Haemolysis by Sendai Virus: Involvement of a Virus Protein Component

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SUMMARY

Treatment of Sendai virus with 0.3% tri(n-butyl)phosphate and 0.1% Tween 80 led to the disintegration of virus envelopes and to the formation of envelope-derived particles (EP) consisting of 'rosettes' endowed with surface projections and of spikeless envelope remnants. EP had a higher buoyant density in CsCl gradients than intact virus (1.29 compared with 1.20 g/cm³); their sedimentation coefficient was 190 S. EP agglutinated red blood cells and contained neuraminidase. Their haemolytic activity was greatly increased in comparison with intact virus. Three virus polypeptides and traces of an additional protein were detected in EP by polyacrylamide gel electrophoresis. EP retained approximately 30% of choline-containing phospholipids present in the virus envelope. The haemolytic activity of EP and of intact virus was destroyed by treatment with either trypsin or diisopropylfluorophosphate. Insolubilised trypsin failed to alter the haemolytic activity. Proteolytic cleavage of RBC membrane components occurred during haemolysis.

INTRODUCTION

The fact that lysolecithin was identified as one of the phospholipid components of Sendai virus and that treatment of the virus with a preparation of lysolecithinase destroyed the virus haemolysin, led to the postulate that lysolecithin was the mediator of haemolytic activity (Rebel, Fontanges & Colobert, 1962; Barbanti-Brodano, Possati & La Placa, 1971). It is difficult to reconcile this conclusion with the following findings: (a) Virus treated under appropriate conditions with trypsin lacked haemolytic activity, although its haemagglutinin (HA) titre, neuraminidase activity and morphological features remained unaltered (Neurath, 1963; Neurath et al. 1973). (b) About 0.1 μg of lysolecithin is present in 1000 haemagglutinin units (H.A.U.) of Sendai virus grown in chick embryos (calculated from data of Blough & Lawson, 1968). About 20 H.A.U./ml of Sendai virus are usually sufficient to cause partial haemolysis of a 10% suspension of chicken red blood cells (RBC) (for example, see Neurath et al. 1972b). At least 10 μg/ml of lysolecithin (i.e. 5000 times more than present in 20 H.A.U. of virus) are required to cause minimal detectable haemolysis. (c) Influenza viruses, which do not induce haemolysis, also contain lysolecithin (0.17 μg/1000 H.A.U. of the A/PR8 strain; (Blough et al. 1967)).

Therefore, a protein component distinct from HA and neuraminidase seems essential for

Sendai virus-induced haemolysis, although a possible role of virus lipids in this process cannot be ruled out. We describe here attempts to identify this protein component and to determine its location in the virus particles.

METHODS

**Purification and radioactive labelling of virus.** Sendai and B/MASS/66 influenza viruses were propagated in chick embryos and purified as described before (Neurath et al. 1973). Components from uninfected allantoic fluid, submitted to the same purification procedure, were used in some control experiments. Sendai virus was labelled *in vivo* by injecting embryos at the time of infection with 10 μCi/embryo of a mixture of [14C]-amino acids (algae hydrolysate; 1 mCi/mg; International Chemical and Nuclear Corporation, Irvine, California), with 10 μCi/embryo of either choline-methyl-[3H]-chloride (0.55 Ci/mmol) or d-glucosamine-6-[3H]-hydrochloride (3.6 Ci/mmol) (both from New England Nuclear, Boston, Massachusetts). For *in vitro* labelling, 0.2 ml samples of a virus suspension (75,000 H.A.U./ml) were mixed with 10 to 20 μl of either diisopropyl-1,3-[14C]-fluorophosphate (200 μCi/ml; 0.6 μCi/μg) or diisopropyl-1-[3H]-fluorophosphate (1 mCi/ml; 4.9 μCi/μg) and incubated at 45 °C for 30 min. Both reagents were dissolved in propylene glycol and obtained from New England Nuclear. The suspensions were diluted with 0.8 ml of 0.13 M NaCl, 0.01 M tris-acetate, pH 7.0 (TS) and centrifuged at 22,000 rev/min for 20 min in the rotor SW 39 of an L4 preparative ultracentrifuge (Spinco Division, Beckman Instruments, Palo Alto, California). The pellets were resuspended in 1 ml of TS, and recentrifuged. The final pellets were used for further analysis.

**Preparation and labelling of red blood cell (RBC) ghosts.** Components of RBC membranes were labelled by treating either intact human O RBC or ghosts obtained therefrom with N-ethyl-1-[14C]-maleimide (NEM) (9.8 μCi/μmol) or with [14C]-formaldehyde (0.33 μCi/μg; both from New England Nuclear). Ghosts were obtained: (a) by lysing 0.06 ml of pelleted RBC in 4.0 ml of 0.01 M tris-acetate, 5 x 10⁻⁴ M CaCl₂, pH 7.5 (TCa), (b) by haemolysis with hydroxyethyl ethylenediaminetriacetate (HEDTA)-treated Sendai virus (Neurath et al. 1972b), or (c) by the method of Dodge, Mitchell & Hanahan (1963).

The labelling of RBC with [14C]-formaldehyde was performed at 0 °C essentially as described by Rice & Means (1971). A suspension of RBC (25% v/v; 0.25 ml) in 0.075 M phosphate, 0.072 M NaCl, pH 8.5 (PB) was mixed with 50 μl of 0.04 M [14C]-formaldehyde. Five minutes later, 40 μl of sodium borohydride (5 μg/ml) in successive 10 μl portions were added, followed by 50 μl one min later. The RBC were washed 4 times with 25 ml each of 0.025 M phosphate, 0.072 M NaCl, pH 7.2 (PBS) and lysed using procedure (a). The ghosts were pelleted by centrifuging at 18,000 rev/min for 20 min in the rotor SW 39, washed with 4 ml of TCa, recentrifuged and suspended in 0.5 ml of TS. The suspension was incubated for 20 min at 37 °C and centrifuged as before. The pelleted ghosts were suspended in 0.5 ml of TS and used for further experiments. For labelling with NEM, 0.25 ml of a 25% suspension of RBC were treated with 10 μl of the reagent (100 μCi/ml) for 1 h at 0 °C. The labelled RBC were washed and lysed as described above.

Haemoglobin-free ghosts from 1 ml of packed RBC, prepared by procedure (c) were suspended in 4 ml of 0.005 M tris-acetate, 0.001 M EDTA, pH 7.4, and labelled with either 0.1 ml of [14C]-NEM or with 0.6 ml of 0.04 M [14C]-formaldehyde. The labelling with NEM was performed at 37 °C; otherwise the same conditions were used as described above. The labelled ghosts were washed 4 times with 40 ml of TS and centrifuged at 18,000 rev/min for 40 min in a Servall RC-2 centrifuge (I. Sorvall, Inc., Norwalk, Connecticut). The final pellets were resuspended in 4 ml of TS.
Electrophoresis in polyacrylamide gels. The polypeptides from labelled Sendai virus and from labelled RBC ghosts, each suspended in 0.025 M-phosphate buffer, pH 7.35, with 2.5 % SDS and 2.5 % 2-mercaptoethanol, were separated by electrophoresis in 8 and 5 % polyacrylamide gels, respectively. The conditions for electrophoresis have been described (Neurath et al. 1972a, 1973).

In some experiments, the polypeptides from intact virus or from envelope-derived-particles (EP), obtained by disruption of the virus with tri(n-butyl)phosphate (TNBP) were dansylated following the method of Talbot & Yphantis (1971). Twenty µl of 10 % dansyl chloride in acetone were added to 1.0 ml suspensions containing either virus or EP in 0.02 M-phosphate buffer, pH 8.2, and 2.5 % (w/v) SDS. The mixtures were boiled for 3 min. The dansylated proteins were separated by gel filtration on Sephadex G-25 from the unbound dye and submitted to electrophoresis after boiling for 90 s in the presence of 2.5 % (v/v) mercaptoethanol. The gels with the fluorescent bands were photographed essentially as described by Talbot & Yphantis (1971).

Density gradient sedimentation. Rate zonal sedimentation was performed in 5 to 50 % sucrose gradients (total volume 4 ml) in TS at 18,000 rev/min. Samples for isopycnic banding were centrifuged at 35,000 rev/min for 24 h on preformed CsCl gradients (density range 1.165 to 1.325 g/cm³) in 0.01 M-tris-acetate, pH 7.0. The rotor SW39 or SW65 was used. Sedimentation coefficients (s₂₀,₅) were calculated by the method of McEwen (1967).

Disruption of virus with TNBP. Tween 80 and TNBP were added to virus suspensions (5000 to 20,000 H.A.U./ml) to achieve final concentrations of 0.1 and 0.3 % (v/v), respectively. The suspensions were mixed for 45 min at 22 °C. Then, 1 ml samples were layered on top of 1 ml of a 10 % sucrose solution in TS, and the tubes were centrifuged at 22,000 rev/min for 45 min in the rotor SW39. EP, recovered in the pellets, were resuspended in TS.

Treatment with pronase. One ml of a solution containing pronase (1 mg/ml; prefiltred through a 0.45 µm Millipore filter) was added to 5000 H.A.U. (in 0.1 ml) of Sendai virus. After incubation for 1 h at 37 °C the suspension was layered on top of 1 ml of a 10 % sucrose solution in TS, and the tubes were centrifuged at 22,000 rev/min for 45 min in the rotor SW39. EP, recovered in the pellets, were resuspended in TS.

Gel filtration. Virus particles and virus components labelled with radioactive diisopropyl-fluorophosphate (DFP) were separated from unbound radioactive reagent (or the degradation product thereof) by gel filtration on 4 ml columns of Sephadex G-25 (Pharmacia, Uppsala, Sweden).

Treatment with unlabelled DFP. Equal volumes of a suspension of Sendai virus (2000 to 100,000 H.A.U./ml) and of solutions containing DFP (0.006 to 0.1 M) in TS were mixed and incubated for 30 min at 45 °C. The neuraminidase and haemolytic activities and HA titres of the treated viruses were determined without prior removal of unreacted DFP, which did not interfere in any of the tests.

Virus-induced release of radioactivity from labelled RBC ghosts. Equal volumes (0.25 ml) of a suspension of either [³⁵S]-NEM- or [³⁵S]-formaldehyde-labelled ghosts and of suspensions containing HEDTA-treated Sendai virus (100 to 2500 H.A.U.) were mixed and incubated at 37 °C. At suitable time intervals, 0.5 ml samples were withdrawn and centrifuged at 26,000 rev/min for 15 min in the rotor SW39. The percentage of released radioactivity was computed from the determined radioactivity of the supernatant fluids and of the resuspended pellets.

Extraction of lipids. Lipids from [³⁴C]-choline- or [³⁴C]-DFP-labelled virus particles (or EP) were extracted by chloroform–methanol 2:1 (v/v). Equal volumes of the virus suspension and of the organic solvent were mixed for 20 min at 22 °C. The phases were separated by centrifuging and the radioactivity counted.
Fig. 1. Particles obtained by treatment of Sendai virus with pronase or with 0.3% TNBP-0.1% Tween 80. (a) Pronase-treated particles, stained with 0.5% ammonium molybdate. (b) to (f) Particles formed after treatment of the virus with Tween-TNBP, stained with 1% sodium phosphotungstate. Spiked particles in the process of detachment from remnants of an empty virus envelope are shown in (c). Particles with 'fuzzy' spikes and with easily distinguishable long and short spikes are shown in (d), and (e), (f), respectively.
**RESULTS**

**Disruption of Sendai virus with TNBP and formation of envelope-derived particles (EP) with enhanced haemolytic activity**

It has been recently reported (Shimizu, Hosaka & Shimizu, 1972) that haemolytic activity was present in EP obtained by re-association of the envelope components released by disrupting Sendai virus with the detergent Emasol at pH 10. However, no comparison was made between the haemolytic activity of EP and intact virus. Preliminary experiments revealed that preparations of TNBP-disrupted virus also caused haemolysis and we therefore studied the properties of EP, obtained in this way.

Two kinds of EP were distinguished by electron microscopy: ‘rosettes’ endowed with surface projections and large remnants of virus envelopes (Fig. 1b to f). EP had an average sedimentation coefficient ($s_{20,w}$) of 190, determined from the distribution of HA and haemo-
Fig. 4. Separation of dansylated polypeptides from SDS-disrupted Sendai virus (right) and from envelope-derived particles obtained by disruption of the virus with TNBP (left).

Lytic activity in fractions after rate zonal sedimentation (Fig. 2). Their average buoyant density (in CsCl) was 1.29 g/cm³; the density of intact virus particles was 1.20 g/cm³ (Fig. 3). Polyacrylamide gel electrophoresis of EP purified by rate zonal sedimentation revealed the presence of three major polypeptides (numbered 0, 2 and 4 in order of their increasing electrophoretic mobility) and of traces of another polypeptide no. 5 (Fig. 4). Polypeptide
Table 1. Effect of DFP on the haemagglutinin, neuraminidase, and haemolytic activity of Sendai virus

<table>
<thead>
<tr>
<th>Final concentration of DFP (μ-mol/ml)</th>
<th>Neuraminidase</th>
<th>HA</th>
<th>Haemolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.0</td>
<td>91.5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>94.5</td>
<td>100</td>
<td>4.6</td>
</tr>
<tr>
<td>6.25</td>
<td>98.0</td>
<td>100</td>
<td>31.4</td>
</tr>
<tr>
<td>3.12</td>
<td>100.0</td>
<td>100</td>
<td>71.5</td>
</tr>
</tbody>
</table>

3, the major protein component of intact virus and constituent of the ribonucleoprotein (RNP), was poorly separated from polypeptide 2 during electrophoresis of dissociated virus (see also Neurath et al. 1973). The RNP and RNP fragments were not detected in the preparation of EP. Therefore, polypeptide 3 was considered to be also absent.

The haemolytic activity of EP was substantially higher (5- to 500-fold with various virus preparations) than that of the original virus. Unfortunately, it could not be determined whether the haemolytic activity was carried by the rosette-like EP, by the large remnants of virus envelopes or by both of these structures. These two kinds of EP were not separated from each other sufficiently either by rate zonal and isopycnic density gradient sedimentation, or by chromatography on DEAE-Sephadex.

The EP retained about 30% of the neuraminidase activity of intact virus; only 2% of neuraminidase became solubilized and released from EP during treatment of the virus with TNBP.

The fact that the density of EP was substantially higher than that of intact virus suggested that the lipid content of EP was much lower as compared with virus particles. However, the EP contained 29% of the radioactivity originally present in [3H]-choline-labelled virus. When [3H]-choline-labelled EP were extracted with chloroform-methanol, 2:1, 66% of the residual radioactivity was recovered in the organic phase and 33% in the interphase. Thus, EP did contain phospholipids with a choline moiety; the relatively high buoyant density of EP must be attributed to the selective removal of other lipids.

Effect of proteolytic enzymes on the haemolytic activity of EP

To establish whether or not a protein component of EP was essential for the haemolytic activity, EP were treated with trypsin or pronase. The haemolytic activity corresponding to 15000 H.A.U. of EP was destroyed by incubation with crystalline trypsin (1 mg/ml) for 3 min at 37°C. The HA-titre and neuraminidase remained unchanged even after 15 min of incubation. The effect of further prolonged incubation at this concentration of enzyme was not investigated. The minimal concentration of trypsin at which a 200-fold (or greater) reduction of haemolytic activity occurred within 1 h at 37°C was 0.031 mg/ml, corresponding to five enzyme units/ml. Similar results were obtained with intact virus. The haemolytic activity of EP or intact virus was also destroyed by treatment with pronase (1 mg/ml) for 3 min at 37°C. Treatment of EP or virus with insolubilised trypsin (9 mg/ml; Miles-Yeda Limited, Rehovot, Israel; specific activity 3 units/mg) for 1 h at 37°C failed to alter the haemolytic activity. It was concluded that a virus protein component, not accessible to the action of insolubilized trypsin, was essential for the haemolytic activity of EP and of intact virus.
Table 2. Comparison of haemolysis caused by lysolecithin before and after treatment with 0.02 M-DFP at 45 °C for 30 min

<table>
<thead>
<tr>
<th>Concentration of lysolecithin (µg/ml)</th>
<th>Percentage haemolysis by Untreated lysolecithin</th>
<th>Percentage haemolysis by DFP-treated lysolecithin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>94.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>47.0</td>
<td>64.0</td>
<td>68.0</td>
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<td>23.5</td>
<td>24.0</td>
<td>22.0</td>
</tr>
<tr>
<td>11.75</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>5.85</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Lysolecithin (at each concentration shown in table) was treated with DFP, which alone had no effect on the extent of haemolysis.

Fig. 5. Rate zonal sedimentation (18,000 rev/min for 45 min) in 5 to 50% sucrose gradients in TS of [14C]-DFP-labelled control material from uninfected allantoic fluid (A), B/MASS/66 influenza virus (B), pronase-treated (C) and intact (D) Sendai virus. Empty columns, HA; shaded area, radioactivity. Fraction 1 = bottom of gradient.
Haemolysis by Sendai virus

Fig. 6. Polyacrylamide gel electrophoresis of components from SDS-disrupted and mercaptoethanol-reduced Sendai virus. Bottom: ●, [14C]-amino acid-labelled virus; ○, [14C]-amino acid-labelled virus treated with HEDTA, adsorbed on RBC ghosts and incubated 2 h at 37 °C; shaded area, [14C]-glucosamine-labelled virus. Middle: [14C]-amino acid-labelled virus treated with pronase. No peaks of radioactivity were observed with pronase-treated [14H]-glucosamine-labelled virus. Top: [14C]-DFP-labelled virus.

Effect of diisopropylfluorophosphate (DFP) on the haemolytic activity

It was shown previously (Neurath, 1965) that Sendai virus reacted with DFP and caused the cleavage of the bond between fluorine and phosphorus. Results summarized in Table 1 show that the haemolytic activity of the virus was destroyed after the latter reacted with DFP at 45 °C for 30 min (inactivation was not observed at 37 °C). The HA-titre, neuraminidase activity and infectivity remained essentially unaltered. Similar results were obtained when EP (which had no infectivity) were treated with DFP.

Treatment of lysolecithin, the alleged virus component responsible for virus-induced
haemolysis (Rebel et al. 1962; Barbanti-Brodano et al. 1970), with DFP under the same conditions failed to affect the lytic properties of this lysophosphatide (Table 2).

Incorporation of radioactivity into virus particles during their reaction with DFP labelled in its alkyl moiety

Experiments were performed to determine whether or not the diisopropylphosphoryl (DIP) group had become attached to a virus component(s) during the reaction of the virus with DFP. Since the haemolytic activity was destroyed by DFP, a DIP-binding component would perhaps be identical with haemolysin.

Sendai virus was treated with DFP radioactively labelled in its alkyl moiety and submitted to rate zonal sedimentation. The distribution of radioactivity and HA in the gradient coincided, except for some radioactive material recovered in the top fractions of the gradient (Fig. 5 D). This indicated that the DIP group was incorporated into at least one virus component. The average number of DIP molecules incorporated per H.A.U. of virus was $1.7 \times 10^9$ (determined from 5 experiments; range $1.4 \times 10^9$ to $1.9 \times 10^9$). Radioactivity was adsorbed to RBC at $0^\circ$ C and eluted during their subsequent incubation at $37^\circ$ C for 2 h. Approximately 20% of the label was released from the virus during elution from RBC. Similar experiments with control material from uninfected allantoic fluid or with B/MASS/66 influenza virus failed to yield evidence for specific incorporation of DIP (Fig. 5 A and B; note difference in scale on left ordinate between Fig. 5 B and D).

Virus particles pretreated for 1 h at $37^\circ$ C with pronase (1 mg/ml) incorporated approximately the same amount of radioactive DIP as did intact virus particles (compare Fig. 5 C and D). The pronase-treated particles failed to agglutinate RBC and did not have neuraminidase activity. They lacked the virus glycoprotein components 2 and 4 (compare Fig. 6,
middle, with solid line and shaded area of Fig. 6, bottom). The particles appeared spikeless when observed under the electron microscope (Fig. 1a). The gamma-globulin fraction (7 mg/ml) from an antiserum against pronase-treated virus, but not from normal rabbit serum, caused a 50% inhibition of haemolysis induced by 800 H.A.U./ml of intact virus. The antiserum did not contain detectable haemagglutination- or neuraminidase-inhibiting antibodies. This result suggested that haemolysin was immunologically distinct from haemagglutinin and neuraminidase and that it still was present in the pronase-treated particles which also retained the DIP-binding component.

Attempts to identify a virus polypeptide binding the diisopropyl-phosphoryl group

In order to determine whether or not any of the virus polypeptides was involved in binding the DIP group, virus treated with [14C]- or [3H]-DFP was electrophoresed in polyacrylamide gels containing SDS. Radioactivity was recovered in a single peak, near the top of the gel, which did not coincide with the position of any major polypeptide recovered in the gel after electrophoresis of [14C]-amino acid-labelled virus (Fig. 6, top) but may have coincided with polypeptide o (Fig. 4). Unfortunately, it was difficult to interpret this result because of two additional findings: (a) when [14C]-DFP treated virus was disrupted with 2.5% SDS (in the presence or absence of 2.5% mercaptoethanol) and submitted to gel filtration on Sephadex G-25, only 10 to 20% of the radioactive label was recovered in the fractions corresponding to the void volume of the column. The major part of label was detected in fractions corresponding to low mol. wt. material. (b) Surprisingly, when radioactive DFP was submitted to gel electrophoresis, the radioactivity became distributed across the gel in a pattern similar to that shown on Fig. 6, top.
Release of the diisopropylphosphoryl group during disruption of the virus with TNBP

Previous results suggested the possibility that haemolysin and a DIP-labelled component might be identical. Therefore, the fate of the DIP-label after disruption with TNBP of virus which had been treated with radioactive DFP was investigated. The EP retained only 2 to 4% of the label originally present in the intact virus. The rest of the radioactivity was recovered in supernatant fluids after sedimenting EP. The major part of the label was released in the form of low mol. wt. material (Fig. 7).

The release of soluble radioactive material upon disruption of virus particles pretreated with [3H]- or [14C]-DFP may be explained by one of the following alternatives: (i) that the DIP-group is not bound irreversibly, and that the disruption of the virus for some reason enhances the liberation of DIP; or (ii) that lipophilic DFP merely becomes trapped in the lipid layer of the virus envelope. The following results clearly support the first alternative: (a) a partial release of radioactivity is always observed with DIP-labelled intact virus (Fig. 7); (b) this release is enhanced by treatment of the virus with unlabelled DFP: 45 and 76% of the label was released during incubation of the virus for 15 min at 45 °C in the absence and presence of 0.025 M-DFP, respectively; (c) only 20% of radioactivity is recovered in the organic phase after extraction of the labelled virus with chloroform–methanol, 2:1; with free DFP, 91% of the radioactivity is recovered in the organic phase; and (d) the release of the DIP-group from virus particles is pH-dependent: 85, 57, 30 and 33% of radioactivity are released from [3H]-DIP-labelled virus during incubation for 15 min at 37 °C at pH 3, 5, 7 and 10, respectively.
Evidence for cleavage of RBC membrane polypeptides during haemolysis by Sendai virus

The virus-associated haemolytic activity was destroyed by DFP, a reagent affecting enzymes which contain serine sites at catalytic positions. Some proteases belong to this category of enzymes (Matheja & Degens, 1970). Therefore, it was of interest to determine whether or not proteolysis of RBC membrane components would occur during Sendai virus-induced haemolysis.

Preliminary experiments revealed that treatment of labelled RBC ghosts, prepared by method (a) with Sendai virus resulted in solubilization of a considerable portion of total radioactive material (Figs. 8, 9). These ghosts retained about 1% of haemoglobin, originally present in RBC, before they reacted with the virus. The electrophoretic mobility in polyacrylamide gels of the radioactive material released from the ghosts was identical to the mobility of free haemoglobin, which was the most prominent radioactive component of the ghosts. It was concluded that the virus caused the release of residual haemoglobin which remained bound to the ghosts after lysis of RBC by method (a).

Additional experiments were carried out with haemoglobin-free ghosts, prepared by method (b). The amount of radioactive material released from virus-treated ghosts exceeded the amount liberated from control ghosts incubated at 37°C. Incubation of the ghosts, even in the absence of virus, led to partial solubilization of radioactive material and to a
decrease in the amount of high mol. wt. material recovered in the top fractions after polyacrylamide gel electrophoresis of the ghosts. For example, in one experiment 70 and 33% of total radioactivity became solubilized after treatment of ghosts for 90 min at 37 °C with HEDTA- and DFP-treated virus (with no haemolytic activity), respectively. Polyacrylamide gel electrophoresis of the insoluble residue from these ghosts revealed the disappearance of a high mol. wt. component from the ghosts treated with haemolytic virus (Fig. 10, top).

Significant differences were revealed between the polypeptide patterns of ghosts prepared by method (b) and those obtained after virus-induced haemolysis (Fig. 10, bottom). In this experiment, 1.75 times less [14C]-N-ethylmaleimide became incorporated into the virus-treated ghosts, although equal amounts of RBC were used for their preparation. The decreased incorporation of label into treated ghosts may be ascribed to the solubilization of some membrane components during virus-induced haemolysis. One of the virus polypeptides (no. 5) also became labelled under the conditions used (additional virus polypeptides were labelled significantly only when the virus was pretreated with reagents cleaving disulphide bonds; Neurath et al. 1973).

Virus polypeptides did not seem to become cleaved during interaction of virus particles with RBC (compare solid and dotted line in Fig. 6, bottom).

**DISCUSSION**

EP obtained by disruption of Sendai virus with TNBP cause haemolysis. Subvirus haemagglutinins (SVHA) resulting from treatment of the virus with diethylether lack this property (Sokol & Neurath, 1962). Therefore, a detailed comparative analysis of EP and SVHA may lead to the elucidation of the nature of haemolysin. EP seem to have the following distinct features: presence of phospholipids and of a high mol. wt. protein component o; occurrence of two morphologically distinct kinds of particles, i.e. of mostly round rosettes and of large envelope remnants. Each of these particles adsorb to RBC. SVHA mostly consist of elongated spiked particles (Neurath et al. 1973). Similar elongated particles observed by Hosaka (1970) in preparations of disrupted virus also failed to cause haemolysis. It is possible that polypeptide o is the DIP-binding component of the virus envelope and the carrier of haemolytic activity. This possibility is supported by the finding that low levels of haemolysis-inhibiting antibodies are detected in sera of rabbits immunized with portions of gels containing polypeptide o. Why this polypeptide species became clearly discernible only when dansylated virus, but not [14C]-amino acid-labelled virus, is electrophoresed is unknown. Relatively high levels of radioactivity are detected in gels at a position corresponding to polypeptide o when disrupted [3H]-glucosamine-labelled virus is electrophoresed. Treatment of the virus with trypsin does not cause a significant change in the electrophoretic mobility of polypeptide o. It cannot be excluded that a minor protein component, undetectable by polyacrylamide gel electrophoresis under the conditions used here, may be essential for haemolysis.

Pretreatment of virus particles with pronase failed to decrease the binding of the DIP group to treated particles (which did not cause haemolysis) in comparison with intact virus. Electron microscopy of the treated particles suggested that they are devoid of surface projections. It seemed unlikely that the disappearance of projections occurred during negative staining of the protease-treated particles with ammonium molybdate (see Neurath et al. 1973). Assuming that (a) pronase does remove the surface projections; (b) there is only a single type of DIP-binding site; and (c) this site is located on the virus component essential for the haemolytic activity, one may postulate that this component is part of the
virus envelope proper. Some portion of this component (including the DIP binding site) is probably hidden within the envelope, while another part is exposed on the surface of the envelope and thus is accessible to proteolytic cleavage by trypsin, which leads to the destruction of haemolytic activity. The disruption of the envelope by detergents or organic solvents would expose the DIP binding sites to an altered, more polar environment, favouring the dissociation of the DIP group. The site attacked by trypsin is probably located on the surface of the envelope in spaces between the surface projections. The fact that insolubilized trypsin failed to decrease the haemolytic activity seems to be in agreement with this hypothesis. Although the penetration of the relatively small trypsin molecules into the space between spikes may probably proceed without steric hindrance, it becomes difficult to explain the attachment of much larger antibody molecules to the same parts of the envelope. Additional studies will be required to explain the inhibition of haemolysis by antibodies which do not cause inhibition of haemagglutination or of neuraminidase.

The proposed localization of haemolysin within the virus envelope would preclude the initiation of haemolysis during the first stages of adsorption of the virus to RBC. In fact, a closer, irreversible interaction of virus with RBC seems to be required for haemolysis (Neurath et al. 1972b).

The incomplete disappearance of polypeptide 4, and the appearance of an additional peak of radioactivity, observed when pronase-treated [14C]-amino acid-labelled virus is dissociated with SDS and electrophoresed (Fig. 6), as compared with the elimination of peaks of radioactivity when [14C]-glucosamine-labelled virus is submitted to the same treatments, remains unexplained.

There is a lack of firm evidence supporting the role of lysolecithin in virus-induced haemolysis. Barbanti-Brodano et al. (1971) did not show whether or not treatment of the virus with lysolecithinase (leading to the destruction of haemolysin) actually decreased the lysolecithin content of the virus envelope. Since haemolysin is destroyed by trypsin (Neurath, 1963; Fontanges, Garrigue & Colobert, 1964) and other proteases (own unpublished data), the eventual presence of protease contaminants in the preparation of lysolecithinase may have been responsible for inactivation of haemolysin. On the other hand, the presence of lipids (phospholipids) seems to be essential for the preservation of haemolytic activity. Since lipids are derived from host cells and are components of both haemolytic and non-haemolytic viruses, their role is probably analogous to the function of phospholipids in sustaining the activity of membrane-bound enzymes (Mavis, Bell & Vagelos, 1972).

Because the haemolysin is inactivated by DFP, it seems possible that a virus protease is responsible for haemolysis. This possibility is supported by a recent report dealing with proteinase activity in purified viruses (Holland et al. 1972). Virus-induced changes in the distribution of polypeptides after electrophoresis of SDS-disrupted [14C]-N-ethylmaleimide-labelled RBC ghosts are considered as evidence for proteolytic cleavage. The polypeptide distribution corresponding to untreated ghosts (Fig. 10, bottom) is similar to that reported by Carraway & Shin (1972). Proteolysis also occurred to a lesser degree during incubation of ghosts (but not RBC) in the absence of virus. Because of the presence of a proteinase in human RBC membranes (Moore et al. 1970), it is not possible to determine, unequivocally, whether the virus-induced proteolysis was the cause or the consequence of RBC membrane lysis. DFP also inactivates enzymes responsible for the transfer of phosphate groups (Matheja & Degens, 1971). The activity of some of these enzymes, unlike that of proteases, is more likely to depend on the presence of phospholipids. Perhaps the study of the effect of Sendai virus on phosphorylation of RBC membrane components (Williams, 1972) will lead to the elucidation of the mechanism of virus-induced haemolysis.
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