Effect of Enzymes on the Attachment of Influenza and Encephalomyocarditis Viruses to Erythrocytes

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SUMMARY
Encephalomyocarditis (EMC) and influenza viruses attach to human erythrocytes causing haemagglutination of the cells. Sialoglycoproteins, containing predominantly glycophorin A, from these cells behave as soluble virus receptors and inhibit haemagglutination by both viruses. Removal of 43% of the sialic acid from erythrocytes with neuraminidase prevented their haemagglutination by EMC virus while loss of 40% of glycophorin sialic acid destroyed its inhibitory properties against this virus. However, about 80% of the sialic acid had to be removed from erythrocytes or from glycophorin to achieve the same results for influenza virus. Trypsin treatment of erythrocytes or glycophorin had little effect on haemagglutination or inhibition involving either virus, although the glycopeptides released contain up to 70% of the total sialic acid, and despite the fact that glycophorin was drastically reduced in size as shown by SDS–polyacrylamide gel electrophoresis. It is concluded that not all of the sialic acid present in erythrocyte sialoglycoprotein receptors is involved in attachment of EMC or influenza viruses and that the attachment sites on erythrocytes for these viruses are not identical.

INTRODUCTION
The attachment of viruses to cell surfaces as a prelude to penetration is generally regarded as a specific process (for review, see Lonberg-Holm and Philipson, 1974). It is a puzzle, therefore as to why many different viruses fail to attach to cells from which sialic acid has been removed, thus implying a low specificity in the process of attachment for these viruses. Even more striking are the reports that the attachment to human erythrocytes of viruses as different as influenza (Kathan et al., 1961) and encephalomyocarditis (EMC) (Enegren & Burness, 1977) viruses probably involves the same sialoglycoprotein receptor, glycophorin (Marchesi & Andrews, 1971). To determine whether influenza and EMC viruses bind to the same active site on glycophorin, we have investigated and report here the effect of trypsin and neuraminidase both on the structure of glycophorin, in situ and free in solution and on its interaction with these viruses.

METHODS
Materials. [3H]acetic anhydride (50 mCi/mmol) and Aquasol-2 were purchased from New England Nuclear; trypsin and lima bean trypsin inhibitor from Worthington Biochemical Corporation; Vibrio cholerae neuraminidase and diithiothreitol from Calbiochem; SDS and lithium diiodosalicylate (LIS) from Sigma; and basic fuchsin (to make Schiff's reagent) and Coomassie Brilliant Blue R-250 from Eastman-Kodak. Outdated human type O blood was obtained from the Canadian Red Cross Blood Transfusion Service.

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Viruses. Growth of the K2 strain of EMC virus in Krebs ascites tumour cells (Sanders et al., 1958) and its purification have been described previously (Burness et al., 1974). The PR-8 strain of influenza virus from a seed culture kindly provided by Dr P. Palese, was grown in the allantoic cavity of 10- to 12-day-old chick embryos (Blaskovic & Styk, 1976).

Haemagglutination (HA) and haemagglutination inhibition tests were performed on microtitre plates as described previously (Enegren & Burness, 1977).

Erythrocyte membranes. These were prepared by hypotonic lysis (Fairbanks et al., 1971).

Glycophorin preparations. These were obtained from human erythrocyte membranes by the lithium diiodosalicylate (LIS)–phenol procedure (Marchesi & Andrews, 1971). The procedure, in brief, consisted of stirring membranes containing 25 mg/ml protein in 300 mM-LIS–50 mM-tris–HCl pH 7.5. After high speed centrifugation, the supernatant fluid was extracted with 50% (w/w) phenol and the aqueous layer dialysed, freeze-dried, ethanol extracted and dialysed once more before storing at -20 °C.

SDS–polyacrylamide gel electrophoresis. This was performed as described by Fairbanks et al. (1971) on samples containing 25 to 50 μg protein which were pre-incubated at 37 °C for 30 min in 1% SDS, 10 mM-tris–HCl pH 8, 1 mM-EDTA, 40 mM-dithiothreitol before electrophoresis for 2 h at 8 mA/gel on 5.6% polyacrylamide in 1% SDS, 40 mM-tris–HCl, 20 mM-sodium acetate, 2 mM-EDTA, final pH 7.4. The gels were stained with Coomassie Blue or periodic acid–Schiff's reagent (Fairbanks et al., 1971) and scanned at 550 nm in a Gilford spectrophotometer fitted with a gel transport accessory.

Protein concentrations. These were measured by the procedure of Lowry et al. (1951).

Sialic acid. This was determined by the thiobarbituric acid method of Warren (1959).

Enzyme digestion of erythrocytes. About 1 ml washed, packed erythrocytes were incubated at 37 °C with 0.4 ml phosphate-buffered saline (PBS) containing either 2 mg trypsin or 20 units neuraminidase. Controls in the absence of enzyme were incubated under similar conditions. After various lengths of time (and the addition of 10 μl 0.1 M-EDTA to stop the enzyme action in the case of neuraminidase), the cells were collected by centrifugation. Sialic acid assays were performed on the supernatant fluids without further treatment for the neuraminidase samples but after hydrolysis with 0.05 M-H2SO4 at 80 °C for 1 h to release sialic acid from the sialoglycopeptides for the trypsin samples. The collected cells were washed with PBS before being used either for haemagglutination and attachment studies or for preparing membranes which were assayed for protein and sialic acid content, and were analysed by SDS–polyacrylamide gel electrophoresis.

Trypsin treatment of glycophorin. Glycophorin preparations containing 360 μg protein in 250 μl were incubated at 37 °C for various times with 5 μl 2% trypsin in PBS. Trypsin activity was stopped by addition of 5 μl 1% lima bean trypsin inhibitor. Controls were treated similarly in the absence of trypsin or with a mixture of lima bean trypsin inhibitor and trypsin. Samples were tested for haemagglutination inhibition and were also analysed by electrophoresis on SDS gels.

Neuraminidase treatment of glycophorin. One ml of a glycophorin preparation containing 2 mg protein was incubated at 37 °C with 400 units neuraminidase in an equal volume of PBS; controls in the absence of enzyme were incubated similarly. After various times of incubation, 200 μl samples were taken and the enzyme inactivated by boiling for 10 min. The preparations were then analysed for free sialic acid and for HA inhibitory activity and were examined by SDS–polyacrylamide gel electrophoresis.

RESULTS

Sialic acid-containing components in erythrocyte membranes

Since influenza and EMC viruses both apparently require sialic acid on the red blood cell surface for attachment (Burness, 1981), it was considered important from the beginning to
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Fig. 1. Scans of SDS-polyacrylamide gel electropherograms of membranes prepared from cells incubated in the presence of neuraminidase for (a, g) 0 min, (b, h) 30 min, (c, i) 60 min, (d, j) 120 min, (e, k) 18 h, or in the absence of neuraminidase (f, l) for 18 h (control). The gels were stained with Coomassie Blue (a to f) or PAS reagent (g to l).

establish the nature of the sialic acid-containing components and the amount of sialic acid present in the membranes of erythrocytes used in these studies. Sialoglycoproteins in particular were examined since they contain most of the erythrocyte sialic acid (Winzler, 1970), less than 2% being found in glycolipids (Beeley et al., 1977).

Human erythrocyte membranes were analysed by electrophoresis on SDS gels which were stained with Coomassie Blue (Fig. 1 a) or with PAS reagent (Fig. 1 g). The Coomassie Blue pattern was complex and similar to that described by Steck (1974) whose nomenclature for labelling the peaks was used (Fig. 1 a). PAS staining, which detects glycoproteins, gave a much simpler pattern than the Coomassie Blue staining; PAS-1 and PAS-2 (nomenclature of Steck, 1974), the dimer and monomer forms of the major erythrocyte sialoglycoprotein, glycophorin A (Marton & Garvin, 1973; Silverberg & Marchesi, 1978) comprised 82.6% ± 0.7 (SE, 4 determinations) of the PAS staining, PAS-3 made up 6.7% ± 0.3 (SE, 4 determinations) and the remaining 10.7% ± 0.9 (SE, 4 determinations) consisted of PAS-4 and other minor components, excluding the variable ‘lipid’ region (Fig. 1 g).
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To release the total sialic acid present, erythrocyte membranes were incubated with 0.05 M-H$_2$SO$_4$ for 1 h at 80 °C. This length of time was considered long enough to liberate all releasable sialic acid since incubation for 1 or 3 h gave identical results. A value of 32.5 μg ± 1.5 (se, 62 determinations) sialic acid/mg membrane protein has been found for the many erythrocyte membrane preparations we have used for various experiments. The membrane preparations studied in the present report contained 33.9 μg sialic acid/mg protein, a value taken to be 100% for comparative purposes in experiments described below.

Incubation of 10$^{10}$ erythrocytes with neuraminidase for 18 h, which liberates 90% of the sialic acid (Fig. 2), released 112 μg sialic acid; this is equivalent to 2.4 x 10$^7$ molecules of sialic acid/cell after corrections for recovery. Assuming approx. 80% of the sialic acid was in glycophorin A, based on SDS–polyacrylamide gel patterns (Fig. 1g) and assuming there are 32 sialic acid residues/molecule (Marchesi et al., 1976), it was estimated there are about 6 x 10$^4$ glycophorin A molecules/cell. The glycophorin used in this report was calculated to contain 26 sialic acid residues/molecule (see below) which leads to an estimate of 7 x 10$^5$ glycophorin A molecules/cell. Previous estimates gave 5 x 10$^5$ (Bretscher, 1971) to 10$^6$ (Winzler, 1970) sialoglycoprotein molecules/cell.

**Effect of neuraminidase on erythrocytes**

**Physical changes**

Erythrocytes were incubated in the presence or absence of neuraminidase for various lengths of time, after which membranes were prepared from the cells and analysed by SDS–polyacrylamide gel electrophoresis using Coomassie Blue (Fig. 1a to f) or PAS reagent staining (Fig. 1g to l). Changes were most apparent in PAS-stained gels. This was to be expected since the PAS reagent stains glycoproteins which are the major sialic acid-containing components present in erythrocyte membranes and which, therefore, would be expected to be affected by neuraminidase. The most obvious changes which began within 30 min of incubation were that the quantity of PAS-1 was greatly diminished and that the major PAS-staining components became more heterogeneous (Fig. 1h to k) compared with membrane samples from unincubated cells (Fig. 1g) or from cells incubated in the absence of enzyme (Fig. 1l). Changes were not so apparent in Coomassie Blue-stained gels although a minor component in the band 4.5 region, marked with an arrow in Fig. 1 (b to e), became more prominent. The fast moving, sharp peak evident particularly in the 60 min samples (Fig. 1c, i) was due to contamination with haemoglobin polypeptide chains. The changes in PAS-stained gels compared with the lack of change in Coomassie Blue-stained gels suggests that the major components revealed with Coomassie Blue neither contain sialic acid nor correspond to the PAS-positive components.

**Chemical and biological changes**

Erythrocytes were treated with neuraminidase for various lengths of time and then separated from the supernatant fluid by centrifugation. The sialic acid present in the supernatant fluid and that released by acid hydrolysis of membranes prepared from the enzyme-treated erythrocytes was measured. It was found that sialic acid was released at a constant rate for up to 2 h of incubation whether supernatant fluids or membranes were considered (Fig. 2). At this time, about 70% of the total or 80% of the neuraminidase-releasable sialic acid was found in the supernatant fluid while about 21% neuraminidase-releasable sialic acid was membrane-associated. Overnight incubation with enzyme was required to achieve maximum release which was equivalent to about 90% of the total sialic acid estimated by acid hydrolysis.
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Fig. 2. Effect of neuraminidase on erythrocytes: chemical and biological changes. Cells were incubated with neuraminidase for various lengths of time, collected by centrifugation and the sialic acid in the supernatant fluid measured (O--O). The treated cells were then used for HA titration and for the preparation of membranes which were analysed for sialic acid content (O--O). Results are expressed as percent of maximum detected. O----O, EMC virus; 0----0, influenza virus.

After 45 min of incubation with neuraminidase, or when about 43% of the total sialic acid was released from the cells, human erythrocytes were no longer agglutinated by EMC virus (Fig. 2). When ³H-labelled EMC virus was tested, 20 min incubation of cells with neuraminidase, which released about 22% of the total sialic acid, reduced virus attachment to the minimum attainable levels which was about 8% of non-enzyme-treated control cells (result not shown). Receptors for influenza virus were more resistant to neuraminidase treatment than those for EMC virus and required 120 min incubation or the loss of approx. 80% of cell sialic acid for their destruction (Fig. 2).

Sialic acid-containing components extracted from erythrocyte membranes

Sialoglycoproteins were extracted from erythrocyte membranes with lithium diiodosalicylate–phenol and examined by electrophoresis on SDS–polyacrylamide gels which were stained with Coomassie Blue (Fig. 3a) or with PAS reagent (Fig. 3b). Based on areas under the curves of scans of PAS-stained gels, the following composition was found: PAS-1 plus PAS-2, 86.4% ± 1.4 (se, 5 determinations); PAS-3, 4.5% ± 0.3 (se, 5 determinations); PAS-4 and other minor components, 9.1% ± 1.3 (se, 5 determinations). The similarity of these values to those obtained when erythrocyte membranes were examined directly by SDS–polyacrylamide gel electrophoresis (Fig. 1g) suggests that the procedure extracts the sialoglycoproteins quantitatively. The 'lipid' component seen on PAS-stained SDS gels of erythrocyte membranes (Fig. 1g) was usually absent from sialoglycoprotein preparations (Fig. 3g). Although these preparations contained material in addition to glycoporphin, they are frequently referred to below as 'glycoporphin preparations' for convenience.

Although the number and distribution of components were similar on Coomassie Blue and PAS reagent-stained SDS–polyacrylamide gel electropherograms of erythrocyte sialoglycoproteins (Fig. 3a, g), the Coomassie Blue components cannot strictly be termed PAS-1, PAS-2, etc. so the alternate nomenclature of CB-1, CB-2, CB-3 and CB-4 was adopted. Analysis of scans of Coomassie Blue-stained SDS gels gave the following composition: CB-1 plus CB-2, 84.9% ± 1.8 (se, 5 determinations); CB-3, 5.3% ± 0.7 (se, 5 determinations); CB-4 plus other minor components, 9.8% ± 1.5 (se, 5 determinations). These values were within the range of those obtained for the corresponding PAS-positive
components suggesting that all of the proteins extracted by the LIS-phenol procedure were glycosylated.

Hydrolysis of erythrocyte sialoglycoprotein preparations with 0.05 M-H$_2$SO$_4$ released 571 ± 14 μg (SE, 5 determinations) sialic acid/mg protein. Assuming that the sialic acid/mg protein ratio was the same for all of the sialoglycoprotein present, most of which was glycophorin A (Fig. 3 a, g), it was calculated that there were about 26 sialic acid residues per polypeptide chain of 14300 dalton size, the mol. wt. calculated for glycophorin A from its amino acid composition (Furthmayr, 1977). The maximum possible number of sialic acid residues per glycophorin based on its known structure is 32 (Marchesi et al., 1976).

**Effect of neuraminidase on glycophorin preparations**

**Physical changes**

Sialoglycoprotein preparations were incubated in the presence or absence of neuraminidase for various lengths of time and, after boiling all samples for 10 min to destroy neuraminidase activity (Enegren & Burness, 1977), the products were analysed by SDS-polyacrylamide gel electrophoresis using Coomassie Blue or PAS staining (Fig. 3). It was observed that neuraminidase treatment increased the electrophoretic mobilities of CB-1, CB-2 and CB-3 (Fig. 3 b to e) and of PAS-1, PAS-2 and PAS-3 (Fig. 3 h to k) and caused these components
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Fig. 4. Effect of neuraminidase on glycophorin: chemical and biological changes. Glycophorin preparations were incubated with neuraminidase for various lengths of time after which the enzyme was destroyed by boiling. The preparations were then measured for free sialic acid (●—●) and for HA inhibitory properties against EMC (○—○) and influenza (●—●) viruses.

to become more polydisperse, as indicated by the broadening of the bands on the electropherograms. These changes began within 10 min of addition of the neuraminidase (Fig. 3b, h). Identical results were obtained whether Coomassie Blue or PAS staining was used suggesting that no major hidden non-sialic acid components were present. The sialoglycoproteins were remarkably stable since samples incubated for 18 h in the absence of neuraminidase (Fig. 3f, l) gave similar profiles to unincubated samples (Fig. 3a, g).

The increase in electrophoretic mobility of the sialoglycoproteins following neuraminidase treatment could have arisen by a reduction in the mol. wt. of the components through release of sialic acid, or by cleavage due to possible protease contamination, or alternatively due to change to a more compact conformation resulting from the loss of negatively charged sialic acid groups. Calculation of mol. wt. changes based upon the amount of sialic acid released (Fig. 4) gave values (results not shown) consistent with the changes in electrophoretic mobilities observed.

Chemical and biological changes

Sialoglycoprotein preparations were incubated with neuraminidase for various lengths of time and the amount of sialic acid released compared with that from identical samples hydrolysed with 0-05 m-H₂SO₄. In the first 60 min, 86% of the sialic acid was liberated by neuraminidase at a linear rate, after which time there was a marked decrease in the rate of release and overnight incubation with enzyme was required for total release (Fig. 4).

The ability of neuraminidase-treated glycophorin preparations to inhibit EMC and influenza virus haemagglutination was also measured. Incubation with neuraminidase for 20 min, which caused release of about 40% of glycophorin sialic acid, abolished its HA inhibitory properties against EMC virus (Fig. 4). Loss of HA inhibition against influenza virus was more resistant and required 45 min incubation or release of 76% sialic acid for complete destruction of inhibition (Fig. 4).

Effect of trypsin on erythrocytes

Physical changes

Trypsin treatment of erythrocytes releases sialoglycopeptides (Makela et al., 1960; Winzler, 1969), quantification of which by sialic acid assays was used to follow the course of proteolytic digestion (Fig. 6). Membranes were prepared from erythrocytes which had been
treated with trypsin for various lengths of time, and analysed by SDS–polyacrylamide gel electrophoresis using Coomassie Blue (Fig. 5a to e) and PAS-reagent staining (Fig. 5f to j). Changes were readily visible in the PAS-stained gels within 5 min of incubation by which time the amount of PAS-1 had decreased and a new component with a mobility between that of PAS-1 and PAS-2 had appeared (Fig. 5f). After 60 min incubation, the new component itself had virtually disappeared and the bulk of the material ran with a mobility between that of PAS-2 and PAS-3. In contrast to these changes, PAS-3 appeared to resist trypsin digestion, or alternatively degradation of PAS-1 generated material with the same mobility as PAS-3. Release of sialoglycopeptides was complete by 60 min incubation (Fig. 6) and little change was seen in the PAS-stained electrophoretic profiles of membranes incubated for longer times (Fig. 5g to i).

When SDS gels of trypsin-treated erythrocyte membranes were stained with Coomassie Blue, changes in the profiles were not as easily detected as in PAS-stained gels, due to the complexity of the profiles.
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Fig. 6. Effect of trypsin on erythrocytes: chemical and biological changes. Cells were incubated with trypsin for various lengths of time, collected by centrifugation and the sialic acid present in the supernatant fluid (●—●) measured after acid hydrolysis. The treated cells were then used to measure their ability to adsorb virus and for the preparation of membranes which were analysed for sialic acid content (○—○). ●—●, EMC virus; ○—○, influenza virus.

A change noted early during incubation was the increase in material with a mobility in the region of band 6 (or perhaps band 7 since it was difficult to distinguish between these components with certainty). This change could not be related to changes in the corresponding PAS-stained gels but could have arisen by cleavage of a larger PAS-positive component into smaller components, one of which lacked carbohydrate residues and, therefore, was not visible on PAS-stained gels but was detected by Coomassie Blue staining. Other changes seen on Coomassie Blue-stained gels were the destruction of band 3 and an increase in material in the region of band 4.5 of the gels (Fig. 5a to d). Although PAS-1 and band 3 have similar mobilities (compare Fig. 1a, g), and both are destroyed by trypsin treatment, the changes caused by trypsin treatment seen in this region on Coomassie Blue-stained gels required longer incubation times than those seen by PAS staining and were most likely unrelated to each other.

Controls consisting of membrane preparations from erythrocytes incubated for 18 h in the absence of trypsin gave SDS–polyacrylamide gel electrophoresis patterns (Fig. 5e, j) similar to unincubated controls (Fig. 1a, g).

Chemical and biological changes

Trypsin treatment of erythrocytes released sialoglycopeptides rapidly in that within 5 min of enzyme addition, 20% of the total cell sialic acid, or 30% of trypsin-releasable sialic acid, was found in the supernatant fluid after the cells had been removed by centrifugation (Fig. 6). Release of sialoglycopeptides reached a plateau after 60 min of incubation when 60% of the cell sialic acid was found in the supernatant fluid. Overnight incubation showed that 70% of the total cell sialic acid could eventually be liberated by trypsic digestion.

Trypsin-treated erythrocytes gave haemagglutination patterns which were difficult to interpret. In the absence of virus, the cells yielded very small ‘buttons’ or none at all in the haemagglutination trays. In the presence of virus, the HA patterns were abnormal and endpoints were difficult to read. Nevertheless, it was clear that trypsin-treated erythrocytes were agglutinated by EMC and influenza viruses, the titres obtained being generally the same or higher than for untreated controls. Because of the difficulties in using trypsin-treated cells in haemagglutination titrations, the biological properties of the cells were tested as follows. Trypsin-treated and untreated erythrocytes were mixed with virus and after 1 h to allow virus...
to adsorb, the cells were collected by centrifugation and the loss of virus from the supernatant fluid measured by haemagglutination assays with untreated human or sheep erythrocytes. This procedure showed that cells incubated with trypsin even for 18 h still bound EMC or influenza virus as effectively as untreated cells even though 70% of the cell sialic acid was released by the treatment (Fig. 6).

The effect of trypsin on the biological properties of erythrocytes was also followed using radioactive EMC virus. In this case, a difference between trypsin-treated and untreated cells was detected, the enzyme reducing attachment to about 50% of untreated controls within 1 h of treatment. Incubation with trypsin for longer times, even overnight, failed to reduce EMC virus attachment below 50% of controls (result not shown).

**Effect of trypsin on glycophorin**

**Physical changes**

Glycophorin preparations were incubated with trypsin for various lengths of time after which the digestion was terminated by addition of lima bean trypsin inhibitor in an amount
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Fig. 8. Effect of trypsin on glycophorin: biological changes. Glycophorin preparations were incubated with trypsin for various lengths of time, the enzyme activity stopped by addition of lima bean trypsin inhibitor and the HA inhibitory properties of the preparations measured. Results are expressed as a percentage of the maximum inhibition detected. O, EMC virus; ●, influenza virus.

previously determined to inhibit trypsin activity completely. Control samples were incubated in the absence of trypsin or in the presence of a mixture of trypsin and trypsin inhibitor. The digests were analysed by SDS–polyacrylamide gel electrophoresis using Coomassie Blue (Fig. 7a to d) or PAS-reagent staining (Fig. 7e to i).

Coomassie Blue staining was not particularly useful since the trypsin and trypsin inhibitor, which were also stained by the procedure, masked the smaller peptides. Nevertheless, it was clear that glycophorin preparations incubated with trypsin were degraded within 15 min to fragments amongst which was a small amount of material with the mobility of CB-2 (Fig. 7a). Incubation for longer times caused the disappearance of the CB-2-like material and the production of small peptides which ran in the trypsin–trypsin inhibitor region of the gels (Fig. 7b, c). PAS-stained gels showed similar changes in that within 15 min of incubation, PAS-1 disappeared and material with the mobility of PAS-2 became evident (Fig. 7e). Incubation for longer times caused reduction in the amount of PAS-positive components detected on the gels (Fig. 7f to h) although the same amount of material was analysed in all cases. Controls incubated with trypsin plus trypsin inhibitor showed no degradation of CB-1 or PAS-1 but an unidentified component with a mobility between that of CB-4 and CB-2 (or PAS-4 and PAS-2) was noted both on gels stained with Coomassie Blue (Fig. 7d) or with PAS reagent (result not shown). A control incubated for 21 h in the absence of trypsin or inhibitor lacked this unidentified component and gave a pattern similar to unincubated samples on gels stained with PAS reagent (Fig. 3).

Chemical and biological changes

The method used to follow the release of sialoglycopeptides from erythrocytes (Fig. 6) could not be applied to glycophorin preparations because of the difficulty of separating sialoglycopeptides from sialoglycoproteins; both would yield sialic acid on acid hydrolysis. Similarly, because the products of trypsin treatment could not readily be separated from trypsin, it was necessary to terminate enzyme activity by addition of lima bean trypsin inhibitor rather than use centrifugation. It was demonstrated that the trypsin inhibitor, itself, had no effect on the biological properties of glycophorin since preparations incubated in the absence of trypsin, or in the presence of enzyme plus trypsin inhibitor were equally effective at HA inhibition (result not shown). In contrast, incubation of glycophorin preparations with trypsin for 15 min reduced the HA inhibitory properties against influenza and EMC viruses. Unexpectedly, however, the inhibitory properties against both viruses recovered and, in fact, became enhanced above controls on prolonged incubation (Fig. 8), in some cases becoming 2 to 4 times higher than the original. Although haemagglutination inhibition for influenza virus
was more sensitive to inactivation by 15 min trypsin treatment than that for EMC virus in the experiment shown in Fig. 8, in other experiments EMC virus haemagglutination inhibition was more sensitive.

**DISCUSSION**

Three observations in the present report are particularly significant in trying to understand the nature of the interaction of EMC and influenza viruses with erythrocytes. The first observation was that differences exist in the rate of neuraminidase inactivation of receptors both *in situ* and in soluble form for EMC virus compared with those for influenza virus. This differential effect of neuraminidase clearly shows that these viruses do not require identical sialic acid residues for attachment. Thus, the perplexing problem of the apparent low specificity of attachment of viruses as different as EMC and influenza viruses posed at the beginning of this report has to some extent been resolved because the viruses do show specificity. However, the exact differences in structure between the attachment sites on the erythrocyte receptors for EMC virus compared with that for influenza virus have yet to be determined.

The second observation considered to be significant was that removal of about 40% of the erythrocyte sialic acid by neuraminidase inhibited EMC virus haemagglutination whereas loss of 70% by trypsin treatment had no similar effect on haemagglutination by EMC or influenza viruses. This shows that not all erythrocyte sialic acid is required for virus attachment but, nevertheless, there appears to exist a sub-set of sialic acid residues which are critically important for binding these viruses. Glycophorin contains 26 (reported here) to 32 sialic acid residues (Marchesi *et al.*, 1976); our results allow estimation of the maximum required for attachment. Neuraminidase treatment of erythrocytes prevents influenza virus haemagglutination when 80% of the sialic acid is released or when less than 5 to 6 residues remain per molecule. Erythrocytes treated with trypsin were still haemagglutinated by either EMC or influenza virus even when 70% of the sialic acid was released, or when there were about 8 to 9 residues per glycophorin. The exact number of sialic acid residues required for virus attachment and their location for EMC and influenza virus respectively is under investigation.

A third observation of significance was that trypsin treatment of glycophorin caused a transient loss in its HA inhibitory properties against EMC and influenza viruses but this recovered on more prolonged incubation. This suggests that fragments of glycophorin have HA inhibitory properties and gives rise to the hope that HA inhibitory active sialoglycopeptides can be isolated and characterized bringing closer an understanding of the fine structure of the active site for virus binding.

Failure of certain viruses to attach to cells from which sialic acid has been removed is generally assumed to prove that such viruses attach directly to sialic acid residues on the cell surface. An alternative hypothesis has recently been proposed that sialic acid plays an indirect role being required to maintain the receptor in the correct configuration, and that neuraminidase treatment destroys this configuration, thus indirectly destroying the attachment site (Burness, 1981). The experiments described in this report were not designed to distinguish between these alternative hypotheses. However, it is considered that the transient inactivation and recovery of the biological properties of glycophorin by trypsin is more readily explained as an effect on the configuration of this receptor than on a change in a binding site containing sialic acid.

Evidence has been presented that gangliosides, which contain sialic acid, can serve as receptors for paramyxoviruses (Haywood, 1974; Holmgren *et al.*, 1980; M. A. K. Markwell, personal communication) although Wu *et al.* (1980) claim that glycoproteins and not gangliosides are the natural receptors for these viruses. Some of the results described here,
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particularly those at the cellular level, would apply whether the receptors for EMC and influenza viruses were ganglioside or sialoglycoprotein. However, the finding that glycophorin preparations gave analogous results to those at the cellular level strongly suggests that sialoglycoprotein per se serve as receptors. It is possible that the results obtained with the glycophorin were due to contamination with gangliosides. This seems unlikely since although analysis of membranes by SDS-polyacrylamide gel electrophoresis revealed the presence of material behaving like glycolipid (see Fig. 7f to g), the glycophorin preparations analysed by the same technique contained little or no glycolipid (Fig. 5g to f). In addition, we have shown previously that removal of material behaving like glycolipid from glycophorin preparations either by affinity chromatography or by chloroform-methanol extraction had little effect or even enhanced the receptor-like properties of the preparations for EMC and influenza viruses (Pardoe & Burness, 1980). Furthermore, the receptor for EMC virus on Krebs ascites tumour cells is sensitive to both neuraminidase and trypsin treatment suggesting involvement of sialoglycoproteins (S. L. Sheppard & A. T. H. Burness, unpublished results). For these reasons, we believe the simplest explanation of the results described in the present report is that the changes observed are by way of sialoglycoproteins, particularly when it is considered that glycolipid sialic acid comprises less than 2% of the total in human erythrocytes (Beeley et al., 1977).

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