Proteins Specified by Influenza Virus in Infected Cells: Analysis by Polyacrylamide Gel Electrophoresis of Antigens Not Present in the Virus Particle

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SUMMARY

Five polypeptides were found when purified influenza virus was analysed by electrophoresis on polyacrylamide gel. Previous work has shown that hyperimmune convalescent antibody prepared in mice and adsorbed with purified influenza virus which had been disrupted with ether still gave precipitin lines in immunodiffusion tests with extracts of cells infected with influenza virus. These presumed non-structural antigens, labelled with radioactive amino acids, were precipitated with the virus-adsorbed antibody preparation. A comparison of the precipitate and purified virus by polyacrylamide gel electrophoresis revealed three polypeptides which differed from the polypeptides of the virion. These were probably virus specific non-structural proteins.

INTRODUCTION

Peritoneal fluids from hyperimmunized mice which had recovered from infection with influenza virus gave a number of precipitin bands in immunodiffusion tests with extracts of chick embryo cells infected with influenza virus. Some precipitin bands could still be demonstrated after the fluids had been extensively absorbed with disrupted virus and were therefore possibly formed by antigens not contained in the virus particle (non-structural antigens) (Dimmock, 1969). This conclusion may be contested on the grounds either of inefficient absorption or that virus particle precursors in the infected cell may possess antigenic determinants lacking in the complete particle.

Non-structural proteins specified by poliovirus have been detected by electrophoresis in polyacrylamide gels of infected cell extracts which were treated with detergent (Summers, Maizel & Darnell, 1965). These workers distinguished non-structural from structural proteins by co-electrophoresis of infected cell extracts with disrupted purified virus labelled with a different isotope.

The molecular weight of influenza virus ribonucleic acid is estimated to be \(2.7 \times 10^6\) (Pons & Hirst, 1968). It should therefore be possible to detect all of the proteins specified by this relatively small amount of nucleic acid in extracts of infected cells. Summers et al. (1965) were able to repress host cell protein synthesis with metabolic inhibitors such as actinomycin D before infection with poliovirus. This would not be suitable for influenza virus since its multiplication is inhibited by actinomycin D during the early part of the cycle (Barry, Ives & Cruickshank, 1962). We circumvented...
this problem by combining polyacrylamide gel electrophoresis with an immunological technique. Radioactively labelled virus-specific antigens were removed from extracts of infected cells by indirect antibody precipitation (Scharff, Shatkin & Levintow, 1963) and the resulting precipitate was electrophoresed together with purified virus labelled with a second isotope. Because we used the antibody preparation after absorption with partially disrupted purified virus, only the presumed non-structural antigens should have been precipitated. Before electrophoresis the precipitate was treated with sodium dodecyl sulphate, which has been shown to dissociate antigen-antibody complexes (Cebra, Givol & Katchalski, 1962). We were able to demonstrate that the precipitate contained proteins differing in size from the structural proteins of the virus.

METHODS

Cells. Chick embryo fibroblasts were prepared from 10- or 11-day-old embryos and grown in Eagle's medium containing 5% calf serum and 0.05% sodium bicarbonate.

Virus. The WSN of influenza A0 was used. Stocks were prepared by inoculating about 10^8 p.f.u. into the allantoic cavity of 11-day-old chick embryos and incubating them at 35°C for 1 day. Virus was concentrated by differential centrifugation and stored at -60°C.

Infectivity titrations. Chick embryo fibroblast monolayers were washed once to remove serum and inoculated with serial dilutions of virus. The monolayers were overlaid with 1% (w/v) agar in Hank's saline containing 0.1% lactalbumin hydrolysate and 0.01 M-tris and incubated at 35°C. Neutral red was added on the 3rd day and the plaques were counted on the 4th day after inoculation.

Haemagglutinin assay. Haemagglutinin (HA) was titrated by twofold dilutions in 0.25 ml. volumes in plastic trays and detected by the addition of 0.5% chicken erythrocytes. The end point was estimated by interpolation between complete agglutination and absence of agglutination.

Antibody. The preparation of hyperimmune convalescent peritoneal fluid against WSN in mice was described by Dimmock (1969). Mice were first infected with virus and subsequently inoculated at intervals by the intraperitoneal route with infected mouse lung tissue emulsified with Freund's complete adjuvant. This procedure stimulated the production of peritoneal fluid which was extracted by paracentesis. After the fluid had been repeatedly absorbed with purified WSN, which had been treated with ether to expose the internal antigen, no antibody to the haemagglutinin, neuraminidase and internal antigens of the virus could be detected (Dimmock, 1969). Peritoneal fluid absorbed in this way gave up to five precipitin bands with extracts of infected chick embryo fibroblasts. No precipitin lines were obtained with uninfected cells and the absorbed fluid did not react with uninfected cells in indirect fluorescent antibody staining.

Preparation of radioactive virus and antigen. Chick embryo fibroblast monolayers comprising about 2 x 10^8 cells were prepared in 140 mm. Petri dishes. The monolayers were washed once and inoculated with virus at a multiplicity of 10 p.f.u./cell. After 45 min. at room temperature, unadsorbed virus was removed and the monolayers washed three times with Hank's saline buffered with 0.05% sodium bicarbonate. Uninoculated cultures were treated identically. Eagle's medium containing [3H] or [14C]-L-phenylalanine and L-leucine (The Radiochemical Centre, Amersham, England) was then added. Each culture contained 200 μc in 0.2 μmoles of each of the
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\(^{3}H\) amino acids or 22 µc in 0.07 µmoles of each of the \(^{14}C\) amino acids in 10 ml of medium. Monolayers were incubated at 35\(^{\circ}\)C for 12 hr. Cells were scraped from the glass surface into the medium and centrifuged at 500 g for 5 min. Virus in the supernatant fluid was purified as described below. The cell pellet was washed once in phosphate buffered saline and resuspended in phosphate buffered saline diluted tenfold. The cells were thoroughly broken using a probe powered by an ultrasonic generator (MEL Equipment Co. Ltd, England). Cell debris was removed by centrifugation at 120,000 g for 3 hr.

**Precipitation of virus antigens with antibody.** Since virus antigens did not form a visible precipitate with the absorbed peritoneal fluid the soluble complexes were precipitated with anti-mouse γ-globulin. Because we thought it likely that this procedure would cause co-precipitation of some labelled cellular material we decided to introduce a preliminary precipitation with rabbit antiserum to uninfected chick embryo fibroblast cells and an equivalent amount of anti-rabbit γ-globulin. This had the advantage that labelled host cell material was selectively removed before precipitation of virus antigens. The supernatant fluids obtained after high-speed centrifugation of infected and control cells were pervaporated to 0.5 ml. and incubated for 3 hr at room temperature with 0.01 ml. of the rabbit antiserum. One ml. of goat anti-rabbit γ-globulin (Microbiological Associates Inc.) was then added and the mixture incubated for 2 hr at room temperature and overnight at 4\(^{\circ}\). The flocculent precipitate was centrifuged at 10,000 g for 30 min. under a layer of paraffin to which lipid adhered. A clear supernatant fluid was obtained by puncturing the side of the tube.

For the precipitation of WSN antigens, 0.03 ml. of peritoneal fluid previously absorbed with purified virus was incubated with the supernatant fluid for 2 to 3 hr at room temperature. An equivalent amount (0.3 ml.) of goat anti-mouse γ-globulin (Microbiological Associates Inc.) was added and the incubation continued for a further 3 hr. The flocculent precipitate was centrifuged at about 1000 g and washed 4 times with 2 ml. of phosphate buffered saline to remove unprecipitated radioactivity. The pellet was resuspended in 0.3 ml. of 0.15 M-NaCl and 0.01 M-phosphate buffer pH 7.4 and stored at −12\(^{\circ}\).

**Purification of radioactively labelled virus.** Concentrated virus obtained by differential centrifugation of either allantoic fluid or of tissue culture fluid was purified through a 10–40 % (w/v) sucrose gradient. About 10\(^{6.4}\) haemagglutinating units (HAU) of this virus was added to the fluid from the labelled infected cultures which contained about 10\(^{6.7}\) HAU. To control possible contamination of virus by radioactively labelled cell material, the same amount of carrier virus was added to the fluid obtained from the labelled non-infected culture. Both lots of virus were then purified in parallel by sedimenting the virus by centrifugation and subsequently centrifuging it on a 10 to 40 % (w/v) sucrose gradient at 16,000 rev./min. for 40 min. in the SW 39 rotor of a Beckman Spinco Model L ultracentrifuge. Fractions (0.4 ml.) were taken from the bottom of the tube and assayed for HA and radioactivity. In the gradient containing radioactive virus the peak of HA and radioactivity occurred in the same fraction. In the control gradient no peak of radioactivity was associated with the peak of HA. Fractions containing the peak of HA from both gradients were diluted with 0.15 M-NaCl and precipitated with 3 vol. of acetone. Precipitates were stored at −12\(^{\circ}\) in 0.2 ml. of water.

To ensure that virus purified by sucrose gradient centrifugation was not contaminated by non-virus radioactive materials a further purification step was undertaken. Labelled
viruses mixed with unlabelled virus grown in tissue culture was purified by sucrose gradient centrifugation. The fraction containing the peak of HA was then centrifuged in a gradient consisting of a mixture of sucrose and potassium D-tartrate from 21 % (w/v) sucrose and 13.5 % (w/v) tartrate to 26 % (w/v) sucrose and 17 % (w/v) tartrate (Griffith, 1968). This was centrifuged at 27,500 rev./min. for 90 min. in the SW39 rotor. Peaks of HA and radioactivity coincided and four fractions containing most of this material were pooled, dialysed to remove sucrose and tartrate and precipitated with acetone.

**Preparation of samples for electrophoresis.** Virus precipitated with acetone or precipitates of antigen + antibody were dissolved in 1 % (w/v) sodium dodecyl sulphate, 0.1 % dithiothreitol, and 0.5 m-urea and were heated in a water bath at 80°C for 10 min. Tris + phosphate buffer, pH 6.7, was added to a final concentration of 0.06 M, sucrose to 20 % (w/v) and phenol red to 0.00025 %.

In some experiments reduced sulphydryl groups were carboxymethylated by the method of Duesberg et al. (1968). Virus or antigen + antibody precipitates were dissolved in 0.1 M-tris pH 8.1, containing 10 M-urea, 1 mM-versene and 1 % (w/v) sodium dodecyl sulphate; solid dithiothreitol was added to 2.4 % (w/v). The mixture was incubated at 37°C for 3 hr and then recrystallized iodacetamide was added to a final concentration of 0.3 M. After 15 min. at 37°C the carboxymethylated proteins were precipitated with acetone and the resulting precipitate prepared for electrophoresis as described above.

**Polyacrylamide gel electrophoresis** followed the method of Davis (1964) which was modified for use with sodium dodecyl sulphate. The gels contained 7, 10.5 or 14 % (w/v) acrylamide monomer and 0.35, 0.525 and 0.7 % bis-acrylamide respectively. Recrystallization of the monomers had no effect on the results obtained. The monomers were made up to the appropriate concentrations in a solution of 0.375 M-tris chloride pH 8.9 containing 0.03 % 'TEMED', 0.075 % ammonium persulphate, 0.1 % sodium dodecyl sulphate and 0.5 M-urea. The gels were polymerized under water in 10 × 0.7 cm. glass tubes and then pre-electrophoresed for 4 hr at 10 mA/tube between electrode reservoirs containing 0.375 M-tris chloride pH 8.9, 0.1 % sodium dodecyl sulphate and 0.05 M-urea.

Dithiothreitol interferes with polymerization and was introduced after pre-electrophoresis by extruding the gels and soaking for 2 days in two changes of 0.375 M-tris chloride pH 8.9, containing 0.1 % sodium dodecyl sulphate, 0.5 M-urea and 0.1 % dithiothreitol.

Before electrophoresis the gels were sucked back into the tubes. The samples were layered on top of the gel and were then overlaid with 0.025 M-tris + glycine pH 8.3 containing 0.1 % sodium dodecyl sulphate, 0.05 M-urea and 0.01 % dithiothreitol. This buffer was used in the electrode tanks.

Electrophoresis was started at 5 mA/tube and the current was increased to 10 mA/tube when phenol red had cleared from the sample zone. Thereafter electrophoresis was continued until the phenol red marker had run about 7 cm. Gels containing radioactively labelled proteins were extruded with a calibrated screw-driven plunger and 0.7 mm. sections were sliced off with a razor blade (Watson, 1969). Preliminary experiments showed that satisfactory elution of proteins from 10.5 and 14 % acrylamide gels was achieved only if the gel slices were crushed and frozen in 0.5 ml. volumes of 0.025 % bovine serum albumin in water, thawed and shaken gently overnight at 37°C.
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With 7% acrylamide gels it was found that satisfactory elution could be achieved by the same process but without crushing the gel slices. Using these methods 75-80% of the applied radioactivity could be recovered from all the gels.

After addition of dioxane based scintillation fluid, radioactivity was measured in a Packard Tricarb scintillation counter under standard conditions for double-label counting. Background levels of radioactivity were determined using two gel fractions cut in front of the phenol red marker band. Similar fractions after addition of standard amounts of 14C- or 3H-labelled protein were used to compute the carry over of counts between the channels which had been adjusted to count mainly 3H or 14C disintegrations. The crushed gel segments in 0.025% albumin solution had a negligible quenching effect on the counts of both isotopes under the conditions used. Electrophoresis of single-labelled isotope material showed that the carry over correction was valid for all the fractions. Sample loads of purified virus were adjusted to contain about 20,000 counts/min. which corresponded to less than 50µg. of protein determined by the method of Lowry et al. (1951). The maximum load of antigen which could be applied was limited by the amount of protein present in the preparation. When sample loads in excess of 200µg. antigen + antibody protein were co-electrophoresed with purified virus the peaks of structural proteins in the latter were spread out with consequent loss of resolution. Accordingly the sample load of antigen + antibody was adjusted to 200µg. protein. This corresponded to 10,000 to 17,000 counts/min. of isotope in the three batches used in this way. Reference proteins were electrophoresed similarly and gels were stained overnight in 0.25% Coomassie Brilliant Blue (R 250 I.C.I. Ltd) in a solvent comprising methanol + water + acetic acid (5:5:1 by vol.). Unbound stain was then removed by repeated changes of the same solvent.

Critique of method of electrophoresis. Most workers have used a continuous buffer system for electrophoresis of samples treated with sodium dodecyl sulphate rather than the discontinuous buffer system of Ornstein (1964) and of Davis (1964) used here. Preliminary experiments were made with a continuous buffer system but this did not give the resolution required to distinguish structural and non-structural protein, whereas the discontinuous system offered a better method for this purpose. It should be emphasized, however, that comparable patterns were obtained from purified virus using both continuous and discontinuous systems. Further, comparable results have been obtained with the two systems for both adenovirus protein (W. G. Laver & D. H. Watson, unpublished results) and herpes virus protein (D. J. Robinson & D. H. Watson, in preparation) although in both the bands were sharper and resolution better in the discontinuous system.

RESULTS

Purification of virus

The peak HA fraction after a sucrose gradient centrifugation of a mixture of unlabelled and labelled tissue culture fluid containing virus had a protein content of 0.8 mg./10^6 HAU. Further purification by sucrose-tartrate gradient centrifugation gave a peak fraction containing 0.3 mg. protein/10^6 HAU. This compares with the values of 0.25 to 1.0 mg./10^6 HAU obtained by Laver (1962) for purified samples of several strains of influenza virus and the figure of 0.4 mg./10^6 HAU calculated from the dry weight of the particle using data quoted by Hoyle (1968). The increased purification by the additional tartrate gradient is apparently due largely to removal of unlabelled
host cell protein since the ratio of radioactive counts/HAU was only decreased by a factor of 1.4. Following these results only virus grown in tissue culture which had been purified on sucrose and tartrate gradients was electrophoresed when protein was to be detected by staining.

Precipitation of labelled antigen

The ratio of radioactivity precipitated from infected and control preparations varied between 4.9 and 6.7. This ratio was not improved by additional washing of the precipitate or by disrupting it in an ultrasonic bath and allowing it to reform. About 100,000 counts/min. were precipitated from a preparation of infected cells.

Separation of virus structural proteins by polyacrylamide gel electrophoresis

It is convenient to describe the results of electrophoresis of purified virus alone although we shall illustrate the results by an experiment in which antigen labelled with $^{14}$C was electrophoresed with purified virus labelled with $^3$H. In fact similar results were achieved when the virus was electrophoresed alone. A refractile band was always seen moving behind the marker phenol red band. This band was apparently related to the presence of sodium dodecyl sulphate and was present even if samples containing no protein were electrophoresed. No proteins were found running in front of this band. Reference proteins were electrophoresed in parallel gels in all experiments and their positions are marked on Fig. 1 (a), (b), (c) which show the results obtained with 7, 10.5 and 14% gels respectively. In 10.5% gels five peaks labelled V1-V5 in Fig. 1 (b) were reproducibly observed in many experiments on several different batches of virus. We believe that the front running peak, coincident with the refractile boundary, was artefactual and represented an accumulation of small material which was not retarded by the gel, since in the 14% gel (Fig. 1(c)) it was not observed, although ribonuclease which was also coincident with the refractile boundary in the 10.5% gel appeared behind it in the 14% gel. In the 14% gel depicted in Fig. 1(c) V2 was not separated from V1 and V4 was not observed although they were both resolved in some other 14% gels. In 7% gel as shown in Fig. 1(a) V1, V2, V3 and V4 were again observed although V5 was apparently now included in the peak running at the refractile boundary.

Two other features were observed in the pattern of virus structural polypeptides in 10.5% gels: first, a shoulder on the cathodal side of V5 (marked X in Fig. 1(b)) and secondly, a small peak on the cathodal side of the refractile boundary peak (marked Y in Fig. 1(b)). These were not reproducibly resolved as clear peaks in all the 10.5% gels and we cannot therefore be certain that they represent real polypeptide peaks. We shall refer again to these components in the next section. The same pattern of peaks was observed on virus which had been purified further on tartrate-sucrose gradients. V1, V2, V3 and V5 were also detected by staining a gel following electrophoresis of this virus (Plate I). The stained gels also showed a component on the cathodal side of V5 and barely separated from it, which possibly corresponded to X. The pattern of peaks was unaffected by prior extraction of virus lipids (Ada & Perry, 1954), by heating the sample at 100° for 2 min. before running or by carboxymethylation of the protein before electrophoresis.

Finally, Fig. 1 (b) shows the result of gel electrophoresis of an unlabelled virus
Electrophoresis of influenza virus on a 10.5% polyacrylamide gel. The virus was grown in chick embryo cells and purified by centrifugation on sucrose and sucrose-tartrate gradients. The gel was stained with Coomassie Brilliant Blue. The scale corresponds to the fraction numbers in Fig. 1(b). Bands can be distinguished corresponding to peaks in Fig. 1 marked V1, V2, V3 and V5 and also to the material at the refractile band (RB). There is an additional band just above V5 which may represent peak X in Fig. 1.
sample to which labelled uninfected tissue culture fluid had been added before purification. No polypeptide peaks were observed in the region of the virus polypeptides.

**Separation of non-structural proteins in polyacrylamide gel electrophoresis**

For the success of the experimental approach we have used, disruption of the antigen + antibody precipitate must produce antigen behaving in electrophoresis as would the original unprecipitated antigen. In a preliminary experiment we therefore partially disrupted a sample of labelled virus with ether and precipitated it using immune peritoneal fluid which had not been absorbed with purified virus. After disruption and electrophoresis the same pattern of peaks was observed, showing that no new peaks were generated after interaction of the structural polypeptides with antibody.

Figures 1(a), (b), (c) show the results obtained on electrophoresis of the precipitate formed by the reaction of 14C labelled antigen with the immune peritoneal fluid adsorbed with partially disrupted virus. In the 10.5% gel (Fig. 1(b)) three polypeptide peaks were observed which were not coincident with any virus peak: these have been labelled N1, N2 and N3. In addition there were peaks coincident with V1, V2, V3, V4, X and Y. The pattern was unaffected by carboxymethylation of the protein. Also shown in Fig. 1(b) is the result of parallel electrophoresis of the precipitate obtained with labelled uninfected cell material. The only peaks observed were one at the same position as the refractile boundary and another at the same position as V3. The position of the slower moving peak at V3 is regarded as fortuitous since it has less radioactivity than the peak of the same mobility from infected cells.

In the 14% gel (Fig. 1(c)) N1 and N2 were again observed but N3 was not resolved. There was a 'plateau' in the region of fractions 60 to 70 which could have represented a number of poorly separated peaks. However, the level of counts in this region did not sufficiently exceed the counts in the corresponding region of the control for us to be sure that this region truly represents virus specific material.

Electrophoresis on 7% gel (Fig. 1(a)) showed a clear separation of N1 from V1 although in this gel N2 and N3 were presumably included in the peak at the refractile boundary.

In summary we observed five structural and three non-structural polypeptides. There are possibly two other structural polypeptides.

**Molecular weight estimation**

Like Shapiro, Vinuela & Maizel (1967), we found that the distances migrated in the gels by the reference proteins were linearly related to the logarithms of their molecular weights. Accordingly we attempted to estimate the molecular weights of the virus-specific polypeptides by this method. Although we are aware that theoretical objections can be made to this procedure, we found both labelled samples and reference proteins behaved reproducibly in gels of any one batch but there were small variations between batches. Accordingly reference proteins and labelled samples were always electrophoresed in parallel gels of the same batch. Since the refractile band contains all material which is not filtered in the gel, molecular weights were not estimated on proteins in this region.

Molecular weights of V1–V5 and N1–N3 were obtained from a number of experiments on gels of different pore size (Table 1). A range of values was observed and a
number of measurements made for each protein on any particular gel. The coefficient of variation of the molecular weights of virus structural proteins determined on 10 % gels ranged from 11 to 13 % for V1, V2, V3 and V5 and was 20 % for V4.

Fig. 1. For legend see foot of facing page.
Table I. Molecular weights of influenza virus specific proteins estimated from electrophoretic mobilities

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<th>Protein</th>
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<th>Non-structural</th>
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DISCUSSION

The combination of immune precipitation with polyacrylamide gel electrophoresis has provided a powerful means of distinguishing non-structural from structural polypeptides. The value of the technique is emphasized by the fact that electrophoresis of infected cell extracts revealed some of the structural but none of the non-structural polypeptides. Three non-structural polypeptides have been shown to be specified by the virus in infected cells. Dimmock (1969) demonstrated up to five presumed non-structural antigens by immunodiffusion methods. Several factors may contribute to this apparent discrepancy. Thus, we do not know how many antigenic determinants may result from combinations of the three non-structural proteins we have recognized and the method we have used would fail to distinguish polypeptides of similar sizes. Alternatively, some of the polypeptides we have characterized as minor components of the virus may really be contaminating non-structural components, although we were...
unable to remove them on further purification. A similar number of structural polypeptides has been observed in the closely related fowl plague virus by Joss et al. (1968).

The total molecular weight of all the virus specific components is over 650,000 even when X and Y are excluded. The nucleic acid of the virus should be capable of specifying protein whose total molecular weight is only 300,000, a figure which is exceeded by the total molecular weight of the structural polypeptides. However, this figure may be regarded as approximate, until the molecular weight of the RNA is determined by a method in which its secondary structure is considered. The determination of molecular weights of unknown proteins from their behaviour in polyacrylamide gel electrophoresis is open to the objection that not all proteins may conform to the empirical relationship of Shapiro et al. (1967). Until the observed values can be confirmed by other methods it would seem prudent to accept them with some caution.

Some of the smaller non-structural polypeptides may of course represent cleavage products of larger molecules as found with poliovirus (Summers & Maizel, 1968). Pulse-chase experiments should allow this to be checked.

It seems unlikely that spurious results have arisen from protein aggregation because we have failed to remove larger components by heating at 100° or by carboxymethylation of reduced sulphhydryl groups. Further when material from the peak fractions of V1 and V2 was re-electrophoresed, we observed a single peak for each in the appropriate position with no peaks in other parts of the gel. It seems improbable that the presence of lipids in the virus envelope affected our observations since prior extraction of lipid did not affect the pattern of peaks observed in subsequent electrophoresis. However, influenza virus also contains carbohydrate, bound covalently to coat protein (Laver & Webster, 1966), whose effect on the observed number and mobility of polypeptides remains a matter for speculation.

We have demonstrated the presence of some non-structural proteins in cells infected with influenza virus. We hope to separate the proteins and to determine their role in virus multiplication.

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