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ZONAL DENSITY GRADIENT ELECTROPHORESIS OF INFLUENZA A VIRUS

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Summary. — Enzymatic activities associated with influenza A virus purified by sedimentation in sucrose density gradient were removed from the virions by sucrose density gradient electrophoresis. A rapidly migrating electrophoretic fraction contained both structural viral polypeptides and chick embryo allantoic fluid polypeptides.

Key words influenza virus; virus-associated enzymes; virus purification; density gradient electrophoresis

Introduction

Preparations of influenza virus purified by sedimentation in sucrose density gradients contain enzymatic activities occurring in plasma membranes of host cells, like ribonuclease (Rosenbergová and Pristašová, 1972; Wiegers and Drzeniek, 1973; Arora *et al.*, 1976), phosphodiesterase (Pristašová and Rosenbergová, 1974) and nucleoside triphosphatases (Drzeniek and Kaluza, 1972; Oxford, 1973). These enzymes could not be removed from the virions by currently employed sedimentation techniques.

The electrophoretic mobility of influenza virus was studied by several authors (Bourdillon, 1940; Miller *el al.*, 1944; Matheka and Giess, 1965; Krivjanská *et al.*, 1978). Ruttkay and Ivaničová (1965) analysed influenza A virus by moving boundary electrophoresis and found that the purified virus preparation was electrophoretically heterogeneous, migrating in several fractions. These findings suggested that the virus could be freed from the cellular contaminants based on different electrophoretic mobilities.

In the present study we purified influenza virus by sucrose density gradient electrophoresis; one of the electrophoretic virus fractions was separated and characterized in some detail.

Materials and Methods

Influenza virus A/Singapore/1/57 (H2N2) was propagated in 10-day chick embryos. The allantoic fluid was harvested after two days of incubation at 35 °C, filtered through glass wool and clarified by centrifugation for 30 min at 10 000 × g. The virus was concentrated in two ways. (*a*) After centrifugation for 60 min at 40 000 × g the virus was resuspended in 0.02 M phosphate buffer, pH 7.2, containing 0.1 M NaCl (PBS); the suspension was clarified at 10 000 × g for 15 min.

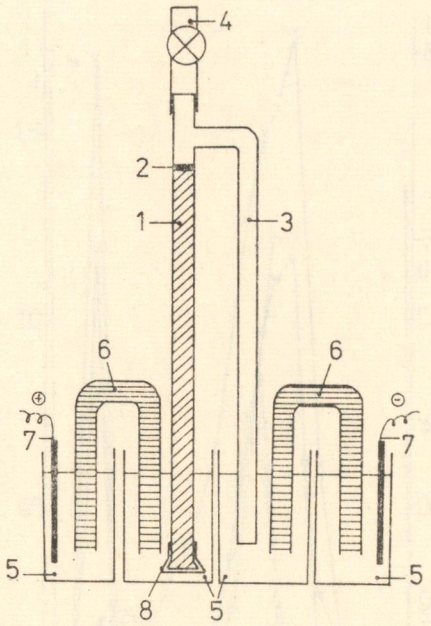

Fig. 1.

Schematic illustration of the separation device for sucrose density gradient electrophoresis
1 — separation glass tube (9—14 mm inner diameter) with sucrose density gradient; **2** — sample; **3** — side-connecting tube; **4** — rubber tube with clamp; **5** — electrolyte; **6** — U-tubes; **7** — electrodes; **8** — cellophane membrane

(*b*) Chicken red blood cells were added to clarified allantoic fluid to a final concentration of 2%. After 1 hr adsorption at 4 °C and two washings with cold PBS the virus was eluted for 2 hr at 37 °C into 1/10 volume of PBS. Then followed sedimentation for 1 hr at 40 000 × g and resuspension in PBS.

Purification of virus. After sedimentation in a 25 and 50% (w/w) discontinuous sucrose gradient for 90 min at 40 000 × g the virus zone was separated, diluted 1 : 2 with PBS and centrifused for 1 hr at 40 000 × g. The pelleted virus was resuspended in a small volume of PBS and further purified by sedimentation in a 20—55% (w/w) continuous sucrose density gradient for 4 hr at 190 000 × g. The virus zone was collected, sedimented for 1 hr at 40 000 × g and the pellet resuspended on 0.01 M phosphate buffer pH 7.6 containing 0.06 M NaCl (further on NaCl-phosphate). Sodium azide was then added to a final concentration of 0.05%.

The haemagglutinating (HA) activity of the virus was assayed as described by Rosenbergová and Pristašová (1972).

Uninfected allantoic fluid harvested from 12-day chick embryos was subjected to the same procedures as the virus (concentration and "purification" by sedimentation in a sucrose density gradient).

Protein concentration was estimated according to Lowry *et al.* (1951).

Sucrose density gradient electrophoresis. The simple apparatus used is schematically illustrated in Fig. 1. The separation tube was filled with 30 ml of linear 20—40% (w/w) sucrose density gradient in NaCl-phosphate and immersed into a vessel containing 40% sucrose in NaCl-phosphate. The side-connecting tube was immersed into a vessel containing NaCl-phosphate. Both vessels were connected by bridges (1% agar in saturated KCl solution) with other vessels filled with saturated KCl solution, into which Pt electrodes were imersed. The virus preparation containing 2% sucrose was layered in 0.5 ml volumes on the gradient and overlaid with NaCl-phosphate. Electrophoresis lasted for 18—22 hr at 5 °C, at 200 V and 8—12 mA. One-ml fractions were collected into tubes after piercing the cellophane membrane at the bottom of the separation tube.

Enzyme assays. Ribonuclease activity was estimated by formation of acid-soluble products of cellular RNA prepared from Ehrlich ascites tumour cells as described (Rosenbergová and

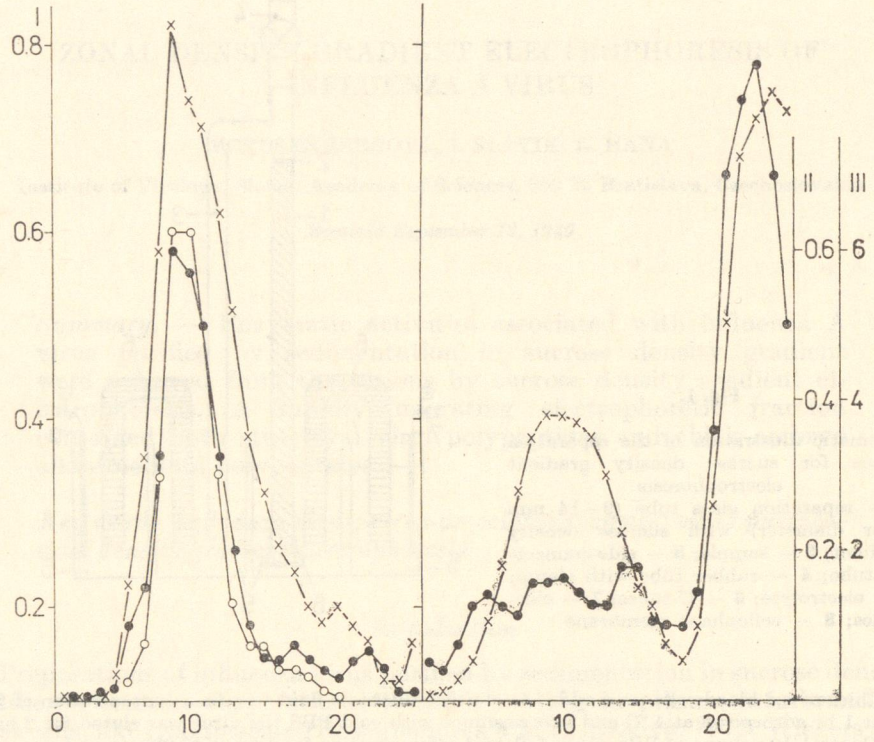

Fig. 2.

Sedimentation profiles of influenza virus (left) and uninfected allantoic fluid (right) in linear 5—20% (w/w) sucrose density gradients

Sedimentation from the left to the right.

Abscissa: fraction number.

Ordinate I: absorbance ($A_{280nm} - A_{310nm}$; ×); ordinate II: ribonuclease activity ($A_{260\ nm}$ of the enzymatic reaction products; ●); ordinate III: HA units per ml × 10^{-4} (○)

Pristašová, 1972). Phosphodiesterase activity was assayed according to Sinsheimer and Koerner (1952) using bis-p-nitrophenyl phosphate as substrate.

Polyacrylamide gel electrophoresis (PAGE) was carried out as described by Slávik *et al.* (1976). Viral proteins were solubilised by 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol in 0.01 M phosphate buffer pH 7.2 and fractionated on 8% gels. Sixty or two hundred micrograms of protein were applied on gels stained respectively with Coomassie brilliant blue R-250 and by a modified Schiff's procedure in which sodium instead of potassium pyrophosphate was used (Zacharius *et al.*, 1969). The absorbances of the stained gels were monitored on a Joyce-Loebl Chromoscan densitometer at 620 and 465 nm respectively.

Results

We followed the purity of influenza virus at various steps of the purification procedure by estimating the HA titre, absorbance at 280 nm and the ribonuclease and phosphodiesterase activities and by polypeptide analysis by PAGE.

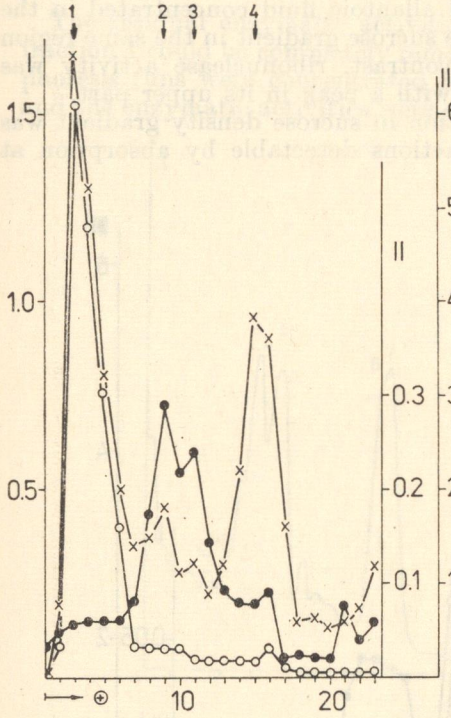

Fig. 3.

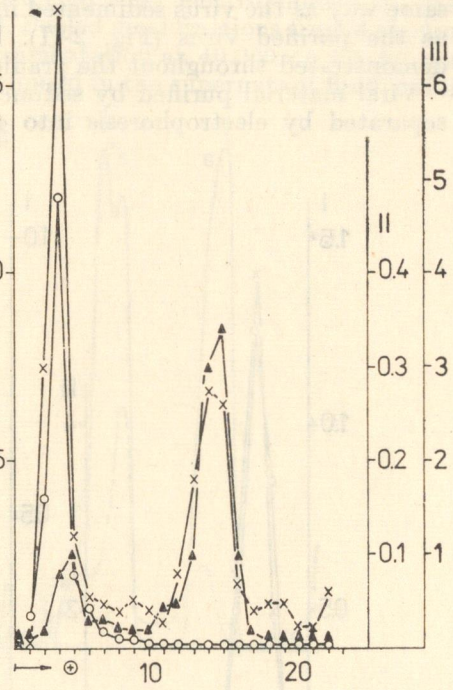

Fig. 4.

Electrophoretic profile of influenza virus purified on a sucrose density gradient
Ordinates I: absorbance ($A_{280\ nm} - A_{310\ nm}$; ×)
Ordinates II: *Fig.* 3 — ribonuclease activity (absorbance of the enzymatic reaction products at 260 nm; ●) *Fig.* 4 — phosphodiesterase activity (absorbance of the enzymatic reaction products at 400 nm; ▲)
Ordinates III: HA units per ml × 10^{-4} (○)
Abscissae: fraction numbers.
Arrows 1—4: fractions 1—4, see text.

The two enzymatic activities occur in plasma membranes of uninfected chick embryo chorioallantoic membrane cells (Pristašová, 1980) and were also found in chick erythrocytes from which they could be released by heating at 37 °C in 0.14 M NaCl (data not shown).

The sedimentation profile of influenza virus purified by sedimentation in sucrose density gradient is shown in Fig. 2-I. Virus and ribonuclease activity co-sedimented in a broad peak. The sedimentation profile of the virus and ribonuclease activity remained unchanged after repeated sedimentation of the virus in sucrose density gradient. The peak of thus purified virus was not symmetrical and a shoulder in fractions 12—14 suggested a heterogeneity

of the sedimented material. Uninfected allantoic fluid concentrated in the same way as the virus sedimented in the sucrose gradient in the same region as the purified virus (Fig. 2-II). By contrast, ribonuclease activity was demonstrated throughout the gradient with a peak in its upper part.

Viral material purified by sedimentation in sucrose density gradient was separated by electrophoresis into 4 fractions detectable by absorption at

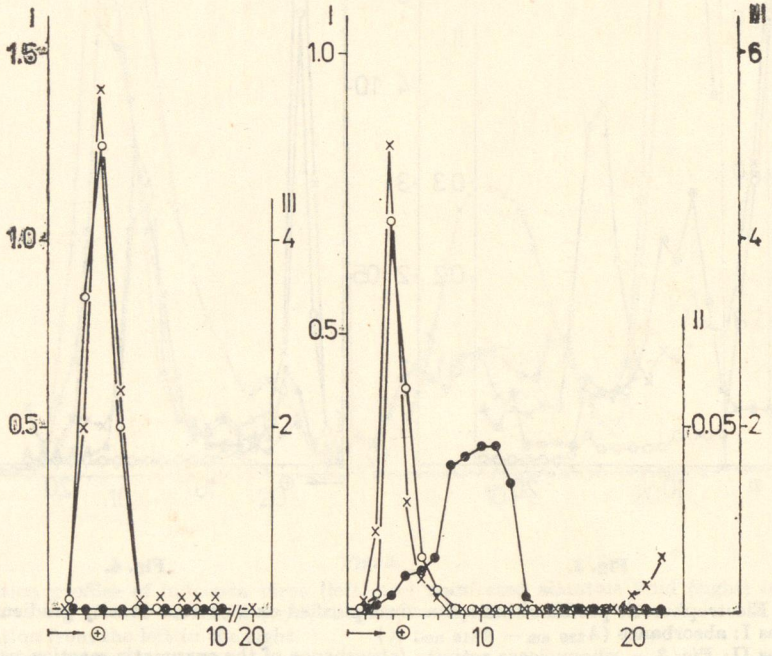

Fig. 5.

Fig. 6.

Fig. 5. Re-electrophoresis of influenza virus (pooled fractions 1 — see Fig. 3).
Fig. 6. Electrophoretic profile of influenza virus purified by adsorption on to and elution from erythrocytes and by sucrose density gradient centrifugation.
Abscissae and ordinates as in Fig. 1.

280 nm (Fig. 3). Most of the HA activity coincided with fraction 1 which contained undamaged virions as shown by electron mictoscopy. About 10—15% of the total HA activity occurred in the other fractions. The ribonuclease activity migrated more quickly than the virus, but low activities of the enzyme were detected throughout the gradient. The proportion of the electrophoretic fractions varied with various virus preparations. Fractions 1 and 4 were present in all preparations, the distribution of fractions 2 and 3 varied. Most of the phosphodiesterase activity migrated with fraction 4, but a minor part still contaminated the viral fraction 1 (Fig. 4).

The residual enzymatic activities, detected after electrophoresis in viral fraction 1 could be completely removed. Pooled viral fractions from 4 electrophoresis runs were concentrated by sedimentation at 40 000 × g for 90 min and the enzymatic activities were determined in the supernatant fluid and in

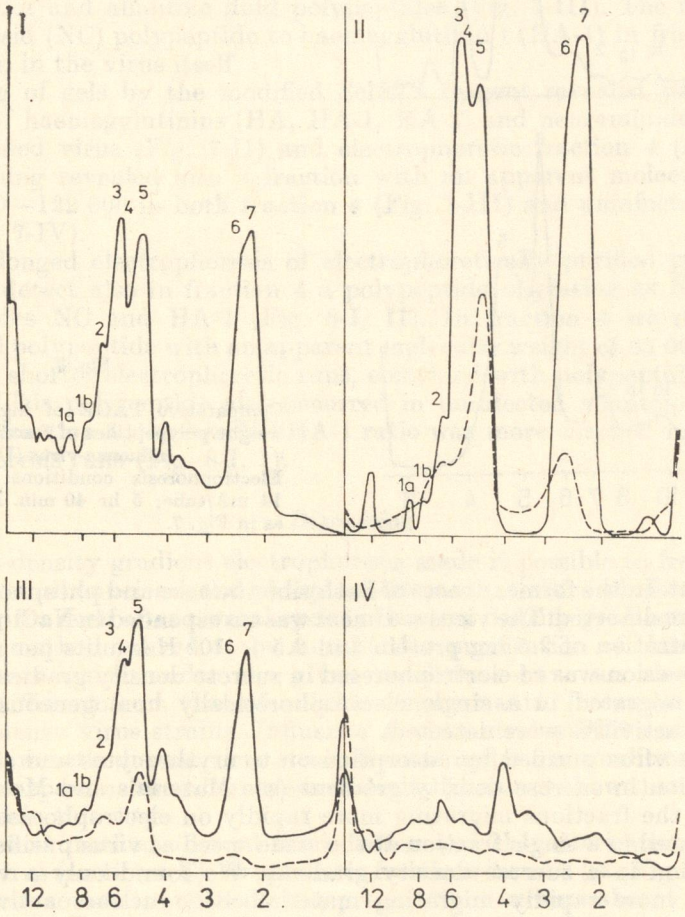

Fig. 7.

PAGE of
I — influenza virus purified by sucrose density gradient centrifugation
II — influenza virus purified by sucrose density gradient electrophoresis
III — fraction 4 (see Fig. 3)
IV — uninfected allantoic fluid
Densitometric resords of 8 cm gels strained with Coomassie brilliant blue R-250 (———) or fuchsin-sulphite (— — —). Electrophoresis conditions: 2.5 V/cm, 4.5 mA/tube; 17 hr. Abscissae: apparent molecular weight × 10^{-4}; ordinates: absorbance. Structural virus polypeptide peaks designated by numbers: 1a, b — P protein; 2 — HA; 3 — NC; 4 — NA; 5 — HA-1; 6 — HA-2; 7 — M protein.

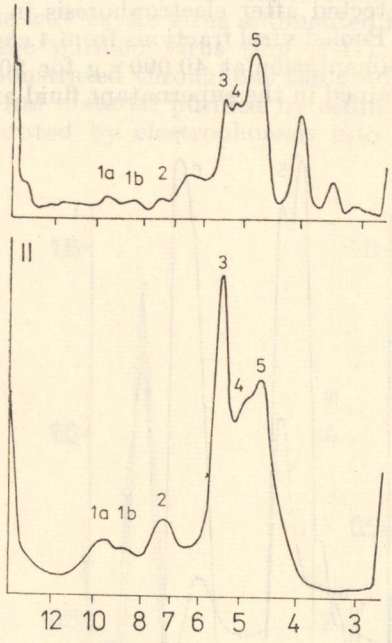

Fig. 8.

Comparative PAGE of high molecular weight polypeptides of fraction 4 (I) and influenza virus (II)
Electrophoresis conditions: 7.5 V/cm; 14 mA/tube; 5 hr 40 min. Designations as in Fig. 7.

the sediment. In the former, traces of both ribonuclease and phosphodiesterase activity were detected. The virus sediment was resuspended in NaCl-phosphate to a concentration of 2.5 mg protein and 2.5×10^5 HA units per ml; 0.5 ml of this suspension was re-electrophoresed in sucrose density gradient (Fig. 5). The virus migrated in a single electrophoretically homogeneous fraction; no enzyme activities were detected.

Influenza virus purified by adsorption on to erythrocytes and subsequent centrifugation in sucrose density gradient (see Materials and Methods) was freed from the fractions migrating more rapidly on electrophoresis (Fig. 6). Virus migrated in a single fraction at the same speed as virus purified only by sedimentation in a sucrose density gradient. We found only a very small amount of more rapidly migrating material. Ribonuclease activity again migrated more rapidly than the virus.

The polypeptides of influenza virus purified by sedimentation and electrophoresis in sucrose density gradients were subjected to PAGE and compared with polypeptides of the electrophoretic fraction 4 (see above) and those of uninfected allantoic fluid "purified" in the same way as the virus.

In viral material purified only by sedimentation in sucrose density gradient we found in addition to polypeptides migrating at the same speed as viral structural polypeptides also contaminating polypeptides with apparent molecular weights of 41 000, 37 000 and 32 000 and numerous polypeptides in the range of molecular weights from 100 000 to 140 000 (Fig. 7-I). We

observed similar polypeptides in the uninfected allantoic fluid (Fig. 7-IV). Virus purified by sucrose density gradient electrophoresis was not contaminated by allantoic fluid polypeptides; some preparations contained a polypeptide with a molecular weight of 120 000—130 000 (Fig. 7-II). In preparations of the rapidly migrating fraction 4 we detected both viral structural polypeptides and allantoic fluid polypeptides (Fig. 7-III). The ratio of the nucleocapsid (NC) polypeptide to haemagglutinin 1 (HA-1) in fraction 4 was lower than in the virus itself.

Staining of gels by the modified Schiff's reagent revealed bands at the location of haemagglutinins (HA, HA-1, HA-2) and neuraminidase (NA) in both purified virus (Fig. 7-II) and electrophoretic fraction 4 (Fig. 7-III). This staining revealed also a fraction with an apparent molecular weight of 120 000—122 000 in both fraction 4 (Fig. 7-III) and uninfected allantoic fluid (Fig. 7-IV).

On prolonged electrophoresis of electrophoretically purified preparations we could detect also in fraction 4 a polypeptide migrating as NA between polypeptides NC and HA-1 (Fig. 8-I, II). In fraction 4 we detected an additional polypeptide with an apparent molecular weight of 65 000 (Fig. 8-I) which, on shorter electrophoretic runs, coincided with polypeptides HA, NA nad NC. This polypeptide also occurred in uninfected allantoic fluid (Fig. 7-IV). In fraction 4, the low NC:HA-1 ratio was more marked in prolonged electrophoresis runs (Fig. 8-I, 1).

Discussion

Sucrose density gradient electrophoresis made it possible to free influenza A virus from ribonuclease and phosphodiesterase activities which co-sediment with the virus even after several cycles of sucrose density gradient centrifugation. The method proved to be suitable for purification of those influenza virus strains which under the given conditions have an electrophoretic mobility different from that of cellular contaminants. It cannot be applied to all influenza virus strains. Influenza A virus strains PR8 and WSN, the same as Newcastle disease virus strain Kansas under our conditions migrated at the same speed as the ribonuclease activity (data not shown) and could not be purified in this way.

The present method allowed the isolation and characterisation of electrophoretic virus fractions. Electrophoretic fractions 2 and 3 (see Fig. 3) occurred in variable amounts in purified virus preparations or were absent from them. We analysed, therefore, the electrophoretic fraction 4 representing about 20% of the total virus preparation. The HA activity of this fraction related to absorbance unit ($HA/A_{280\ nm}$) was 2.6×10^3, i. e. one order of magnitude lower than in the virus fraction in which it was 4×10^4. About 80% of polypeptides of fraction 4 were represented by viral structural polypeptides. The mutual ratio of structural polypeptides in fraction 4 resembled that in purified virus with the difference that the amount of NC polypeptide was significantly lower. The fact that adsorption on to and elution from erythrocytes resulted in removal of fraction 4 from the virus preparation means that

this fraction is either not adsorbed on to or not eluted from the erythrocytes. It can be assumed that the HA activity of fraction 4 could be masked or that the function of glycoproteins is changed.

The mobility of all electrophoretic fractions of influenza A virus in zonal sucrose density gradient electrophoresis was in accordance with the results obtained in moving boundary electrophoresis by Ruttkay and Ivaničová (1965). The present results indicate that fraction 4 contains both viral and cellular polypeptides. The elucidation of the structural organization of fraction 4 requires further study.

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