PHYSICAL AND BIOLOGICAL PROPERTIES OF INFLUENZA VIRUS COMPONENTS OBTAINED AFTER ETHER TREATMENT*

BY FRED M. DAVENPORT, M.D., RUDOLF ROTT, D.V.M., AND WERNER SCHÄFER, D.V.M.

(From the Max Planck Institute für Virusforschung, Tübingen, Germany, and the Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor)

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Since Hoyle first demonstrated that influenza virus could be split with ether, a number of publications have appeared concerning the properties of the subunits derived thereby (1–13). Nevertheless, at present, knowledge of the morphologic and biologic properties of such split products is still inadequate; in part because the biologic and serologic investigations reported were, in general, not carried out with fractions of well defined physical properties.

Meanwhile, Schäfer and colleagues have extensively studied the subunits of another agent of the myxovirus group, i.e. the virus of fowl plague, and by using a modification of Hoyle's method have established the following information. Fowl plague virus can be degraded with ether to yield two distinct components. The smallest, designated originally as g antigen, but herein after referred to as internal s antigen, is an RNA-containing subunit which basically appears to be a small spherical particle about 10 to 15 mμ in diameter. In preparations of internal s antigen, elongated filaments are commonly found composed apparently of 2 to 6 such particles. In the course of these investigations, it was shown that internal s antigen derived from fowl plague virus exhibited by complement fixation a close serologic relationship to suspensions of internal s antigen obtained from the FM1 strain of influenza A'. The large subunit, designated hemagglutinin since it causes hemagglutination, is a spherical particle with a diameter of about 30 mμ. It contains carbohydrate and protein, and carries the receptor-destroying enzymatic activity of that virus. Further, it appears to contain the essential antigens needed to confer protection by vaccination (14–18).

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The purposes of the present investigation were to isolate, to define physically, and to compare serologically and biologically those subunits of influenza A and of fowl plague viruses that can be separated by applying the methods of ether fractionation and of purification that were developed for the study of fowl plague virus. This report summarizes the data obtained.

**Materials and Methods**

**Virus.**—The strains of influenza virus used were chosen from the collection of the Strain Study Center, Commission on Influenza, School of Public Health, Ann Arbor. They were: swine 1976 (1931), PR8 (A-1934), FM1 (A' -1947), PR301 (A' -1954), and AA/23 (Asian-1957). The strain of fowl plague (KP) virus used was the “Rostock” strain maintained by the Max Planck Institut für Virusforschung, Tübingen, Germany.

**Sera.**—1. Influenza antisera: (a) paired ferret antisera obtained from bleedings performed before and 2 to 3 weeks after infection were made available by the Virus Laboratory, Ann Arbor. (b) paired rabbit sera were prepared at Tübingen against preparations of the hemagglutinins using vaccines of the mineral oil emulsified type (19). Bloods were obtained before and 8 weeks after vaccination.

2. Fowl plague antisera: (a) Mouse serum, for complement fixation tests. Pooled fowl plague serum of mice was used, prepared according to the method of Schäfer (20). As a control, a pool of serum from normal mice of the same highly inbred strain was employed. (b) Chicken serum, for hemagglutination inhibition. Serum obtained after vaccination and subsequent infection with the “Rostock” strain was employed (21). Sera of normal chickens served as a control.

All sera were heated at 56°C. for 30 minutes before testing. The sera of mice were preserved in 0.05 per cent phenol.

**Preparation of Subunits.**—Purified concentrates of each virus were prepared from infected allantoic fluid by adsorption on and elution from chicken erythrocytes, followed by differential centrifugation. Treatment of the concentrates with ether at 37°C. for 8 or more hours, and separation and concentration of the subunits was performed according to the method of Schäfer and Zillig (14). The internal s antigen preparations were free from hemagglutinin as demonstrated by testing with chicken erythrocytes.

**Hemagglutination and Hemagglutination Inhibition Tests.**—These were carried out by a standard method (22) using plastic plates and a 1 per cent suspension of chicken erythrocytes. Non-specific inhibitors were inactivated by treatment of sera with three volumes of M/90 KI0 at room temperature for 1 hour. Periodate was then neutralized by the addition of an equal volume of 1 per cent glycerol in saline.

**Complement Fixation Tests.**—The micro method used was in principle that of Fulton and Dumbell (23). Chessboard experiments were performed varying complement and serum, while using a standardized amount of antigen. Each antiserum was tested with the different hemagglutinins and internal s antigens. Antiserum were diluted in twofold steps. The dilution factor of complement was 1.3. As complement, guinea pig serum preserved according to Witte was employed (24).

Each antigen was standardized against its homologous antiserum by determining the amount of complement fixed by varying dilutions of antigen in the presence of excess antibody. The dilution of each antigen that bound a standard amount of complement (1.6 mm.4) was chosen as the “standard antigen amount.”

**Electron Microscopy.**—Electron micrographs were taken by an electrostatic AEG-Zeiss instrument. Preparations were first fixed on grids in OsO4 vapor, then washed three times with distilled water, and after drying in air, shadowed with platinum-rhodium at an angle of about 25°.
Analytical Ultracentrifugation.—A Phywe air-driven analytical centrifuge equipped with the Lamm scale was used.

Electrophoresis.—Electrophoresis was performed in a Tiselius apparatus. For experimental conditions see Schäfer and Zillig (14).

Determination of Neuraminidase Activity.—The neuraminidase activity was determined according to Mohr (25). A suspension of sheep erythrocyte stromata, containing 220 µg. of neuraminic acid per ml. served as substrate. One enzyme unit corresponds to 1 µg. of neuraminic acid liberated from 1 ml. of the substrate suspension.

Solutions.—Saline refers to 0.15 M NaCl in 0.02 phosphate pH = 7.2.

EXPERIMENTAL

Morphology of the Strains of Influenza Virus Investigated as Observed by Electron Microscopy.—Preparations of influenza viruses are not identical morphologically. For example, it is well known that an unusual proportion of filamentous forms is observed with certain strains. Obviously the split products of atypical forms might be different from those of the usual spherical virus units. Therefore, it was necessary to establish the morphology of each strain before ether treatment. The electron micrographs obtained showed in all cases predominantly the familiar spherical forms of approximately 70 to 100 mÅ in diameter (Fig. 1). Filaments were present in preparations of all strains, but in low frequency. In some preparations of fowl plague, of PR8 and especially of PR301, (Fig. 2), dumbbell-shaped particles were found. It is believed that similar forms have not previously been described. The significance of this variation has not yet been clarified, but it represents an intriguing problem to investigate further.

Electron Microscopic Appearance of the Split Products.—Electron micrographs of the split products of the various influenza viruses and of fowl plague virus were indistinguishable. The presence of dumbbell-shaped forms had no discernible effect upon the appearance of the subunits. In each case internal s antigen preparations appeared as beaded filaments of variable length (Fig. 3). The filaments seem to be composed of small, spherical particles about 10 to 15 mÅ in diameter arranged linearly. Discrete particles of that size and shape are also found.

In contrast, preparations of the hemagglutinin particles were relatively homogeneous. A representative grid is reproduced in Fig. 4. The spherical particles have a diameter of about 30 mÅ.

These findings are in remarkable agreement with those reported previously (and repeated in the present study) concerning the morphology of the subunits of fowl plague virus (15). They differ from those recently reported on influenza virus by Paucker, Birch-Andersen, and VonMagnus (13) in two minor respects. The preparations of hemagglutinin obtained by those investigators were inhomogeneous in size and shape, although the predominant form was a spherical particle about 30 mÅ in diameter. Furthermore, in their preparations of internal s antigen, the beaded appearance of the filaments and the
basic small spherical forms of 10 to 15 m\(\mu\) in diameter were not apparent. In considering these discrepancies it seems important to bear in mind that the method of extraction of virus with ether used by Paucker, Birch-Andersen, and VonMagnus was less intensive than that used in the present investigation, since they extracted at room temperature for 1 hour, while in the present studies, extraction at 37°C. for a minimum of 8 hours was the rule. The possibility also exists that fine details of the morphology of internal s antigens were lost in the process of preparing the electron micrographs published by these investigators. Although comparisons were not made using both schemes for fractionation, the explanation proffered for the minor discrepancies found is that the longer extraction at higher temperature is probably the more efficient method.

*Text-Fig 1. Sedimentation characteristics of FM1 internal s antigen. Scale distance: 5 cm., 30,000 r.p.m. (35).*

_Sedimentation and Electrophoretic Characteristics of Internal s Antigen._—To
compare further the physical similarities of the internal s antigens obtained by fractionation of influenza A viruses and of fowl plague virus, a concentrate of internal s antigen, was prepared from a large pool of FM1-infected allantoic fluid. In Text-fig. 1 the sedimentation characteristics of this concentrate are depicted. The results are analogous to those previously found with fowl plague virus (14). In the case of FM1 three components could be recognized. The slowest moving component sedimented at an $S_{20}$ value of about 17S. The fastest at an $S_{20}$ value of about 43S. The third gradient did not separate clearly and therefore the corresponding values could not be calculated. The heterogeneity observed in the centrifugal field indicates that the inhomogeneity found by electron microscopy was not an artifact produced by preparation of the grids. Electrophoretically only one gradient was observed, migrating with a velocity of about $-5.5 \times 10^{-5}$ cm$^2$ X V$^{-1}$ sec$^{-1}$ in 0.5 per cent NaCl M/100 phosphate buffer solution (ionic strength about 0.1 pH 7.0). The velocity of this gradient was like that of the corresponding gradient described using a preparation of internal s antigen derived from the Rostock strain of fowl plague (14).

**TABLE I**

<table>
<thead>
<tr>
<th>Material examined</th>
<th>Hemagglutinin derived from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swiss</td>
</tr>
<tr>
<td>Hemagglutinin Virus</td>
<td></td>
</tr>
<tr>
<td>HA units/ml.</td>
<td>16,000</td>
</tr>
<tr>
<td>Neuraminidase units ml.</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>330</td>
</tr>
</tbody>
</table>

Enzymatic Activity of the Hemagglutinin of Different Influenza Strains.—Several workers have shown that the enzymatic activity of influenza viruses is carried by the hemagglutinin (2, 7). In the case of fowl plague virus it was shown that the neuraminidase activity of the intact virus particle, was coupled with the hemagglutinin fraction obtained by ether treatment and concentrated by subsequent centrifugation (14, 32). Using the same procedure the hemagglutinin of some strains of influenza virus unexpectedly failed to elute spontaneously or did so poorly even though incubated at 37°C for 6 hours. In these cases, elution of the hemagglutinin was achieved by the addition of receptor-destroying enzyme$^1$ and incubation at that temperature for 3 hours. In all instances the corresponding viruses showed good enzymatic activity (cf. Table I). Because of these intriguing findings, differences in the enzymatic activity of the various influenza hemagglutinins were quantitatively studied.

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$^1$ Supplied by Behringwerke, Marburg, Germany.
using the neuraminidase test of Mohr (25). The results are shown in Table I. From the values derived, Asian and swine virus hemagglutinin appeared to possess the highest neuraminidase activity. The activity of PR8 hemagglutinin was distinctly lower. That of FM1 and PR301 strains was practically non-existent. The findings were not investigated further; therefore at present it cannot be decided whether the enzymatic activity of each virus is inherently different, is coupled to the corresponding hemagglutinin with a different degree of affinity, or whether the enzymatic activity of these strains was degraded at different rates by the ether treatment used. The last explanation is thought to be more likely. Hoyle has also observed degradation of enzymatic activity after ether treatment (2).

Other Biologic Activities of Preparations of Influenza Subunits.—Infectivity for eggs in terms of intact virus activity was found to be either completely abolished or reduced by more than 10 to 100 millionfold in samples of the hemagglutinin and internal s antigens. Regularly, hemagglutinin preparations titered 4 to 8 fold higher with guinea pig erythrocytes than with chicken erythrocytes. The hemagglutinins derived from all strains were heat-stable when incubated at 56°C for 30 minutes. These findings are in agreement with those recorded previously (2). Not previously reported is the finding that suspensions of either hemagglutinin or internal s antigen were non-toxic for mice when given intracerebrally or intranasally in high concentration.

Serologic Properties of Subunits of Influenza and Fowl Plague Viruses—Hemagglutinin.—The unique antigenic diversity of the influenza strains studied, as measured by hemagglutination inhibition, offered the opportunity to

### TABLE II
**Similarity of Hemagglutination Inhibition Antibody Levels Determined in Convalescent Antisera with Virus and with the Corresponding Hemagglutinin**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Anti Serum</th>
<th>Swine 1931</th>
<th>PR8 1934</th>
<th>FM1 1947</th>
<th>PR301 1954</th>
<th>Asian AA/23 1957</th>
<th>KP (Rostock)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>V</em></td>
<td>H</td>
<td>V</td>
<td>H</td>
<td>V</td>
<td>H</td>
<td>V</td>
</tr>
<tr>
<td>Swine</td>
<td>1024</td>
<td>—</td>
<td>2048</td>
<td>—</td>
<td>32</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>PR8</td>
<td>—</td>
<td>—</td>
<td>2048</td>
<td>2048</td>
<td>8</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>FM1</td>
<td>16</td>
<td>32</td>
<td>16</td>
<td>32</td>
<td>2048</td>
<td>2048</td>
<td>32</td>
</tr>
<tr>
<td>PR301</td>
<td>—</td>
<td>—</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>1024</td>
</tr>
<tr>
<td>Asian</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>256</td>
</tr>
<tr>
<td>KP</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* V, virus.

H, hemagglutinin component of virus.

—, antibody not detected in antiserum diluted 1/8.
discover whether the method employed for fractionation and purification of hemagglutinins had altered the antigenic specificity of the products. Three possibilities were entertained. The extraction and purification procedures might either degrade the reactivity of the hemagglutinin, release and make apparent antigens submerged when the intact virus particle was used for testing, or have no influence upon the results of hemagglutination-inhibition measurements. To examine these questions, simultaneous hemagglutination inhibition tests were performed with virus concentrates and the hemagglutinins derived therefrom using appropriate specific sera. In no case was inhibition caused by normal serum at a dilution of 1:8. The results with the convalescent antisera are shown in Table II.

TABLE III
Specific Complement Fixation Activity of the Various Hemagglutinins Related to HA Units and N Content

<table>
<thead>
<tr>
<th>Hemagglutinin derived from:</th>
<th>N/ml in gm.</th>
<th>HA titer (− log)</th>
<th>Dilution yielding 1 CHS standard dose</th>
<th>Calculated HA titer/standard dose</th>
<th>N ag/standard dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine</td>
<td>12.0 × 10⁻⁴</td>
<td>4.5</td>
<td>1/320</td>
<td>97</td>
<td>0.37</td>
</tr>
<tr>
<td>PR8</td>
<td>41.0 × 10⁻⁴</td>
<td>5.1</td>
<td>1/1000</td>
<td>125.9</td>
<td>0.41</td>
</tr>
<tr>
<td>FM1</td>
<td>28.8 × 10⁻⁴</td>
<td>4.8</td>
<td>1/512</td>
<td>123.5</td>
<td>0.56</td>
</tr>
<tr>
<td>PR301</td>
<td>6.4 × 10⁻⁴</td>
<td>3.6</td>
<td>1/64</td>
<td>63.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Asian</td>
<td>5.6 × 10⁻⁴</td>
<td>3.9</td>
<td>1/64</td>
<td>124.0</td>
<td>0.87</td>
</tr>
<tr>
<td>KP</td>
<td>40.0 × 10⁻⁴</td>
<td>5.4</td>
<td>1/2000</td>
<td>126.0</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Clearly, the results demonstrate that the isolated hemagglutinins react in the hemagglutination inhibition test with homologous and heterologous antisera to essentially the same degree and extent as do the corresponding strains of virus. No evidence for release of hidden antigens or of degradation of the characteristic major antigens of each strain by ether treatment was obtained. Fowl plague virus and its hemagglutinin failed to exhibit cross-reactions with any of the influenza strains tested. The titer found with fowl plague virus was lower than that obtained with the hemagglutinin. This result is not characteristic. In a subsequent experiment with the same serum it could be shown that there was no detectable difference.

The serologic specificity of the hemagglutinin was next examined by complement fixation. The standard dose found for each hemagglutinin in the respective experiments is correlated with HA-units and amounts of N in Table III. It appears that the standard doses of the various hemagglutinins contained within reasonable limits, similar concentrations of HA units and of nitrogen.

The results of the chess board complement fixation tests are demonstrated in Text-fig. 2 in which the serologic relations of the various antigens can be
Text-Fig. 2. Results of chessboard complement fixation tests obtained with the various hemagglutinins.
estimated from the areas circumscribed by the respective curves. The areas were measured by planimetry and the degree of complement fixation activity then expressed as index numbers. The reaction with the homologous antigen is designated as index 1. Each of the other index numbers was calculated by dividing the area obtained when a heterologous antigen was used by the area obtained with the homologous antigen. Since cross-reactions were not reciprocal, for convenience appropriate pairs of index numbers were averaged (23). The values calculated are shown in Table IV.

As can be readily seen by inspection of the curves presented in Text-fig. 2, the homologous hemagglutinin shows in each case a much stronger reaction than the heterologous. Thus the hemagglutinin subunit obtained reacted by complement fixation in a highly strain specific manner. The mean index values calculated emphasize this fact (Table IV). In the reactions with heterologous sera the index decreases in general to a value between 0.10 and 0.25. Some indication of a closer antigen relation is observed between Asian and PR8, and between Asian and KP viruses for which index numbers 0.40 and 0.41 were found. No corresponding crossing was observed in hemagglutination inhibition. The results of hemagglutination inhibition and complement fixation reactions fit well in the case of FM1 and PR301 where, by complement fixation, a mean index value of 0.39 was found.

One might question that the cross-reactions observed were caused by some internal s antigen contained as a contaminant in the preparations of hemagglutinin. However, this component was not found in electron micrographs of the preparations nor was nucleic acid demonstrable spectrophotometrically. Hence the cross-reactions observed are interpreted as a demonstration of the sharing of common antigens among the hemagglutinins of the type A viruses studied. The strong cross-reaction found by complement fixation between

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Swine</th>
<th>PR8</th>
<th>FM1</th>
<th>PR301</th>
<th>Asian</th>
<th>KP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine</td>
<td>1</td>
<td>0.19</td>
<td>0.17</td>
<td>0.08</td>
<td>0.14</td>
<td>0.11</td>
</tr>
<tr>
<td>PR8</td>
<td>*0.19</td>
<td>1</td>
<td>0.22</td>
<td>0.24</td>
<td>0.40</td>
<td>0.17</td>
</tr>
<tr>
<td>FM1</td>
<td>0.17</td>
<td>0.22</td>
<td>1</td>
<td>0.39</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>PR301</td>
<td>0.08</td>
<td>0.24</td>
<td>0.39</td>
<td>1</td>
<td>0.29</td>
<td>0.13</td>
</tr>
<tr>
<td>Asian</td>
<td>0.14</td>
<td>0.40</td>
<td>0.17</td>
<td>0.29</td>
<td>1</td>
<td>0.41</td>
</tr>
<tr>
<td>KP</td>
<td>0.11</td>
<td>0.17</td>
<td>0.17</td>
<td>0.13</td>
<td>0.41</td>
<td>1</td>
</tr>
</tbody>
</table>

* Ratio of complement fixation expressed as the arithmetic mean of corresponding index numbers.

TABLE IV

Serologic Comparison by Complement Fixation of Hemagglutinins of the Influenza A and Fowl Plague Strains

- Ratio of complement fixation expressed as the arithmetic mean of corresponding index numbers.
Asian, PR8, and KP hemagglutinins was unexpected. The findings suggest that the hemagglutinins of these viruses may be more closely related than heretofore was appreciated.

*Internal s Antigens.*—It has generally been accepted that the soluble antigen derived from infected chorio-allantoic membrane or its counterpart, the internal s antigen of influenza A viruses, does not react strain-specifically in complement fixation tests (26-29, 11). Yet there is at least one reason for challenging this view by further experimentation. This resulted from the concept that the internal s antigen is the nucleoprotein that directs the infected cell to produce a virus with the unique serologic properties that characterize its hemagglutinin. Hence, if the hemagglutinins of the progeny were serologically different, one might expect that the parent internal s antigens would be different and that the difference might be serologically distinguishable under the proper conditions.

To explore this possibility, complement fixation tests were performed with the internal s antigen preparations in the same manner as with the hemagglutinins. Each antiserum was tested with standard doses of the various internal s antigen suspensions.

The correlations between standard doses of internal s antigens and amounts of nitrogen can be seen in Table V. It is apparent that the preparations of swine, PR8, FM1, and PR301 internal s antigen possessed similar specific complement-fixing activities with respect to N content. Those of Asian and of KP viruses were somewhat more potent.

The results found with internal s antigens in the chessboard complement fixation test (Text-fig. 3) were somewhat different from those obtained with hemagglutinins. In the reactions with hemagglutinins the complement fixation activity of the homologous antigen is always markedly superior to those of the heterologous, whereas with internal s antigens the differences between the homologous and heterologous preparations were less prominent. Nevertheless, it is clear that the strongest reaction was with the homologous internal s antigen.

### Table V

<table>
<thead>
<tr>
<th>Internal s antigen derived from:</th>
<th>N/ml. in gm.</th>
<th>Dilution yielding 1 CF standard dose</th>
<th>N μg./standard dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine</td>
<td>10.4 × 10^-5</td>
<td>1/300</td>
<td>0.34</td>
</tr>
<tr>
<td>PR8</td>
<td>13.2 × 10^-5</td>
<td>1/300</td>
<td>0.44</td>
</tr>
<tr>
<td>FM1</td>
<td>16.8 × 10^-5</td>
<td>1/350</td>
<td>0.47</td>
</tr>
<tr>
<td>PR301</td>
<td>6.0 × 10^-5</td>
<td>1/64</td>
<td>0.93</td>
</tr>
<tr>
<td>Asian</td>
<td>9.6 × 10^-5</td>
<td>1/500</td>
<td>0.19</td>
</tr>
<tr>
<td>KP</td>
<td>23.6 × 10^-5</td>
<td>1/2500</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Text-Fig. 3. Results of chessboard complement fixation tests employing the various internal s antigens.
According to Text-fig. 3 the most pronounced antigenic differences from the other strains was observed with swine and KP antigens.

Table VI, in which the mean values of indices are recorded, shows the relationships more exactly. It is obvious that in general much stronger cross-reactions were found between the internal s antigens than between the hemagglutinins. With internal s antigen preparations index values of 0.40 to 0.80 are predominant, whereas with the hemagglutinins most indices ranged between 0.10 and 0.25. Values as low as 0.32 were found in the cross-reactions with internal s antigens derived from swine and the influenza strains of human origin. KP antigen apparently takes a position between, with indices ranging from 0.29 to 0.62.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Swine</th>
<th>PR8</th>
<th>FM1</th>
<th>PR301</th>
<th>Asian</th>
<th>KP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine</td>
<td>1</td>
<td>0.37</td>
<td>0.32</td>
<td>0.36</td>
<td>0.40</td>
<td>0.62</td>
</tr>
<tr>
<td>PR8</td>
<td>*0.37</td>
<td>1</td>
<td>0.69</td>
<td>0.61</td>
<td>0.77</td>
<td>0.62</td>
</tr>
<tr>
<td>FM1</td>
<td>0.32</td>
<td>0.69</td>
<td>1</td>
<td>0.61</td>
<td>0.73</td>
<td>0.62</td>
</tr>
<tr>
<td>PR301</td>
<td>0.36</td>
<td>0.61</td>
<td>0.61</td>
<td>1</td>
<td>0.81</td>
<td>0.29</td>
</tr>
<tr>
<td>Asian</td>
<td>0.40</td>
<td>0.77</td>
<td>0.73</td>
<td>0.81</td>
<td>1</td>
<td>0.45</td>
</tr>
<tr>
<td>KP</td>
<td>0.62</td>
<td>0.62</td>
<td>0.62</td>
<td>0.29</td>
<td>0.45</td>
<td>1</td>
</tr>
</tbody>
</table>

* Ratio of complement fixation expressed as the arithmetic mean of corresponding index numbers.

From the results of complement fixation as presented in Text-fig. 3 and Table VI one may conclude that all the influenza internal s antigens tested have a distinct proportion of their antigenic structure in common.

It might be questioned whether the apparent serologic specificity found with the internal s antigen suspensions used could be an artifact traceable to the presence of small contaminating amounts of the corresponding hemagglutinin which also reacts strain specifically in the complement fixation test (see above). That possibility seems largely excluded by the finding that one standard dose of the several hemagglutinins contained a minimum of 64 and a maximum of 131 hemagglutinating units, and it will be recalled all internal s antigen preparations were devoid of hemagglutinating activity. Hence, the conclusions seem justified that in these complement fixation tests a serologic difference in the internal s antigens, which are the nucleoproteins (2, 14) derived from each strain of virus studied, is being measured. Otherwise the existence of a non-hemagglutinating, serologically strain-specific component must be postulated to explain our results.
To exclude this possibility, internal s antigen of FM1 was purified electrophoretically by separating the material migrating in the gradient. For similar purposes, internal s antigen of swine strain was processed by a further cycle of high speed centrifugation. By preparative electrophoresis the specific complement fixation activity of the FM1 internal s antigen was not changed significantly when related to N content per standard dose (Table VII). Furthermore, neither purification procedure changed the reactivity of the respective internal s antigens in the complement fixation tests, as shown by trials with the homologous and heterologous sera (Table VIII). The arithmetic mean of the index

<table>
<thead>
<tr>
<th>Table VII</th>
<th>Specific Complement Fixation Activity of Internal s Antigen Prepared according to the Normal Scheme and after Electrophoretical Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal s antigen</td>
<td>N/ml in gm.</td>
</tr>
<tr>
<td>FM1 standard</td>
<td>110</td>
</tr>
<tr>
<td>FM1 electroph.</td>
<td>32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table VIII</th>
<th>Arithmetic Means of the Index Numbers from Purified Internal s Antigens Derived from FM1 and Swine Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
<td>Antiserum</td>
</tr>
<tr>
<td>FM1</td>
<td>1</td>
</tr>
<tr>
<td>Swine</td>
<td>0.31</td>
</tr>
</tbody>
</table>

numbers are essentially the same as in the experiment described in Table VI. Thus the differences observed between the various internal s antigens are not readily explained by the presence of impurities.

Our findings are at variance with those of Lief, Fabiyi, and Henle (11) who have reported that internal s antigens prepared from many strains of influenza A were serologically indistinguishable by complement fixation. This discrepancy may be explainable either by the fact that the method of testing used in the experiments reported herein is more sensitive than that employed by Lief et al., that the viruses studied by us were antigenically more limited than the many strains studied by them, or that differences in the intensity and/or duration of ether treatment resulted in preparations of different serologic specificity. It seems highly probable that our success in demonstrating the serologic specificity of the internal s antigens can be attributed in part to the more delicate method used for analysis and to the high degree of refinement of
the suspension tested. Whatever the final explanation may prove to be, the demonstration that differences can be measured serologically in the nucleoproteins isolated from several strains of type A influenza virus is believed to be a unique and important finding. Grist's observation (30) that an element of strain specificity is demonstrable when soluble antigens prepared from infected egg membranes are used to test the acute and convalescent sera of infants, support the interpretation offered.

Comparison of the Antigenicity of the Influenza Viruses and Their Hemagglutinins.—Because influenza viruses have been widely used for protection by vaccination against influenza, and because Schäfer (17) has shown that the hemagglutinin isolated from KP virus induced immunity when used as a vaccine, it was of special interest to test the capacity of these viruses and their corresponding hemagglutinins to induce antibody by vaccination. At this stage of the investigation, materials were in short supply and therefore only preliminary observations could be attempted. For this purpose, a volume of 0.3 ml. of either virus or hemagglutinin was mixed with an equal volume of formalin diluted 1:2000. After holding for several days at 4°C, the mixtures were emulsified mechanically (19) with an equal volume of mineral oil and emulsifier compounded so as to contain 9 parts of the former and 1 of the latter. A single rabbit was inoculated intramuscularly in each thigh with 0.3 ml. of a vaccine. The sera were tested against the isolated hemagglutinins, and the results are reproduced in Table IX. It should be noted that the hemagglutination titer of stock aqueous suspensions used for the vaccines was not uniform. While anti-

<table>
<thead>
<tr>
<th>Test hemagglutinin</th>
<th>Swine 1931</th>
<th>PR8 1934</th>
<th>PR301 1954</th>
<th>Asian AA/23 1957</th>
<th>KP (Rostock)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V H V H V H V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swine 1931</td>
<td>256</td>
<td>8</td>
<td>32</td>
<td>64</td>
<td>---</td>
</tr>
<tr>
<td>PR8 1934</td>
<td>&gt;4096</td>
<td>512</td>
<td>1024</td>
<td>128</td>
<td>---</td>
</tr>
<tr>
<td>PR301 1954</td>
<td>32</td>
<td>64</td>
<td>64</td>
<td>8</td>
<td>---</td>
</tr>
<tr>
<td>Asian AA/23 1957</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>8</td>
<td>2048</td>
</tr>
<tr>
<td>KP (Rostock)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

* V, virus.
H, hemagglutinin component of virus.
—, antibody not detected in antiserum diluted 1/8.
body levels induced by vaccination with the more potent swine hemagglutinin and the Asian virus preparations were higher than those obtained after inoculation of their counterparts, this difference was not found constant with the PR8 vaccines, and the results after vaccination with equivalent amounts of PR301 virus or hemagglutinin seemed the same. From these limited data the suggestion is made that the isolated hemagglutinin might be as effective as the corresponding virus for stimulating hemagglutination inhibition antibodies when given by vaccination.

DISCUSSION

Classification of animal viruses is at present an inexact art in which cognizance has been taken of the morphology of intact viruses, their host range, the disease pictures they produce, their epidemiology, their serologic relationship, etc. The results of the present investigation suggest an additional parameter that may prove to be useful; i.e., the morphology and the reactivity of their component parts. Thus the demonstration that the hemagglutinin and the internal s antigen subunits isolated from strains of influenza A have the same morphology as the corresponding components of fowl plague virus and the finding that the internal s antigen of fowl plague virus cross-reacts serologically with the internal s antigens of each of the known families of influenza A viruses, clearly lends additional support for classifying fowl plague virus in the same category with group A influenza virus (31). According to Rott and Schäfer the same can be stated for virus N (32). At present, only limited information is available concerning the morphology and serology of subunits of other strains of influenza-like viruses isolated from animals; i.e., the horse and duck influenza viruses (32) and NDV (33).

The description given of the morphologic, physical, and biologic properties of the subunits obtained after ether fractionation of these prototype strains of type A influenza viruses represents a comprehensive effort towards investigating their basic relationships. Taken together with the results of other studies on fractionation with ether of influenza and of fowl plague virus (2-18), they are interpreted as follows: influenza A viruses possess a lipid-containing surface which is disrupted by ether yielding at least two well defined subunits. The one, called hemagglutinin is a spherical particle about 30 μ in diameter. It apparently is a carbohydrate protein complex (6, 17) and carries the hemagglutinating and enzymatic activity of the intact virus particle. It induces hemagglutinating inhibiting antibodies when given by vaccination. Since the presence of hemagglutination-inhibiting antibodies correlates well with resistance against influenza, the way seems open for developing refined influenza virus vaccines that would contain a higher proportion of the essential protective antigens of influenza viruses and possibly might produce a lower rate of undesirable febrile reactions. The substance responsible for the "toxic" properties
of influenza viruses is as yet unidentified but the demonstration in the present experiments that the isolated hemagglutinins or internal s antigens were not toxic for mice when given intracerebrally or intranasally lends encouragement for further exploration of that possibility.

The internal s antigens of influenza A viruses are nucleoproteins (6, 17) and appear in suspensions prepared after ether extraction as filaments of variable length, apparently composed of multiples of an approximately spherical unit about 15 m$\mu$ in diameter. Soluble antigen preparations obtained from different strains of group A influenza viruses have previously been considered to be serologically indistinguishable by complement fixation (26–29, 11) but the results of the present investigation establish that the internal s antigens of the viruses studied may be distinguished serologically. Presumably antigenic specificity is conferred by the protein portion of the complex, a conclusion that implies that the different nucleic acids of the several viruses are coupled to antigenically different proteins. Such an event would not be surprising if current concepts are correct that the nucleic acid of viruses, including those of influenza A (34) are the essential infective unit, and therefore might well be expected to be capable of directing the synthesis of their own carrier proteins. Finally, the findings encourage an immunologic approach to the study of nucleoproteins.

**SUMMARY**

The Rostock strain of fowl plague, the swine, A, A', and Asian strains of influenza A as well as their hemagglutinin and internal s antigen subunits obtained after ether splitting, were found to be morphologically indistinguishable when examined simultaneously. Hemagglutinin fractions reacted in a highly strain specific manner when tested by hemagglutination inhibition or by complement fixation using sera obtained after infection. With the same sera internal s antigen fractions were shown to be serologically distinguishable by complement fixation. This observation may stimulate interest in the feasibility of employing immunologic techniques for the study of nucleoproteins. The significance of the findings reported is discussed.

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pestviren als Modell. I. Die komplementbindenden Antigene bei der Klassischen
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EXPLANATION OF PLATE 72

Fig. 1. Shadowed preparation of Asian influenza virus. × 30,000.

Fig. 2. Shadowed preparation of PR301 strain showing dumbbell shaped forms. × 30,000.

Fig. 3. Shadowed preparation of internal s antigen obtained from Asian strain. × 30,000.

Fig. 4. Shadowed preparation of hemagglutinin obtained from swine strain. × 30,000.
(Davenport et al.: Influenza virus components after ether treatment)