by use of the same reaction mixture, as described previously (22). Addition of 100 μg of influenza B/LEE/40 virus protein to the complete *M. lysodeikticus* reaction mixture resulted in 56.5% decrease in incorporation of ^{3}H-TMP, suggesting the presence of DNase in the influenza virus preparations. However, rebanding of influenza B/LEE/40 virus preparations by rate zonal centrifugation in 10 to 40% sucrose, while decreasing DNase activity fivefold, did not result in the appearance of DNA polymerase activity in the virus-containing reaction mixtures.

DISCUSSION

The results of the present study indicate that the number of polypeptides, the molecular weights, and the percentage contribution to the total protein of the influenza B/LEE/40, B/Hong Kong/8/73, B/Victoria/98926/70, and B/Rome/1/67 virus particles are mostly similar to those described for influenza A viruses (12). However, differences were detected in the estimated molecular weight of the NP which was 69,100 in the present study but 53,000 as described by Skehel and Schild for influenza A (26). Values of 60,000 (5, 13) and 64,000 (24) have been described by others for influenza A virus, and it is not clear at present whether these different estimates are a result of varying techniques or represent a true heterogeneity between influenza viruses for this polypeptide. However, experiments with the influenza A recombinant X-31 virus co-migrating with in-

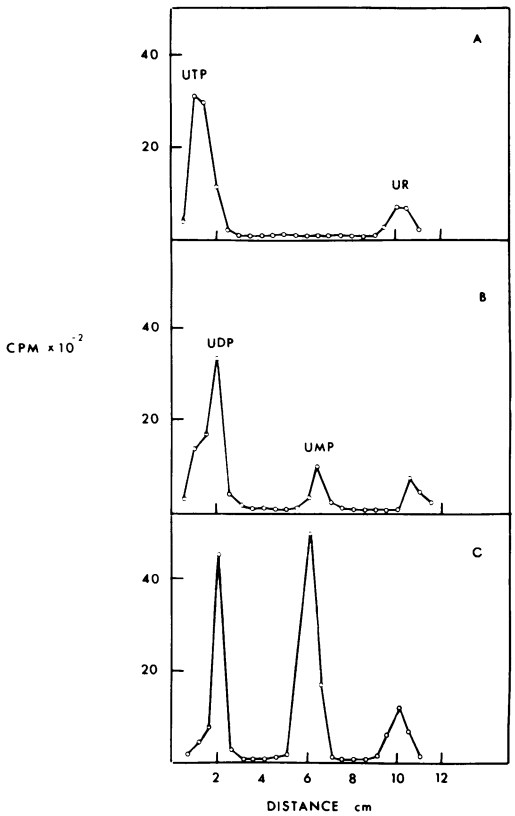

FIG. 6. *Thin-layer chromatography analysis of the reaction products of the UTPase enzyme associated with influenza B/Lee virus. A, Zero time. B, Time 10 min incubation at 37 C. C, Time 30 min incubation at 37 C. Influenza B/LEE/40 virus (10 μg) purified as described in Materials and Methods with a final banding to equilibrium in 25 to 40% (wt/vol) cesium chloride was incubated with 0.1 mM ^{3}H-UTP and 6 mM Mg^{2+} in 50 mM Tris-hydrochloride buffer (pH 8.0) at 37 C. At varying time periods 5 μliters of the reaction mixture was spotted onto silica gel plates together with "cold" nucleotide tri-, di-, and monophosphates as markers, and the plates were developed for 4 h with a solvent of n-butanol (35 vol)-acetone (25 vol)-acetic acid (15 vol)-5% ammonium hydroxide (15 vol)-water (10 vol). Spots were located with a UV light and removed by scraping into a toluene scintillation mixture containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2-(5-phenyloxazolyl)benzene for liquid scintillation spectrometry.*

fluenza B/LEE/40 virus in polyacrylamide gels provided additional evidence for a difference in the molecular weights of the NP polypeptide of the viruses (J. S. Oxford, unpublished data). In these experiments the molecular weights of the NP of B/LEE/40 virus and X-31 virus was 69,000 and 54,000, respectively.

Haslam (8) detected three major and three minor polypeptides in a study of radioisotopically labeled polypeptides of influenza B/LEE/40 virus. More recently a neuraminidase subunit composed of four molecules of glycoprotein of 63,000 daltons has been proposed for influenza B/LEE/40 virus although these authors also detected a polypeptide of 56,000 daltons as a minor contaminant of neuraminidase preparations (18). Earlier studies in the same laboratory indicated that the neuraminidase of influenza B/LEE/40 virus had a molecular weight of 56,000 (8). The results of the present study agree closely with the molecular weight estimate of 63,000 for the neuraminidase polypeptide of influenza B/LEE/40 virus.

RNA-dependent RNA polymerase activity was detected in purified preparations of influenza B/LEE/40 and B/Rome/1/67 viruses, and the optimum conditions for the assay were similar to those described previously for influenza A strains (4, 22, 25). No activity was detected unless detergent was present, whereas enzyme activity was still present in virus "cores" (J. S. Oxford, unpublished data) suggesting that the RNA polymerase enzyme activity was not simply an absorbed contaminant on the surface of the virus. No DNA polymerase activity was detected in the influenza B virus preparations, although a number of different methods were used in an attempt to activate a hypothetical DNA polymerase enzyme. In control experiments, high levels of DNA polymerase activity were detected in microbial DNA polymerase enzyme preparations, indicating that the technique used was relatively sensitive although it is possible that optimum conditions to detect a virus-associated DNA polymerase enzyme were not achieved. The inhibition of influenza virus multiplication in tissue culture by actinomycin D has implicated a role of DNA intermediates in the replication of the virus (1). In addition, an association of a reverse transcriptase with purified preparations of influenza A virus has been reported (W. W. Ackermann, S. T. Barker, B. A. Miller, and H. Kurtz, Bacteriol. Proc., p. 221, 1971), but the enzyme may have been an absorbed contaminant from tissue culture cells.

Other enzyme activities closely associated with the purified influenza B virus preparations were relatively high levels of nucleoside and deoxynucleoside phosphohydrolase activities. The nucleoside phosphohydrolase activity associated with influenza B virus has comparable activity to the enzyme(s) associated with reovirus (11). Also, the nucleoside triphosphatases from influenza B and reovirus have a low specificity and use UTP, ATP, CTP, or GTP as well as deoxynucleotide triphosphates as substrates. A major difference between the en-

TABLE 3. *Nucleoside phosphohydrolase activity of influenza B/LEE/40 virus particles*

Reaction mixture[a]	Time of incubation (min)	% of initial ^{3}H-UTP count[b] as:			
		UTP	UDP	UMP	Uridine
Complete + 10 μg B/LEE/40 virus protein	0	80.9	15.2	1.4	2.4
	30	31.0	22.8	41.7	4.5
Complete + 10 μg B/LEE/40 virus protein + 0.2 mM ATP	30	57.3	36.4	3.4	2.9
Complete + 10 μg B/LEE/40 virus protein + 0.2 mM CTP	30	55.8	35.6	5.3	3.3
Complete + 10 μg B/LEE/40 virus protein + 0.2 mM GTP	30	48.2	38.6	8.9	4.3
Complete + 10 μg B/LEE/40 virus protein + 0.2 mM UTP	30	56.8	34.6	4.9	3.7

[a] Complete reaction mixture contained 0.1 mM ^{3}H-UTP, 50 mM Tris-hydrochloride buffer (pH 8.0), 6 mM Mg^{2+}.

[b] Initial count of ^{3}H-UTP, approximately 20,000 counts/min.

TABLE 4. *Effect of detergents on RNA-dependent RNA polymerase activity of influenza B/LEE/40 virus particles*

Detergent	Optimum % (wt/vol)	^{3}H-UMP incorporated (dpm/mg of B/LEE virus protein/h $\times 10^{-3}$)
Nonidet P-40	0.2	104.8
Tween 20	0.2	40.4
Sodium deoxycholate	0.04	33.2
Sodium dodecyl sulfate	0.02	14.8
Sarkosyl	0.04	12.4
Brij	0.04	40.4
None		1.2

TABLE 5. *Absence of RNA-dependent DNA polymerase activity in influenza B/LEE/40 virus particles*

Reaction mixture	^{3}H-TMP incorporated (dpm/mg protein/h $\times 10^{-4}$)
Complete + DNA polymerase[a] + DNA (6 μg)[b]	82.1
Complete + DNA polymerase	0
Complete + DNA polymerase + DNA (6 μg) + B/LEE virus (100 μg)	35.7
Complete + B/LEE virus (200 μg) + 0.1% Nonidet P-40	0.04
Complete + B/LEE virus (200 μg) + DNA[b] (6 μg)	0.01
Complete + B/LEE virus (200 μg) + yeast RNA (1 μg)	0.03
Complete + B/LEE virus (200 μg) + heat shock (2 min at 50 C)	0
Complete + B/LEE virus (200 μg) + α-chymotrypsin (4 μg) + DNA (6 μg)	0

[a] DNA-dependent DNA polymerase from *M. lysodeikticus*.

[b] Calf thymus DNA.

zymes of influenza B virus and reovirus is that "activation" of enzyme is required for reovirus and the enzyme is associated with reovirus core proteins. For the latter virus the presence of phosphohydrolase enzymes destroying RNA polymerase substrates has been suggested as a possible mechanism for regulating RNA polymerase activity (11).

A low degree of pyrophosphatase activity and a low degree of RNase activity was detected in a proportion of influenza virus preparations tested. These enzyme activities, with the exception of RNA-dependent RNA polymerase, were all present in uninfected egg allantoic fluid, and therefore the presence of enzyme activity in even highly purified preparations of influenza virus could result from minor contamination. Only contamination in excess of 0.5 to 1.0% would be detectable by Coomassie blue-stained gels of polyacrylamide, and although studies with influenza A virus have demonstrated that highly purified virus particles contain less than 1% host cell protein (9) even this degree of contamination could result in significant amounts of cell-derived enzyme activities. Further, the phosphohydrolase and RNase enzymes appeared to be located on the influenza virus surface, since disruption of virus particles by detergents, for example, was not required for activity to be detected, again suggesting their origin as cell-derived contaminants. It has been demonstrated that highly purified poxvirus preparations can adsorb DNA polymerase from cytoplasmic extracts of cells containing the enzyme (28). Others have described ATPase activ-

ity associated with partially purified preparations of myxoviruses (20, 21), and these viruses are known to incorporate fragments of cell membrane in the virus particle coat (9). Detailed serological and enzyme kinetic studies using solubilized phosphohydrolase enzyme(s) will be required to determine unequivocally whether the enzyme is a cell-derived contaminant or is coded by the virus genome.

ACKNOWLEDGMENTS

I would like to thank Gertraud Stange for excellent technical assistance and G. C. Schild and W. G. Laver for supplying influenza virus strains.

LITERATURE CITED

1. Barry, R. D., D. R. Ives, and J. G. Cruickshank. 1962. Participation of deoxyribonucleic acid in the multiplication of influenza virus. Nature (London) **194**:1139-1140.
2. Chakraverty, P. 1971. Antigenic relationship between influenza B viruses. Bull. WHO **45**:755-766.
3. Chen, P. S., T. Y. Toribara, and H. Warner. 1956. Micro determination of phosphorus. Anal. Chem. **78**:1756-1758.
4. Chow, N-L., and R. W. Simpson. 1971. RNA-dependent RNA polymerase activity associated with virions and subviral particles of myxoviruses. Proc. Nat. Acad. Sci. U.S.A. **68**:752-756.
5. Compans, R. W., H. D. Klenk, L. A. Caliguiri, and P. W. Choppin. 1970. Influenza virus proteins. I. Analysis of polypeptides of the virion and identification of spike glycoproteins. Virology **42**:880-889.
6. Dimmock, N. J., and D. H. Watson. 1969. Proteins specified by influenza virus in infected cells: analysis by polyacrylamide gel electrophoresis of antigens not present in the virus particle. J. Gen. Virol. **5**:499-509.
7. Gold, P., and S. Dales. 1968. Localisation of nucleotide phosphohydrolase activity within vaccinia. Proc. Nat. Acad. Sci. U.S.A. **60**:845-852.
8. Haslam, E. A., A. W. Hampson, I. Radiskevics, and D. O. White. 1970. The polypeptides of influenza virus. III. Identification of the haemagglutinin, neuraminidase and nucleocapsid proteins. Virology **42**:566-575.
9. Holland, J. F., and E. D. Kiehn. 1970. Influenza virus effects on cell membrane proteins. Science **167**:202-205.
10. Hoyle, L. 1968. The influenza viruses. Virology monogr., vol. 4. Springer Verlag, Vienna.
11. Kapuler, A. M., N. Mendelsohn, H. Klett, and G. Acs. 1970. Four base specific nucleoside 5'-triphosphatases in the subviral core of reovirus. Nature (London) **225**:1209-1213.
12. Kilbourne, E. D., P. W. Choppin, I. T. Schulze, C. Scholtissek, and D. L. Bucher. 1972. Influenza virus polypeptides and antigens—summary of influenza workshop I. J. Infect. Dis. **125**:447-455.
13. Klenk, H. D., R. Rott, and H. Becht. 1972. On the structure of the influenza virus envelope. Virology **47**:579-591.
14. Laver, W. G. 1964. Structural studies on the protein subunits from three strains of influenza virus. J. Mol. Biol. **9**:109-124.
15. Laver, W. G. 1969. Purification of influenza virus, p. 82. *In* K. Hable and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
16. Laver, W. G., and N. Baker. 1972. Amino acid composition of polypeptides from influenza virus particles. J. Gen. Virol. **17**:61-67.
17. Laver, W. G., N. G. Wrigley, and H. G. Pereira. 1969. Removal of pentons from particles of adenovirus type 2. Virology **39**:599-605.
18. Lazdins, I., E. A. Haslam, and D. O. White. 1972. The polypeptides of influenza virus. VI. Composition of the neuraminidase. Virology **49**:758-765.
19. Mizutani, S., and H. M. Temin. 1971. Enzymes and nucleosides in virions of Rous sarcoma virus. J. Virol. **8**:409-416.
20. Neurath, A. R. 1965. Study on the adenosine diphosphatase (adenosine triphosphatase) associated with Sendai virus. Acta Virol. **9**:313-322.
21. Neurath, A. R., and F. Sokol. 1963. Association of myxoviruses with an adenosine diphosphatase and adenosine triphosphatase as revealed by chromatography on DEAE cellulose and by density gradient centrifugation. Z. Naturforsch. **18**:1050-1052.
22. Oxford, J. S. 1973. An inhibitor of the particle associated RNA-dependent RNA polymerase of influenza A and B viruses. J. Gen. Virol. **18**:11-19.
23. Rosenbergova, M., and S. Pristasova. 1972. Nuclease activity of large RNA viruses. Acta Virol. **16**:1-8.
24. Schulze, I. T. 1970. The structure of influenza virus. I. The polypeptides of the virion. Virology **42**:890-904.
25. Skehel, J. J. 1971. RNA-dependent RNA polymerase activity of the influenza virus. Virology **45**:793-796.
26. Skehel, J. J., and G. C. Schild. 1971. The polypeptide composition of influenza A viruses. Virology **44**:396-408.
27. Stone, H. O., A. Portner, and D. W. Kingsbury. 1971. Ribonucleic acid transcriptases in Sendai virions and infected cells. J. Virol. **8**:174-180.
28. Tan, K. B., and B. R. McAuslan. 1972. Binding of deoxyribonucleic acid-dependent deoxyribonucleic acid polymerase to poxviruses. J. Virol. **9**:70-74.
29. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **234**:1971-1975.
30. Webster, R. G., and W. G. Laver. 1970. Antigenic variation in influenza virus, p. 271. *In* J. L. Melnick (ed.), Progress in medical virology. S. Karger, Basel.