Presence of Haemagglutinin in the Envelope of Extracellular Vaccinia Virus Particles

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SUMMARY

The relationship of vaccinia haemagglutinin (HA) to extracellular enveloped virus (EEV) was examined. EEV banded in cesium chloride gradients at a density of 1.23 to 1.24 g/ml coincident with a peak of HA activity. EEV of an HA+ vaccinia strain showed greater than 90% adsorption to rooster red blood cells (RBCs) as detected by infectivity and ³H-thymidine labelling whereas intracellular naked virus (INV) of the HA+ strain and EEV of an HA- strain failed to show significant adsorption. The adsorption was specifically inhibited by antiserum to vaccinia. Adsorption kinetic experiments demonstrated a lack of temperature dependence on the total amount of EEV adsorbed. No elution of EEV from RBCs could be detected. The capacity of EEV to adsorb to RBCs was found to be stable at 56°C for 30 min.

INTRODUCTION

The production of haemagglutinin during vaccinia infection was first described by Nagler (1942). Early studies showed the vaccinia haemagglutinin (VHA) to be of a lipoprotein nature (Chu, 1948; Stone, 1946) composed of a virus antigen which conferred serological specificity and a lipid which was responsible for attachment to the red blood cell (Burnet, 1946). This duality was recently confirmed by reconstitution of an active VHA from the two components separated by ether/ethanol extraction (Smith, Pratt & Baxby, 1973).

The relationship of the VHA to the infectious particle has been studied by numerous investigators. The dissociation of VHA from vaccinia virus has been demonstrated by the absence of virus particle adsorption to VHA-sensitive red blood cells (Burnet & Stone, 1946; Blackman & Bubel, 1972). Physicochemical studies using differential centrifugation (Burnet & Stone, 1946; Chu, 1948), density gradient centrifugation (Neff, Ackerman & Preston, 1965), column chromatography (McCrea & O’Loughlin, 1959) and polyacrylamide electrophoresis (Weintraub & Dales, 1974) have all demonstrated that the VHA is not a component of the vaccinia virion.

The studies recounted above were performed entirely with intracellular vaccinia virus particles obtained by disruption of infected cells. However, Turner & Squires (1971) and Appleyard, Hapel & Boulter (1971) recently provided evidence that extracellular virus particles were antigenically different from intracellular virus. Furthermore, the latter authors visualized by electron microscopy the presence of an envelope on extracellular virus that was absent from intracellular virus. These two findings encouraged us to explore the possible relationship of VHA to the envelope of extracellular virus particles. In this paper, we refer to the two virus populations of intracellular naked virus and extracellular enveloped virus as INV and EEV respectively.
METHODS

Viruses. The virus strains IHD-J (VHA+) and IHD-W (VHA-) were generously supplied by Dr S. Dales (The Public Health Research Institute, New York, N.Y.). They were grown in roller bottle monolayers of KB cells cultured in Eagle’s MEM plus 5% calf serum. Cells were infected with 5 p.f.u./cell for 1 h at 37 °C and were then incubated in growth medium containing 3 μCi/ml ³H-thymidine for 24 h, at which time the cells and supernatant medium were separated by centrifugation. INV was released from the cells by suspension of the cells in distilled water (2 x 10⁷ cells/ml) for 10 min at room temperature followed by Dounce-homogenization (5 strokes). Nuclei and large debris were removed by centrifugation at 3000 rev/min for 10 min. EEV was concentrated from the growth medium by centrifugation at 10000 rev/min for 45 min in an SW 27 rotor. The resulting pellets were resuspended in PBS. INV and EEV were purified by centrifugation in an SW 40 rotor at 30000 rev/min for 3 h on caesium chloride gradients formed by pre-layering 1.30 (3 ml), 1.25 (4 ml) and 1.20 g/ml (5 ml) caesium chloride solutions. The INV and EEV bands were harvested and dialysed against PBS. DMSO was added to the dialysed virus to a final concentration of 10% and the virus frozen at -20 °C until required.

Antisera. Antisera to IHD-W and IHD-J were produced by scarification of rabbits with INV purified from RK-13 cells as described above for virus grown in KB cells. Each rabbit received 1 x 10⁸ p.f.u. Scarifications with the two strains were performed on different days in separate rooms and the rabbits were housed separately to eliminate cross-immunization. Antisera were collected 35 days post scarification.

Plaque and plaque reduction assays. Tenfold dilutions of virus were made in a Hepes-buffered lactalbumin medium and 0.1 ml plated on to monolayers of RK-13 cells in 35 mm plastic Petri dishes. The dishes were incubated at 37 °C for 1 h with occasional rocking. At the end of the adsorption period, 3 ml of a Hepes-buffered lactalbumin medium containing 5% calf serum and 0.3% agarose was added to each dish and incubation continued at 37 °C. Plaques 1 to 2 mm in diam. were counted at 48 h p.i. without staining.

Neutralization titres (NT) of antisera were determined by plaque reduction. Twofold dilutions of antisera were reacted with an equal volume (0.1 ml) of EEV for 2 h at 37 °C and then plaque as described above. Between 50 and 100 plaques/plate were obtained with untreated EEV. Titres were expressed as the serum dilution giving a 50% plaque reduction.

Haemagglutination (HA) and haemagglutination inhibition (HI) tests. HA titrations were performed by serial twofold dilutions in 0.05 ml PBS in microtitre plates and readings were taken 1 h after the addition of an equal volume of 0.5% rooster RBCs suspended in PBS plus 1% normal rabbit serum (NRS). For determination of HI activity serial twofold dilutions of antisera in 0.025 ml were made and incubated for 1 h at room temperature with 4 haemagglutinating units (HAU) of VHA followed by the addition of RBCs. HA and HI titres are shown as the reciprocal of the last dilution showing clearly demonstrable activity.

Measurement of virus adsorption to RBCs. The following adsorption conditions were used except where otherwise indicated. Washed VHA sensitive rooster and VHA non-sensitive monkey RBCs were suspended at a 10% concentration in PBS (Ca²⁺ and Mg²⁺ free) plus 2% NRS just prior to use. The RBCs were pre-incubated at 37 °C for 10 min before the addition of an equal vol. of ³H-thymidine-labelled virus material. Incubation was then continued at 37 °C for 1 h on a rocking apparatus. All experiments were conducted with between 1500 and 5000 ct/min per reagent mixture (sp. act. 1500 to 3000 p.f.u./ct/min). After incubation the RBCs were sedimented at 1500 rev/min for 4 min and the supernatant fluid carefully removed. The supernatant fluid was sampled for infectivity by removal of 10 µl.
Enveloped vaccinia and haemagglutinin

Fig. 1. Fractionation of a CsCl gradient after centrifugation of EEV at 30,000 rev/min in an SW 40 rotor for 3 h as described in Methods. Fractions were tested for density (■ — ■), infectivity (○ — ○) and HA (■ — ■).

which was diluted in 1.0 ml of PBS + 1% calf serum and frozen until plaque-assayed. The remainder of the supernatant fluid was tested for radioactivity by filtration through 22 μm Millipore filters followed by a 70% ethanol wash. This procedure trapped the virus particles on the filter. The VHA-insensitive monkey RBCs were exposed to virus parallel to the VHA-sensitive rooster RBCs. The specific adsorption is therefore expressed as the percent reduction of supernatant virus by rooster RBCs compared to monkey RBCs.

Electron microscopy. Virus particles from caesium chloride gradients were dialysed against PBS overnight before electron microscopic examination. Particles were applied to carbon-coated formvar films and negatively stained with 1% uranyl acetate, pH 4.5.

RESULTS

Density gradient analysis of EEV and VHA

Virus concentrated from growth medium of roller bottles by pelleting was centrifuged on caesium chloride gradients as described in Methods. Three bands of light-scattering material could be seen. Measured by infectivity and HA activities the material separated into three populations (Fig. 1). The band nearest the bottom of the tube appeared at a density of 1.26 to 1.27 g/ml and contained some infectious virus but no detectable HA. Electron microscopy showed this band to contain naked virus particles (Fig. 2a). These particles represented only 9% of the total infectivity in the gradient. This band was absent from CsCl gradients of virus grown in stationary cultures. The band in the middle of the tube appeared at a density of 1.23 to 1.24 g/ml. It contained 55% of the total infectivity coincident with an HA peak representing less than 10% of the total HA. This population of infectious virus was composed largely of enveloped virus particles (Fig. 2b). The uppermost band appeared at the interface
Fig. 2. Electron micrographs of virus particles from a caesium chloride gradient prepared as described in Methods. (a) Naked virus particles and (b) EEV particles with densities of 1.26 to 1.27 and 1.23 to 1.24 g/ml respectively.

between the starting material and the density gradient and contained the largest portion (75%) of the HA activity in the gradient, but only a small amount (16%) of infectious virus. Thus a small portion of the total VHA had the same density as EEV while the majority of the VHA was separable from EEV.
Enveloped vaccinia and haemagglutinin

Table I. The capacity of EEV and INV particles from VHA+ and VHA- vaccinia strains to adsorb to RBCs*

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Virus strains,†</th>
<th>type of particles</th>
<th>RBCs used for adsorption‡</th>
<th>p.f.u./0.1 ml (\times 10^{-4}) in supernatant§</th>
<th>% adsorption of p.f.u.</th>
<th>ct/min in supernatant</th>
<th>% adsorption of ct/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IHD-J INV</td>
<td>R</td>
<td>46</td>
<td>18</td>
<td>2640</td>
<td>2960</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IHD-W EEV</td>
<td>R</td>
<td>87</td>
<td>14</td>
<td>3150</td>
<td>3630</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>101</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IHD-J EEV</td>
<td>R</td>
<td>2</td>
<td>95</td>
<td>70</td>
<td>1710</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>IHD-J INV</td>
<td>R</td>
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<td>0</td>
<td>2210</td>
<td>2240</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>IHD-W EEV</td>
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<td>74</td>
<td>0</td>
<td>2940</td>
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<tr>
<td></td>
<td>M</td>
<td>64</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>IHD-J EEV</td>
<td>R</td>
<td>2</td>
<td>95</td>
<td>80</td>
<td>1860</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Virus particles and RBCs were incubated with agitation for 1 h at 37 °C before being processed as described in Methods for supernatant infectivity and radioactivity.
† Virus strains were grown and the particles purified as described in Methods.
‡ R = Rooster, M = monkey.
§ Average of 3 Petri plates.

Adsorption of enveloped and naked particles from HA+ and HA- vaccinia strain to rooster RBCs

The coincidence of infectivity and VHA in caesium chloride gradients suggested but did not unequivocally establish the physical association of VHA to EEV. To provide further evidence for this relationship, we determined the capacity of caesium chloride purified EEV to adsorb to RBCs. For the purpose of demonstrating that any EEV-related VHA activity was specific for the envelope component, the naked particles (INV) of the VHA+ IHD-J vaccinia strain were included as a control in the experiments. The possible occurrence of any non-specific adsorption of EEV due to an unknown non-VHA related property of the envelope component was controlled by the inclusion in the experiments of EEV particles from the VHA- IHD-W vaccinia strain. Adsorption was monitored by the removal of infectious virus and 3H-thymidine label from supernatant fractions after sedimentation of the RBCs at the end of the adsorption period. The specificity of the reaction for the VHA-sensitive rooster RBC was established by determination of the adsorption ratio of rooster/monkey. Under the conditions used non-specific virus particle adsorption to the VHA-insensitive monkey RBC compared to virus treated in parallel in the absence of any RBC was usually undetectable (less than 5 %). The results of the two experiments shown in Table 1 clearly indicate that adsorption to rooster RBCs was very low for both the INV particles of the VHA+ IHD-J strain and the EEV particles of the VHA- IHD-W strain. The only significant adsorption of virus particles occurred with EEV of the VHA+ IHD-J strain. All adsorption experiments using EEV particles of the IHD-J strain gave greater than 90 % adsorption. A concomitant removal of HA activity after adsorption was observed (data not shown). These results were interpreted as showing a physical association of VHA to infectious virus and that the VHA activity resides as a surface component of the envelope of extracellular enveloped virus particles (EEV).
Serological specificity of EEV-associated VHA

Since the presence of VHA in the envelope of EEV was clearly detectable by adsorption to red blood cells, it seemed logical that the antigenic specificity of the adsorption reaction could be determined by adsorption inhibition using specific antiserum. The results of one such experiment are shown in Fig. 3. Rabbit antivaccinia serum against IHD-W, IHD-J and NRS at serial twofold dilutions were reacted with the EEV for 2 h before the addition of rooster RBCs and further incubation at 37 °C. Such treatment with antiserum to the HA⁺ IHD-J strain with NT and HI titres of 4000 and 128 respectively but not with NRS caused the complete inhibition of EEV adsorption at a dilution of 1280. The inhibitory effect rapidly diminished on further dilution with 41 % and 78 % EEV adsorption occurring at dilutions of 2560 and 5120, respectively. Antiserum to the HA⁻ IHD-W strain with an EEV neutralizing titre of 2000 but lacking HI antibodies was also tested for inhibitory activity. Although the antiserum was HI⁻ it differed significantly from NRS in the interaction with EEV with inhibitory activity still detectable at a dilution of 160.

Analysis of the interaction between EEV and RBC

The conditions described in Methods for measurement of virus adsorption to RBCs were the result of examination of various parameters (data not shown). It was found that 0.01 M-CaCl₂ in PBS inhibited virus adsorption in agreement with the results of Blackman & Bubel (1972). Addition of NRS to reaction mixtures was found to decrease virus adsorption with increasing NRS concentration in a linear fashion between 1 and 5 % NRS when the reaction mixture was incubated in the absence of agitation. Adsorption was reduced from 80 % (1 % NRS) to 60 % (5 % NRS). This reduction was completely abolished if the reaction mixtures were agitated continuously during incubation and resulted in 90 % adsorption at 1 to 5 % NRS concentration.
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Fig. 4. Adsorption kinetics of $^3$H-thymidine-labelled EEV at varying temperatures. EEV was mixed with RBCs (pre-incubated at the appropriate temperature) and incubated with agitation at 37 °C (□—□), 24 °C (■—■) or 4 °C (▲—▲). Samples were taken at specified time intervals and processed as described in Methods.

The reaction was further characterized by adsorption kinetics experiments as depicted in Fig. 4. The reaction was complete at 4, 24 and 37 °C by 30 min with negligible differences in the total amount of EEV adsorbed. However, a temperature dependence was evident if the reaction mixtures were incubated in the absence of agitation, resulting in twice as much virus adsorption at 37 °C than at 4 °C (data not shown). A temperature dependence was only apparent early in agitated reaction mixtures with greater adsorption evident at 37 °C than at 4 °C. The rapidity and completeness of the adsorption reaction even at 4 °C indicates the high affinity of the VHA for the RBC receptor site.

Further evidence for the high affinity of VHA was acquired by addition of antivaccinia serum to EEV–RBC complexes. If the reaction between VHA and RBC were reversible then the presence of adsorption inhibiting antibodies should prevent the readsorption of the eluted EEV particles. Table 2 shows the results of one such experiment in which adsorption was 85 %. EEV–RBC complexes exposed to NRS gave approximately twice as much supernatant radioactivity as compared to the addition of phosphate-buffered saline. This low unspecific dissociation of EEV–RBC complexes remains unexplained. The addition of rabbit antivaccinia serum gave varying amounts of supernatant radioactivity. Although serum K-42 was able to cause complete inhibition of EEV adsorption to RBCs at a dilution of 320 (data not shown), it did not result in an increase in supernatant radioactivity compared to NRS. Serums K-4 and K-13 did show a low but consistent accumulation of radioactivity in the supernatant fluid. However, this increase was not proportional to the HI or NT titres.
Table 2. The effect of antivaccinia rabbit sera on EEV–RBC complexes

<table>
<thead>
<tr>
<th>Treatment of EEV-RBC* complexes</th>
<th>Ct/min in supernatant* after treatment of EEV-RBC complexes</th>
<th>Vaccinia antibody titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>190</td>
<td>HI</td>
</tr>
<tr>
<td>NRS</td>
<td>420</td>
<td>NT</td>
</tr>
<tr>
<td>K-42</td>
<td>470</td>
<td>128</td>
</tr>
<tr>
<td>K-4</td>
<td>610</td>
<td>128</td>
</tr>
<tr>
<td>K-13</td>
<td>720</td>
<td>1024</td>
</tr>
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</table>

* EEV–RBC complexes were formed by incubation of a 6 ml reaction mixture of EEV with rooster RBCs for 15 min at 37 °C with agitation and then divided into six 1·0 ml portions. A 1·0 ml EEV monkey RBC control reaction mixture was incubated in parallel. The control and one of the 1·0 ml portions were then sampled for supernatant radioactivity with 1620 ct/min for control monkey RBCs and 240 ct/min (85 % adsorption) for the rooster RBCs. To the remaining 5 portions were added 0·1 ml of PBS, NRS or rabbit antivaccinia serum (K-42, K-4 or K-13) and incubation continued for 1 h before being sampled for supernatant radioactivity.

Table 3. The effect of temperature on EEV adsorption to RBCs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>RBC</th>
<th>Ct/min in supernatant</th>
<th>% adsorption of ct/min</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>24 °C 30 min</td>
<td>R</td>
<td>180</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>24 °C 30 min</td>
<td>M</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>56 °C 30 min</td>
<td>R</td>
<td>120</td>
<td>95</td>
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<tr>
<td></td>
<td>56 °C 30 min</td>
<td>M</td>
<td>2720</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24 °C 30 min</td>
<td>R</td>
<td>170</td>
<td>90</td>
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<tr>
<td></td>
<td>24 °C 30 min</td>
<td>M</td>
<td>1740</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56 °C 30 min</td>
<td>R</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>56 °C 30 min</td>
<td>M</td>
<td>1700</td>
<td></td>
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</table>

The effect of heat on the capacity of EEV to adsorb to RBCs

It was previously reported that the tissue lipid haemagglutinin is heat labile while VHA is heat stable (Youngner & Rubenstein, 1962). We therefore studied the thermal stability of the EEV-associated VHA. EEV particles were incubated for 30 min at either 56 or 24 °C followed by the addition of either monkey or rooster RBCs to determine the quantity of VHA which could still be adsorbed. The data in Table 3 reveals the thermostability of EEV-associated VHA. Adsorption was greater than 90 % for both heat treated and untreated EEV.

DISCUSSION

The VHA has been reported not to be related to the infectious vaccinia virus particle (Burnet & Stone, 1946; Chu, 1948; McCrea & O'Loughlin, 1959; Neff et al. 1965; Blackman & Bubel, 1972; Weintraub & Dales, 1974). These studies have, however, always used intracellular virus as the source of virus material. The findings of Appleyard et al. (1971) that extracellular virus possesses an envelope not present on intracellular virus and the suggestion by Boulter & Appleyard (1973) that the HA may be a component of the envelope has reopened the question of the relationship of VHA to infectious virus. We have undertaken to answer this question by determining the capacity of EEV to adsorb to VHA sensitive rooster RBCs compared to the insensitive monkey RBCs.

It was found that a prerequisite for detection of EEV adsorption was the removal of the large surplus of VHA that was non-particle associated and which therefore could compete...
with any EEV associated VHA for the rooster RBC receptor site. We accomplished this by equilibrium centrifugation of concentrated EEV in caesium chloride gradients. This resulted in a peak of infectious EEV at a density of 1.23 to 1.24 g/ml coincident with a peak of HA activity representing less than 10% of the total HA in the gradient. The occurrence of a small quantity of naked virus particles at a density of 1.26 to 1.27 g/ml in addition to EEV in the extracellular fluid is most probably a result of rupture of a few cells in the roller bottle monolayers during virus growth since these particles were absent from growth medium of virus cultivated in stationary cultures. The density difference between naked and enveloped virus agrees with those reported by Boulter & Appleyard (1973). We have confirmed the findings in previous studies (Burnet & Stone, 1946; Blackman & Bubel, 1972) showing the inability of naked virus particles from cytoplasmic extracts (INV) of infected cells to adsorb to rooster RBCs. However, we purified from the extracellular medium a population of enveloped virus particles (EEV) which demonstrated a specific adsorption to RBCs. These results were interpreted as evidence for the physical association of VHA to EEV and that the presence of VHA was specific for the envelope component of EEV.

Antiserum to the HA+ vaccinia strain (IHD-J) with HI and NT antibodies was able to inhibit the adsorption of EEV to RBCs. The sensitivity of the adsorption inhibition assay proved to be greater than the usual haemagglutination inhibition test. However, this increased sensitivity is dependent on the specific activity (p.f.u.: radioactive label) of the 3H-thymidine-labelled EEV and thus can vary in each EEV batch. Although EEV particles from the HA- vaccinia strain (IHD-W) were unable to adsorb to RBCs, antiserum to this strain which was HI+ demonstrated a significant adsorption inhibition activity. A possible explanation for this inhibition could be the interaction of antibodies with surface antigens other than the haemagglutinin and thereby prevent EEV adsorption by stearic hindrance. The question then arises as to why the EEV adsorption inhibition assay is able to detect stearic hindrance whereas the conventional HI test is not. The greater sensitivity of the EEV adsorption inhibition assay may be the basis for this difference. However, a second possibility is that the antigenic arrangement of the cell membrane bound HA and non-HA antigens (used in the HI test) is different from their arrangement in the envelope of EEV particles (used in the adsorption inhibition assay).

The interaction of EEV and rooster RBCs was found to be maximal if the reaction mixture was constantly agitated. Unagitated reactions resulted in reduced adsorption titres with increasing serum concentration or decreased temperature. This is apparently due to a reduction of collision events. Previous workers (Burnet & Stone, 1946; Smith et al. 1973) have employed NRS at 5 to 10% concentrations to eliminate haemagglutination by tissue lipids. Our results and previous studies by Youngner & Rubenstein (1962) have shown that 1% NRS is sufficient to completely eliminate tissue lipid haemagglutination. Higher concentrations of NRS were therefore deemed unnecessary particularly since the EEV used in this study was separated from the bulk of VHA and tissue lipids and the EEV associated VHA activity was heat stable whereas the lipid haemagglutinin is heat labile (Youngner & Rubenstein, 1962).

The high affinity of VHA for the rooster receptor site has been previously reported (Chu, 1948). Our results confirm these findings with regard to EEV-associated VHA. The great rapidity of reaction even at 4°C and the lack of any detectable difference in the total amount of EEV that could be adsorbed in 30 to 60 min at 4 and 37°C with constant agitation indicates the high affinity of VHA for rooster RBCs. Inouye & Norrby (1973) were able to demonstrate that the adenovirus–RBC interaction was reversible. In the presence of anti-fibre antibodies diluted 200-fold, 90% of the virus had eluted in 5 min. Their data were
interpreted as evidence for a steady-state condition. We were only able to detect a minimal elution during a 1 h treatment of previously adsorbed EEV with two rabbit hyperimmune antivaccinia sera. However, another serum containing HI antibodies was unable to detect even a low level of elution. As a result of these experiments, we conclude that the formation of EEV–RBC complexes compared to that of adenovirus is irreversible.

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REFERENCES

(Received 3 November 1975)