Production of Incomplete Pseudorabies Viruses in Enucleated Pig Kidney Cells

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Abstract

Pseudorabies viruses (PrV) were grown in the pig kidney cells which were enucleated with cytochalasin B after infection. When protein synthesis was blocked by the drug, cycloheximide, from the time of infection, viral m-RNA accumulated in the cytoplasm and translation of viral proteins occurred after the removal of both drug and nucleus. A number of viral specific polypeptides were to be modified after synthesis and some of these post-translational modifications of proteins were prevented in the absence of the nucleus. ~10,000 fold decrease of viral titer in enucleated cells in comparison to that in intact cells was observed due to the formation of protein-nonmodified viral particles or incomplete particles (IP).

Co-infection of cells with IP and normal infectious virions resulted in ~100 fold reduction in yield. The lower yield of infectious virus produced by the cells was due to interference by IP. A similar phenomenon was observed when cell cultures were initially infected with standard virus and superinfected with IP. Exposure of IP to standard antiviral swine serum for 1 hr. at 37°C or UV light (20 watts) for 5 min. markedly reduced their interfering ability. It is therefore clear that the interference with the synthesis of infectious virus by IP resided in these particles, which had antigenic properties similar to those of standard pseudorabies viruses. Quantitative assay and qualitative analysis of antigenic proteins of IP were discussed.

Introduction

The role of the host nucleus in the viral replication has been obtained by the application of inhibitors of nuclear functions such as actinomycin D and mitomycin C (1). The function of the nucleus in the regulation and modification of herpesvirus polypeptide synthesis was observed (3). Comparison of the development of a group of animal viruses in enucleate cells was also studied (4).

The development of pseudorabies virus (PrV) in host cells seems to involve a complex system of regulatory mechanisms controlling the production and posttranslational modification of viral specific proteins. In this study, the role of the host nucleus in the replication cycle of PrV has been investigated by the techniques of enucleate cells, using a fungal metabolite, cytochalasin B (9, 2). With such cells it is possible to demonstrate that the development of a normal infectious PrV is dependent on the physical presence of a nucleus. The formation of IP in the cytoplasmic host after enucleation has been demonstrated. Evidence of IP retaining PrV antigenicity and interfering with the growth of standard PrV is also presented.

Materials and Methods

Confluent monolayers of pig kidney (PK) cells were grown in plastic Leighton tubes. The medium used for the growth of cells was Eagle's medium containing 10% fetal bovine serum, 100 μ of penicillin and 170 mg streptomycin per ml at pH 7.4. Cells were infected with

30 PFU per cell of the strain PrV-FH. Virus infectivity was assayed in PK cells by determination of the 50% tissue culture infective dose ($TCID_{50}$) at 48-120 hrs. post-infection.

Cytochalasin B (Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in dimethyl sulfoxide as described by Prescott *et al.* (9). Cell enucleation was carried out using the system described previously (3).

Purification of globulin fraction was performed as described by Kanitz (7). The eluate from the column was finally concentrated 10-fold with Aquacide II. Protein concentration was determined by the biuret method (8). Highly purified bovine serum albumin was used as the standard.

Identification of viral antigens with immunodiffusion tests and quantitative assays with rocket immunoelectrophoresis were conducted according to Sun *et al* (10).

Crossed immunoelectrophoresis was done as described by Vestergaard (12) with a few modifications. Glass plates (9.4 cm by 8.4 cm) were used and were covered with 1.5 mm thick 1% (wt/vol) agarose (Nutritional Biochemicals Corporation), dissolved in a buffer as described previously (11, 14). The first dimensional electrophoresis was performed for 90 min. at 12.7 v/cm of gel and the second dimensional electrophoresis was completed in 15 hr. at 6 v/cm of gel. The plates were then dried, stained, and treated as described (11, 14). In the first-dimensional electrophoresis, a neutral detergent, Triton X-100 (0.5%) and an anionic detergent, sodium deoxycholate (0.05%) were incorporated in the agarose gel. In the second dimensional electrophoresis, the gel contained purified anti-PrV gamma-globulin prepared as described above.

Results

It had been observed that the virus titer detected in the enucleated cell cultures was lower than that detected in the intact cell cultures. As shown in Table 1, the surviving viral fraction was a function of treatment time. Various lengths of time of enucleation gave host cells with various ability to support viral development. After 120 min. of treatment, the virus titer already dropped four log units. These results indicate that the synthesis of PrV viral specific proteins and the

TABLE 1. *Growth of Pseudorabies Virus (PrV) in Enucleated Pig Kidney Cells*

Time of Treatment (minutes)	Virus Titer (TCID 50/ml.)
0	10^6
30	10^4
60	10^3
120	10^2
180	10^2

Cells were infected with PrV-FH at multiplicity of infection (m.o.i.) = 30. The infected cell culture was harvested at 24 hr. of postinfection and assayed in PK-W2E cells.

formation of a complete and normal infectious virus do need the presence of host nucleus. The formation of defective incomplete virus in the enucleated host is possible.

Table 2 showed that the addition of IP grown in enucleated cells (treated for 1 hr. or 2 hr.) to a standard virus inoculum reduced the yield of infectious virus by approximately 100 fold, whether the cells were exposed both simultaneously (co-infection) or in tandem (superinfection). Thus, it was demonstrated that IP contained a factor which interfered with the production of infectious virus.

The effects of incubation with PrV antiserum or of UV light on the interfering ability of IP were also tested. Exposure of IP to standard antiviral serum for 1 hr. at 37°C or UV light (20 watts) for 5 min. completely lost their interfering ability (Table 3). It is therefore clear that the interference with the synthesis of infectious virus by IP resides in incomplete particles, which had antigenic properties similar to that of standard PrV.

TABLE 2. *Capacity of Incomplete PrV Particles to Interfere with the Replication of Standard PrV*

Infection	Virus Titer (TCID 50/ml.)
Standard PrV Control (VC)	10^7
Incomplete Virus Grown in Cells Treated for 1 Hour (EN 1 H)	10^4
Incomplete Virus Grown in Cells treated for 2 hours (EN 2 H)	10^3
VC + EN 1 H (co-infection)	10^5
VC + EN 2 H (co-infection)	10^5
VC 1 hr. EN 1 H (superinfection)	10^5
VC 1 hr. EN 2 H (superinfection)	10^5

Cells were infected with VC at m.o.i. = 10 and IP (1ml) in both co-infection and superinfection. The infected cell culture was harvested at 24 hr. of post-infection and assayed in PK-W2E cells.

TABLE 3. *Effect of UV Light and PrV Antiserum on the Interfering Ability of the Incomplete Viruses*

Infection	Virus Titer (TCID 50/ml.)
Standard PrV Control (VC)	10^7
Incomplete Virus Grown in Cells Treated for 1 hour (E N I H)	10^4
EN 1 H + Antiserum (E N I H S)	no c.p.e.
VC + E N I H S	10^7
VC 1 hr. E N I H S	10^7
E N I H + 5 min. UV irradiation (E N I H U)	no c.p.e.
VC + E N I H U	10^7
VC 1 hr. E N I H U	10^7

ENIHS, cell cultures were infected by 1 ml of IP or ENIH which had incubated for 1 hr. at 37°C water bath with 1:20 dilution of standard PrV antiserum from swine. The infected cultures were washed with phosphate buffer saline to remove unabsorbed virus, as well as PrV antiserum.

ENIHU, cell cultures were exposed to 1 ml of IP or ENIH which had been treated with 20 watts of UV light for 5 min.

An immunodiffusion test (IDT) to analyze the PrV antigen in IP is presented in Fig. 1. Wells 7 in Fig. 1A and Fig. 1B contained positive virus control (PrV grown in intact cells) and tested IP sample (PrV grown in cells enucleated for 2 hr.), respectively. Various dilutions (undiluted to 32x) of anti-PrV antiserum were filled in surrounding wells (1 to 6). The antigenicity of the tested IP sample in Fig. 1B seemed to be stronger than that of the reference positive control (Fig. 1A), for a weakly positive sample always resulted in a precipitin band near the sample well. If the normal swine serum was filled in the surrounding wells, no precipitin bands were observed. It is therefore concluded that these bands are formed by specific antigen-antibody reactions.

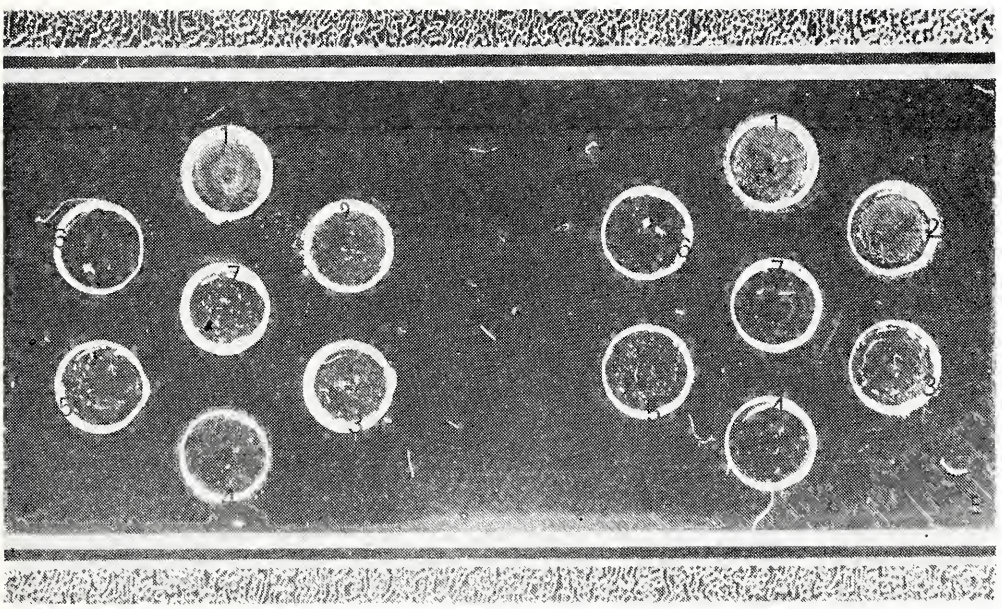

FIGURE 1. *Analysis of PrV antigens in IP by immunodiffusion test. Equal volumes (40 μl) of virus control (Fig. 1A) and IP (Fig. 1B) were used in wells 7. Various dilutions of anti-PrV swine serum were filled in the surrounding wells. Wells 1, 2, 3, 4, 5 and 6 contained undiluted, 2x, 4x, 8x, 16x and 32x of antiserum, respectively.*

The accurate concentration of antigenic proteins in IP was also investigated through the rocket immunoelectrophoretic technique (RIET). A plot of immunoprecipitate distances or areas of PrV standards from RIET are shown in Fig. 2. The concentration of antigen is directly proportional to the height or the covered area by each rocket. IP grown in the enucleated cells did not reduce the amount of viral antigenic polypeptide synthesis as shown in Fig. 3. By measuring the entire covered area of each rocket and comparing it to that of the standard curve (Fig. 2), the antigenicity of IP was determined. The quantitative estimate of antigenic proteins in IP was 60 mg/ml, which was slightly higher than that of the positive virus control (48 mg/ml). Thus, the defectiveness of IP is shown in the lack of infectious ability (Table 1), but not in the antigenic protein formation.

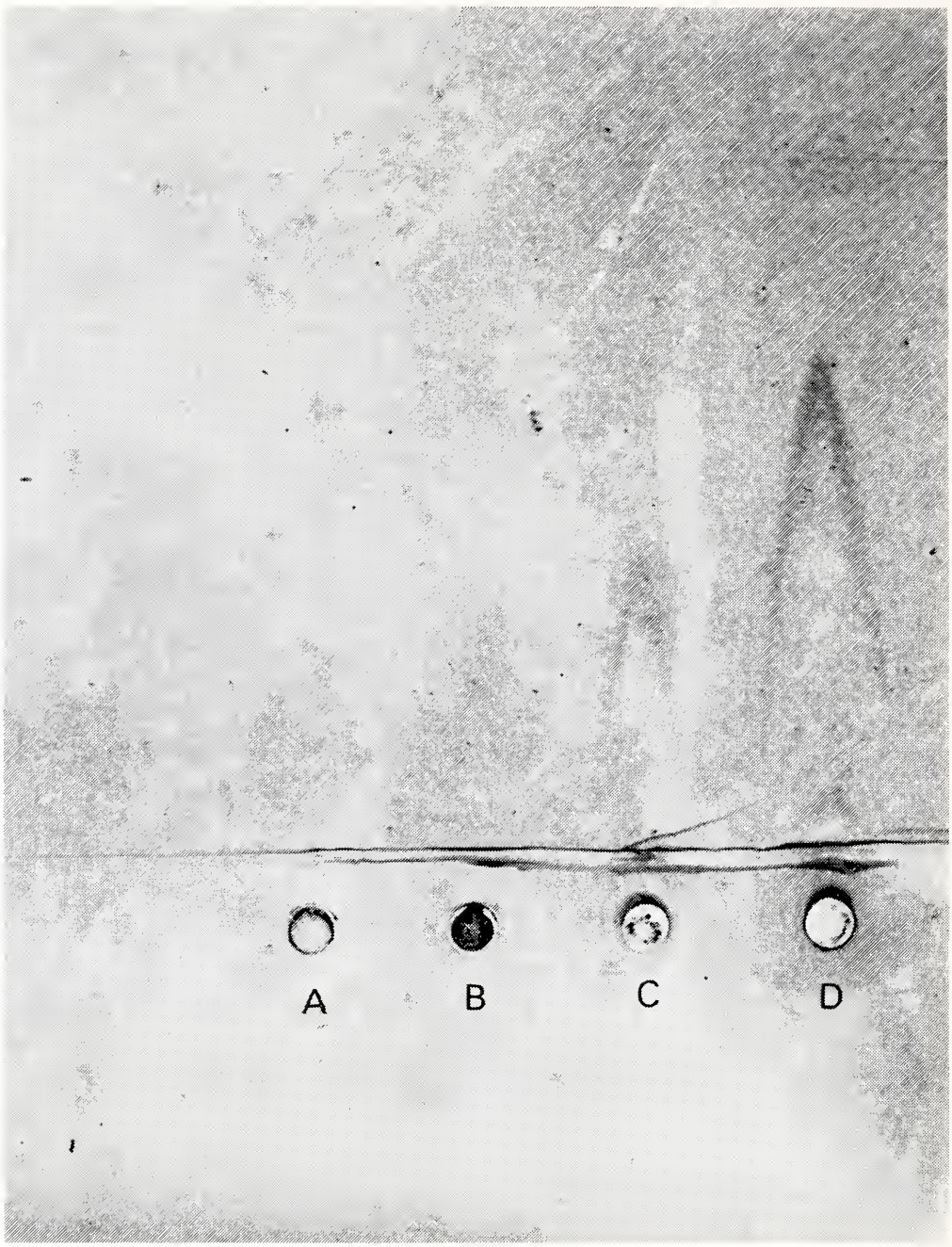

FIGURE 2. *Plots of immunoprecipitate linear spans of PrV standards (0.2mg/λ) from rocket immunoelectrophoresis. It was performed by applying 60V of field strength for 15 hr. (A) 0λ. (B) 6λ. (C) 18λ. (D) 54λ. 1λ = 0.001 ml.*

A demonstration of antigenicity in IP with IDT and a quantitation of its antigenic proteins with RIET were described above. However, identification of individual antigenic determinants presented in the immunoelectrophoretic precipitate profile was accomplished by cross immunoelectrophoresis. Analysis of individual precipitates presented in standard PrV virions is illustrated in Fig. 4. The precipitate pattern revealed four antigenic determinants designated as Ag 1, Ag 2, Ag 3 and Ag 4. The area outlined by each precipitate is proportional

to the amount of the corresponding antigenic determinant, if the concentration of antibodies in the second-dimensional gel is kept constant. Therefore, a quantitative ratio among these four antigenic determinants was determined, Ag 1:Ag 2: Ag 3:Ag 4 = 2:1:2:5. A comparison of the precipitin profile between PrV grown in intact cells and IP from enucleated cells is in progress. Any alteration of the quantitative ratio of these four antigenic determinants in IP is investigated.

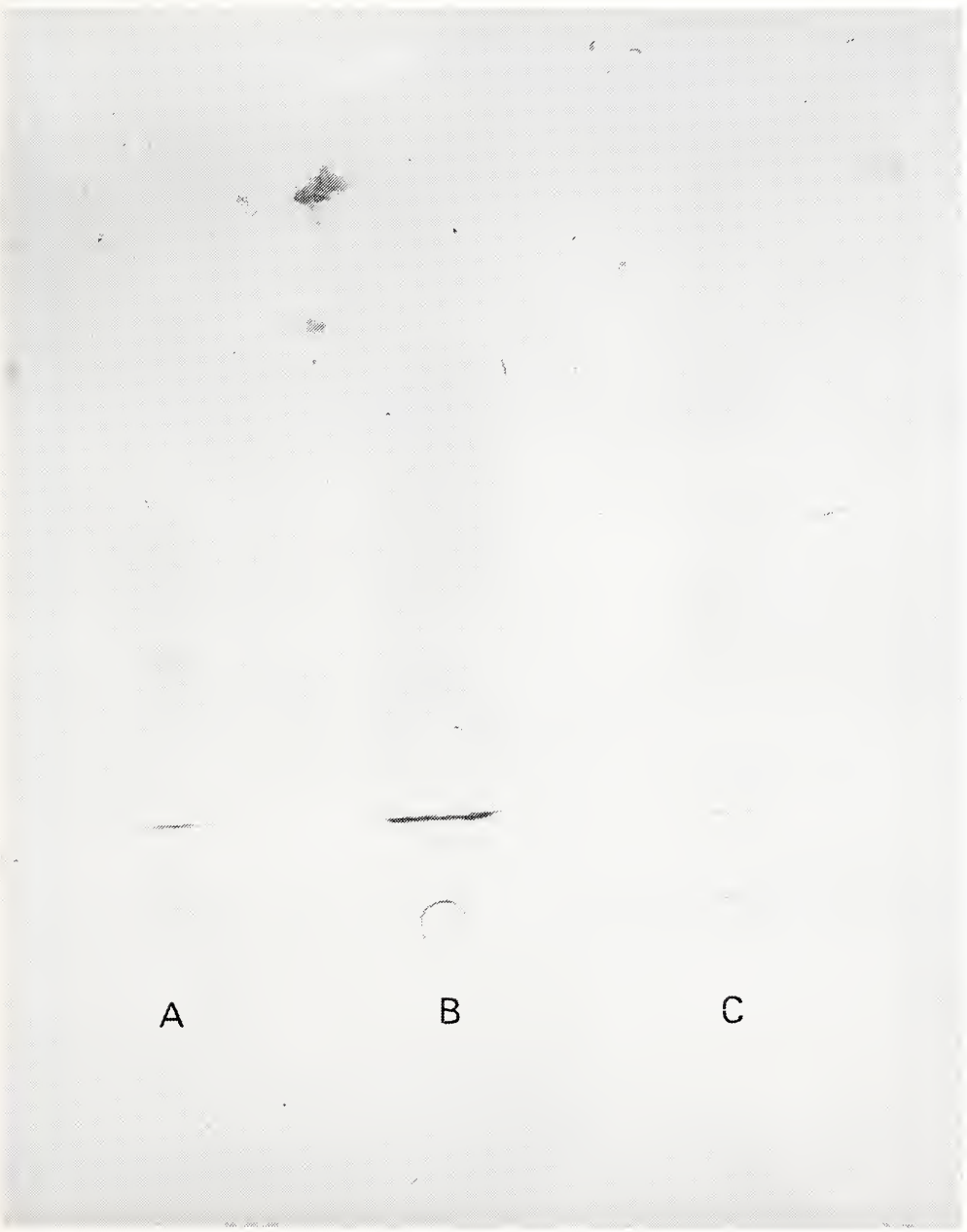

FIGURE 3. *Immunoclectrophoretic quantitation of antigenic protein in IP. (A) Control virus grown in intact cells (50λ), (B) IP grown in enucleated cells (50λ), (C) standard PrV (2mg).*

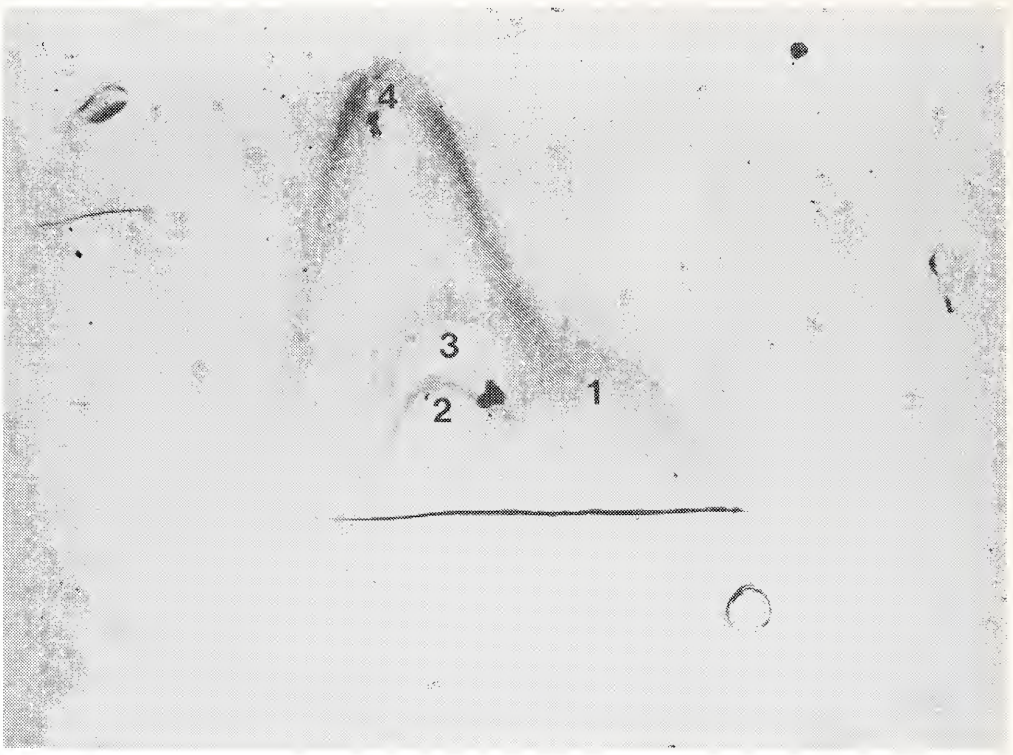

FIGURE 4. *Crossed immunoelectrophoretic patterns of standard PrV precipitating antigens. First dimensional and second dimensional electrophoresis were performed as described in materials and methods. 75λ of standard viral antigen was put into the well. 1: first antigenic determinant (Ag 1); 2: second antigenic determinant (Ag 2); 3: third antigenic determinant (Ag 3) and 4: fourth antigenic determinant (Ag 4).*

Discussion

The formation of IP in enucleated cells could be due to an alteration of the regular protein synthesis pattern, such as inhibitor modification. The pattern of herpesviral protein synthesis in enucleated host differed in several respects from those found in intact cells, was reported (3, 6).

Our results showing that IP when formed in the enucleated cells, lost its infectivity (Table 1), but retained antigenicity (Fig. 1 and 3) are reminiscent of an observation by Von Magnus (13), who found there was formation of non-infectious but hemagglutinating particles of the influenza virus.

The increase in the ability of IP to react with anti-PrV swine serum (Fig. 3) could be due either to the formation of more antigenic polypeptides in the pool of IP for reaction with anti-PrV antibodies or to the retention of critical molecular sizes or protein charges of incomplete virion in comparison with those of mature intact virions, which increase anodal electrophoretic mobility during immunoanalysis.

We have applied both a neutral detergent, Triton X-100 (0.5%) and an anionic detergent, sodium deoxycholate (0.05%) in our two dimensional (crossed) immunoelectrophoresis. The electrophoretic mobility of amphilic proteins in the virus can be altered by the so-called "charge-shift electrophoresis" (5) due to anodic migration, and the res-

olution of antigens is greatly improved in our two-dimensional immunoelectrophoresis by the incorporation of these detergents. This type of combination of "charged-shift" electrophoresis with regular two-dimensional immunoelectrophoresis thus permitted us to have a better identification of antigenic determinants.

Our previous studies of polyacrylamide gel electrophoresis of membrane-bound viral antigenic protein gave four subunits with molecular weights of 61,500, 68,000, 75,000 and 88,000 (11). In this study the crossed immunoelectrophoresis of standard PrV also indicates four antigenic determinants. It is possible that the four subunits we found in the previous study correspond to these four antigenic determinants observed in the two dimensional immunoelectrophoresis.

In summary, we can say that IP differ from the fully active standard virions by the apparent lack of infectivity (Table 1), by their capacity to inferfere with the propagation of infectious virus (Table 2), and by having a slightly larger quantity of antigenic proteins (Fig. 3).

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