Seelig, A., & Seelig, J. (1974) *Biochemistry 13*, 4839–4845.
Seelig, A., & Seelig, J. (1980) *Q. Rev. Biophys. 13*, 19–61.
Shieh, H.-S., Hoard, L. G., & Nordman, C. E. (1981) *Acta Crystallogr. B37*, 1538–1543.
Shimshick, E. J., & McConnell, H. M. (1973) *Biochem. Biophys. Res. Commun. 53*, 446–451.
Slater, J. L., & Huang, C. H. (1988) *Prog. Lipid Res. 27*, 325–359.
Snyder, B., & Freire, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4055–4059.
Sperotto, M. M., Ipsen, J. H., & Mouritsen, O. G. (1989) *Cell Biophys. 14*, 79–95.
Sternin, E., Bloom, M., & MacKay, A. L. (1983) *J. Magn. Reson. 55*, 274–282.
Stockton, G. W., & Smith, I. C. P. (1976) *Chem. Phys. Lipids 17*, 251–263.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954–966.
van Ginkle, G., Korstanje, L. J., van Langen, H., & Levine, Y. K. (1986) *Faraday Discuss. Chem. Soc. 81*, 49–61.
Vist, M. R., & Davis, J. H. (1990) *Biochemistry 29*, 451–464.
Whittall, K. P., Sternin, E., Bloom, M., & MacKay, A. L. (1989) *J. Magn. Reson. 84*, 64–71.
Wolber, P. K., & Hudson, B. S. (1981) *Biochemistry 20*, 2800–2810.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267–287.
Yeagle, P. L. (1988) in *The Biology of Cholesterol* (Yeagle, P. L., Ed.) CRC Press Inc., Boca Raton, FL.

Evidence for a Direct Role for Sialic Acid in the Attachment of Encephalomyocarditis Virus to Human Erythrocytes†

Amir Tavakkol‡ and Alfred T. H. Burness*
Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3V6
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ABSTRACT: Sialic acid residues are required in cellular receptors for many different mammalian viruses. Sialic acid could have a *direct role*, being an integral part of the virus binding site on the receptor. Alternatively, negatively charged sialic acid could have an *indirect role*, being responsible for holding the receptor in the required configuration for virus recognition, for instance, by interacting with positively charged amino acid residues found in the polypeptide chain of receptors. We have investigated the role of sialic acid in virus attachment by studying the interaction of the small RNA virus encephalomyocarditis (EMC) with glycophorin A, its receptor on human erythrocytes. In several experiments, influenza virus A was used for control purposes. Blocking positive charges on glycophorin either in lysine residues by acetylation or in arginine residues with butanedione did not affect its interaction with EMC virus. In contrast, blocking negatively charged carboxyl groups in sialic acid residues by amidation destroyed the ability of glycophorin to inhibit EMC virus attachment suggesting an important role for this part of sialic acid in EMC virus attachment. Removal of the polyhydroxy side chain in sialic acid residues of glycophorin by mild oxidation with periodate followed by reduction with borohydride had little effect on its interaction with EMC virus. Further, sialic acid species with either an acetyl or glycolyl group attached to the amino group on position 5 interacted equally well with EMC virus. We conclude that sialic acid residues play a direct role in attachment of EMC virus to its receptor forming part of the binding site itself, rather than the indirect role of interacting with basic amino acids to maintain the integrity of the attachment site on the receptor.

Many viruses such as influenza virus (Kathan et al., 1961; Marchesi & Andrews, 1971; Allaway & Burness, 1986), encephalomyocarditis (EMC)[1] virus (Enegren & Burness, 1977; Allaway & Burness, 1986), reovirus (Paul & Lee, 1987), and bluetongue virus (Eaton & Crameri, 1989) attach to human red cells by means of glycophorin A, which is the most abundant sialoglycoprotein in the human erythrocyte surface membrane (Furthmayr, 1978). Attachment of all four viruses is inhibited by pretreatment of red cells or glycophorin preparations with neuraminidase (sialidase), suggesting a role for sialic acid in the process.

Sialic acid could have one or two roles in virus attachment (Burness, 1981). It could have a direct role, being the moiety, or at least an important part of the site, to which these viruses bind. Alternatively, negatively charged sialic acid residues could have an indirect role, interacting with positively charged amino acids in glycophorin A holding the receptor in the appropriate three-dimensional configuration for virus binding. An indirect role for sialic acid would allow each virus to bind to its own specific region of glycophorin, but attachment of all would be inhibited by one enzyme, neuraminidase.

An indirect role for sialic acid in virus attachment leads to the prediction that blocking glycophorin A lysine ϵ-amino groups by acetylation or arginine guanidyl groups by butanedione should inhibit attachment of the receptor to viruses. We have tested these predictions in the study reported here. We show that neither treatment affected the interaction of

† Supported by grants from the Canadian Medical Research Council and the Canadian Diabetes Association.
* To whom correspondence should be addressed.
‡ Present address: Department of Dermatology, R6558, Kresge I, University of Michigan Medical School, Ann Arbor, MI 48109-0314.

[1] Abbreviations: EMC, encephalomyocarditis; PBS, phosphate-buffered saline; phosphate–NaCl, 0.1 M NaCl in 0.02 M phosphate buffer, pH 8.0.

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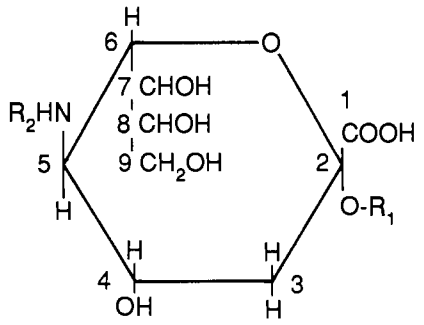

FIGURE 1: Sialic acids: R_1 = H in the free acid or the oligosaccharide side chain terminated by the sialic acid residue in sialoglycoproteins or gangliosides; R_2 = acetyl (CH_3CO) or glycolyl (CH_2OHCO) in commonly occurring sialic acids.

glycophorin A with EMC virus, thus excluding an indirect role for sialic acid in EMC virus attachment.

Since the results were consistent with a direct role in attachment, we next examined which groups in sialic acid residues were involved in virus binding. Modifications to glycophorin sialic acid residues were introduced by direct chemical attack on either the three-carbon long polyhydroxy side chain by periodate–borohydride treatment or the carboxyl group by amidation. In addition, we examined red cells that had attached to the amino group in position 5 of their sialic acid residues (Figure 1), substituents differing from those in human glycophorin. The results suggested that the carboxyl group, but not the polyhydroxy side chain nor the substituents on the amino group in sialic acid, was important for interaction with EMC virus.

Materials and Methods

Materials. Anti-M and anti-N rabbit immune sera were obtained from Ortho Diagnostic Systems, Don Mills, Ontario, *Vibrio cholerae* neuraminidase was from Calbiochem, Aquasol-2, L-[^{3}H]leucine and [^{3}H]acetic anhydride were from New England Nuclear, 2,3-butanedione and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride were from Sigma Chemical Corp., outdated human types O/M and O/N blood were from Canadian Red Cross, St. John's, Newfoundland, fresh bovine and equine blood were supplied by Mr. Roger Bowen, Animal Care Services, Memorial University of Newfoundland, and horse liver alcohol dehydrogenase was a gift from Drs. James Orr and Robert Rimsay.

Glycophorin. This was prepared by subjecting human erythrocyte membranes from outdated, type O/M blood to the lithium diiodosalicylate–phenol procedure of Marchesi and Andrews (1971). No effort was made to purify the individual glycophorin species present (Furthmayr, 1978). The term glycophorin is used in this report to denote human material unless stated otherwise.

Protein and Sialic Acid Concentrations. These were measured by the procedures of Lowry et al. (1951) and Warren (1959), respectively.

Amino Acid Analyses. These were performed in a Beckman Model 121 MB analyzer on samples containing 0.5–1.0 mg of glycophorin hydrolyzed at 110 °C for 24 h in 6 M HCl.

Viruses. The K2 strain of EMC virus was grown in the presence or absence of [^{3}H]leucine in Krebs II ascites tumor cells and purified as described previously (Burness et al., 1974). The PR/8 strain of influenza virus was grown in the allantoic cavity of 10–12-day-old chick embryos (Blaskovic & Styk, 1976).

Virus Attachment and Its Inhibition. About 20 μg (10^4 cpm) of purified ^{3}H-labeled EMC virus was incubated in 0.5 mL of PBS at ambient temperature for 45 min in the presence of about 10 μg of modified or unmodified glycophorin. To the mixture was added 10 μL of packed, washed human erythrocytes and incubation continued for a further 30 min, after which the cells were collected by centrifugation to obtain the supernatant fluid, resuspended in PBS, and again centrifuged. Cells, supernatant, and wash were all treated with 0.5 mL of 30% H_2O_2 to bleach hemoglobin before measurement of their radioactive content in Aquasol-2 in a scintillation counter. Attachment was calculated from the radioactivity in the cell sample expressed as a percentage of the total radioactivity recovered.

Hemagglutination Inhibition. Serial 2-fold dilutions of modified or unmodified glycophorin at a starting concentration of approximately 600 μg of protein/mL were prepared on microtiter plates. To each microtiter well was then added an equal volume (50 μL) containing eight hemagglutinating units of EMC or influenza viruses or anti-M antiserum. The plates were incubated at 4 °C for virus or at ambient temperature for antiserum for 30 min before adding to each well 50 μL of 0.2% (v/v) human types O/M red cells. Dilutions were made throughout in a solution of 4.5% (w/v) glucose–PBS containing 0.1% (w/v) gelatin (1:1). End points were read after keeping the microtiter plates at 4 °C overnight.

Sucrose Density Gradient Centrifugation. About 20 μg (10^4 cpm) of ^{3}H-labeled EMC virus was incubated at ambient temperature for 1 h with 50 μg of modified or unmodified glycophorin in 100 μL of phosphate–NaCl. The mixture was then analyzed by centrifugation on 10–30% (w/v) sucrose gradients in phosphate–NaCl in a Beckman SW 50.1 rotor at 300000*g* for 25 min at 4 °C. Fractions were collected from the top of the gradient by using a Buchler gradient harvester. The pellet at the bottom of the tube was dissolved in 0.2 mL of 1 M NaOH and neutralized with 0.2 mL of 1 M HCl. Fractions were diluted to 1 mL with phosphate–NaCl prior to addition of Aquasol-2 for radioactivity measurements in a scintillation counter.

Acetylation of Glycophorin. The method described by Fraenkel-Conrat (1957) was used as follows. To 1 mg of a glycophorin preparation in 3 mL of saturated sodium acetate at 0 °C was added 20 mg of acetic anhydride followed at 15-min intervals by four further additions each of 5 mg of this reagent; controls were prepared by using either PBS or acetic acid in place of acetic anhydride. After the last addition, the preparations were dialyzed against distilled water overnight, lyophilized, and made up to 0.5 mg of glycophorin/mL in PBS.

Determination of the Extent of Acetylation. The number of lysine residues acetylated was estimated from the loss of ninhydrin-positive ε-amino groups (Fraenkel-Conrat, 1957). In this method, 0.5 mg of acetylated or nonacetylated glycophorin in 0.1 mL of PBS was treated with 1 mL of ninhydrin reagent and the mixture heated in vigorously boiling water for 20 min. After cooling rapidly, 5 mL of 50% (v/v) aqueous isopropyl alcohol was added and the absorbance at 570 nm measured within 10 min. A calibration curve for free amino groups was prepared by using glycine treated similarly.

Amidation of Glycophorin. The method described by Hoare and Koshland (1967) was used as follows. To 2 mg of glycophorin in 8 mL of 6 M guanidine hydrochloride containing 1 M glycinamide was added 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride to 0.1 M and the mixture incubated at 25 °C for 60 min with constant addition of 0.1 M HCl to maintain the solution at pH 4.7. The reaction was terminated by addition of 15 mL of 1.0 M sodium acetate buffer, pH 4.7, and the sample dialyzed overnight against 1

Table I: Effect of Different Treatments on the Ability of Glycophorin To Inhibit Hemagglutination

glycophorin treatment	hemagglutination inhibition[a]		
	EMC virus	influenza virus	MN antiserum
none	256	1024	64
acetic acid	128	2048	128
acetic anhydride	128	2048	<2
none	512	2048	64
butanedione	512	2048	64
none	512	1024	128
amidation[b]	<2	768	<2
none	512	1536	256
periodate 1:1[c]	512	512	96
periodate 2:1[c]	512	<2	48
periodate 3:1[c]	64	<2	<2
periodate 4:1[c]	64	<2	<2

[a] Expressed as the reciprocal of the glycophorin dilution to inhibit one hemagglutination unit. [b] Amidation with glycinamide. [c] Ratios of periodate to sialic acid (see Materials and Methods).

mM HCl, lyophilized, and made up to 0.5 mg of glycophorin/mL in PBS.

When taurine was used to amidate carboxyl groups, the same conditions were used with taurine replacing the glycinamide.

Periodate–Borohydride Treatment of Glycophorin. The method described by Suttajit and Winzler (1971) was used as follows. About 1 mg of glycophorin in 0.14 M NaCl was treated with 2 h at 0 °C in the dark with sodium periodate solution at final concentrations of 1 mol of sialic acid to 1, 2, 3, or 4 mol of periodate. The reaction was terminated by addition of 3 mol of glycerol per mole of periodate used in the reaction. After exhaustive dialysis against 0.14 M NaCl, 1 mL of 0.1 M sodium bicarbonate in 0.14 M NaCl and 0.2 mL of 1 M sodium borohydride were added and, after keeping the mixture for 2 h at 0 °C, acetic acid was added to bring the sample to pH 4.5, after which it was dialyzed against distilled water, lyophilized, and made up to 0.5 mg of glycophorin/mL in PBS.

Butanedione Treatment of Glycophorin. The method of Yankeelov (1972) was used as follows. One milligram of glycophorin, or of horse liver alcohol dehydrogenase as a positive control, was incubated at room temperature for 2 h with 3 mL of 0.5% (w/v) butanedione in 0.05 M sodium borate buffer, pH 8.4. The sample was then dialyzed overnight against borate buffer, lyophilized, and made up to 0.6 mg of protein/mL in phosphate buffer adjusted to pH 8.4 for storage.

Horse Liver Alcohol Dehydrogenase Activity. The ability of unmodified and butanedione-modified enzyme to reduce acetaldehyde in the presence of NADH was measured as described by Ryzewski and Pietruszko (1977).

Results

Effect of Acetylation on Glycophorin. We have shown previously that glycophorin inhibits EMC virus hemagglutination (Enegren & Burness, 1977). To determine if lysine residues are important in this inhibition, we examined the effect of acetylation on glycophorin since it is known that acetylation blocks lysine ϵ-amino groups (Fraenkel-Conrat, 1957). We found that, within the limits of the technique, glycophorin acetylated with acetic anhydride inhibited EMC virus hemagglutination equally as well as glycophorin incubated with acetic acid or PBS to serve as controls (Table I). Similarly, acetylated glycophorin still inhibited influenza virus hemagglutination at the same levels as unmodified glycophorin controls (Table I).

Table II: Effect of Acetylation and Amidation of Glycophorin on Its Ability To Inhibit ^{3}H-Labeled EMC Virus Attachment

glycophorin	% virus attachment	% inhibition of attachment[a]
absent	51.5	
untreated	23.0	55.3
acetylated	22.7	55.9
absent	53.2	
untreated	28.8	45.9
amidated with glycinamide	52.5	1.3
absent[b]	82.9	
untreated	48.6	41.4
amidated with glycinamide	91.7	0
amidated with taurine	89.9	0

[a] 100 − % attachment compared to that in the absence of glycophorin. [b] Virus with higher specific radioactivity was used in this series of experiments.

Table III: Effect of Different Treatments on the Ability of Glycophorin To Cause EMC Virus Aggregation

glycophorin	% ^{3}H virus in pellet	glycophorin	% ^{3}H virus in pellet
absent	3.1	absent	7.0
untreated	78.4	periodate 1:1[b]	30.9
acetylated	72.7	periodate 3:1[b]	5.1
untreated	69.2		
amidated[a]	6.9		

[a] Amidated with glycinamide. [b] Ratio of periodate to sialic acid (see Materials and Methods).

We have also shown previously that glycophorin inhibits attachment of ^{3}H-labeled EMC virus to human erythrocytes (Enegren & Burness, 1977). We found in the present studies that about 52% of the radioactivity in ^{3}H-labeled EMC virus preparations was bound by human erythrocytes, but in the presence of appropriate glycophorin concentrations this was reduced to 23% (i.e., about 55% inhibition) (Table II). We found under similar conditions that acetylated glycophorin still inhibited ^{3}H-labeled EMC virus attachment by about 56% (Table II), suggesting that the amino groups in glycophorin were not important for interaction with EMC virus.

Conditions can be selected to centrifuge ^{3}H-labeled EMC virus to the middle of a sucrose density gradient (Figure 2a) with 3% or so of the radioactivity sedimenting to the bottom of the centrifuge tube (Table III). However, when virus was mixed with glycophorin before centrifugation, the virus peak was lost (Figure 2a), as shown previously (Enegren & Burness, 1977), and about 78% of the radioactivity was found in the pellet (Table III). Similarly, acetylated glycophorin also caused EMC virus aggregation (Figure 2a), resulting in about 73% of the radioactivity being recovered from the pellet (Table III).

Glycophorin A can serve not only as a virus receptor but also as the antigen responsible for MN blood group activity (Hamaguchi & Cleve, 1972). MN antigenic activity is destroyed by acetylation of glycophorin (Lisowska & Duk, 1975). We confirmed that acetylation of glycophorin with acetic anhydride reduced its ability to inhibit NM antiserum hemagglutination to below the level of detection (Table I). This showed that the conditions we used acetylated glycophorin effectively.

Another method to monitor the effectiveness of the acetylation procedure is to measure the number of free amino groups in glycophorin by a ninhydrin procedure (see Materials and Methods) before and after acetylation. Human glycophorin contains five lysine residues (Tomita & Marchesi, 1975) and an NH_2 terminus giving a total of 6 free amino groups per

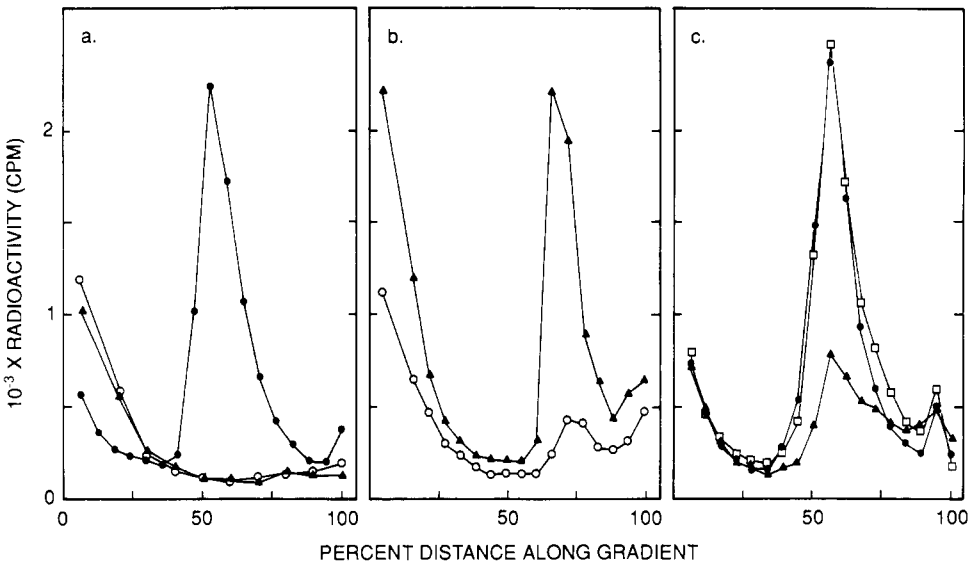

FIGURE 2: Sucrose density gradient centrifugation studies on EMC virus and glycophorin. Approximately 20 μg of ^{3}H-labeled amino acid EMC virus alone or after mixing with about 50 μg of modified or unmodified glycophorin was analyzed by centrifugation on sucrose gradients (see Materials and Methods). The direction of centrifugation is from left to right. (a) Virus alone (●) or mixed with glycophorin treated either with PBS (i.e., unmodified) (○) or with acetic anhydride (▲), which causes acetylation. (b) Virus mixed with unmodified (○) or amidated (▲) glycophorin. (c) Virus alone (●) or mixed with glycophorin treated with sodium periodate at concentrations of 1 mol of sialic acid to 1 (▲) or 3 mol (□) of periodate before reduction with sodium borohydride.

molecule. We found by the ninhydrin procedure 5.5 and 5.4 free amino groups per glycophorin molecule incubated in PBS or acetic acid, respectively, but only 1.2 per acetylated glycophorin.

Effect of 2,3-Butanedione on Glycophorin. Butanedione specifically blocks positive charges on the guanidyl group of arginine (Yankeelov, 1972). We found that butanedione-treated and untreated glycophorin were equally effective at inhibiting hemagglutination by EMC virus, influenza virus, or MN antiserum (Table I). This suggests either that arginine residues are not involved in the interaction of glycophorin with any of these three agglutinins or, alternatively, that butanedione was not modifying arginine residues under the conditions used.

To check this second possibility, we treated horse liver alcohol dehydrogenase with butanedione since this enzyme is known to have arginine in its active center (Lange et al., 1974). We found enzymatic activity was reduced to 9.7% of untreated controls.

Effect of Amidation on Glycophorin. Amidation blocks negative charges on carboxylic acid groups such as those found in aspartic, glutamic, or sialic acids (Hoare & Koshland, 1967). We found that amidation of glycophorin with glycinamide severely affected its inhibition of hemagglutination by EMC virus and, as reported previously (Ebert et al., 1972), by MN antiserum (Table I). In contrast, amidated and untreated glycophorin inhibited influenza virus hemagglutination essentially to the same extent (Table I).

Amidation also affected the ability of glycophorin to block ^{3}H-labeled EMC virus attachment. Untreated glycophorin caused a 46% reduction in the amount of radioactivity attaching to human erythrocytes, whereas amidated glycophorin inhibited attachment by about only 1% (Table II).

Amidation of glycophorin also reduced its ability to cause ^{3}H-labeled EMC virus aggregation as determined by sucrose density gradient sedimentation. As expected, addition of untreated glycophorin caused virus to aggregate, resulting in about 70% of the radioactivity appearing in the pellet at the bottom of the tube (Table III) with a small peak of virus in the gradient (Figure 2b). In contrast, virus mixed with amidated glycophorin behaved identically with that mixed with no glycophorin (Figure 2a); the virus sedimented mostly as a peak found about halfway down the gradient (Figure 2b) with some material at the top of the gradient and about 7% in the pellet (Table III).

Table IV: Glycine Content of Amidated and Untreated Glycophorin

treatment of glycophorin	amino acid	nmol	calcd no. of residues[a]
untreated	phenylalanine	177.6	2
	glycine	516.7	5.8
amidated with glycinamide	phenylalanine	129.3	2
	glycine	1739.7	26.9
amidated with taurine	phenylalanine	57.8	2
	taurine	917.8	31.8

[a] Number of residues calculated by dividing nanomoles of amino acid by nanomoles of phenylalanine and multiplying by 2, the number of phenylalanine residues in glycophorin A (Tomita & Marchesi, 1975).

Determination of the Extent and Sites of Amidation by Glycinamide. Glycophorin A contains 6 aspartic and 12 glutamic acid residues (Tomita & Marchesi, 1975), a carboxy-terminus, and 26–30 sialic acid residues (Pardoe & Burness, 1981; Fukuda et al., 1987), i.e., a total of about 45–49 carboxyl groups. Since amidation potentially affects all carboxylic acid groups, it was important to know which residues were modified.

The number of carboxylic acid groups amidated can be determined by amino acid analysis since the acid hydrolysis required to release amino acids converts the substituent glycinamide into glycine (Hoare & Koshland, 1967). We confirmed there were 6 glycine residues per glycophorin molecule for every 2 phenylalanine residues present (Table IV), as reported previously by Tomita and Marchesi (1975). Analysis of glycophorin amidated with glycinamide revealed about 27 glycine residues for every 2 phenylalanine residues (Table IV). The extra glycine in amidated glycophorin suggests that about 21 carboxyl groups had been blocked.

To determine which residues contained blocked carboxyl groups, we next examined the nature of the sialic acid residues present in amidated glycophorin. Hydrolysis with 0.1 N

H_2SO_4 at 80 °C for 60 min is effective at releasing sialic acids from glycosidic linkages (Schauer, 1987). Acid hydrolysis of amidated glycophorin under these conditions revealed that about 66% of the sialic acid residues were modified, taking into account that amidated residues had only about 7% of the color yield of nonamidated residues (A. Tavakkol and A. T. H. Burness, unpublished results) in the thiobarbituric acid assay (Warren, 1959). Since glycophorin contains 26–30 sialic acid residues, these results suggested that 17–21 must have been amidated, which accounts for most, if not all, of the 21 carboxyls found by amino acid analysis to be blocked. It would seem, therefore, that few if any of the aspartate or glutamate carboxyls were modified and that the changes in biological properties of glycophorin on amidation were due to sialic acid changes alone.

Blocking Carboxyl Groups in Glycophorin with Taurine. Amidation of glycophorin with glycinamide replaced the negatively charged carboxyl group with a neutral group and also added a side chain at carbon atom 1 of the sialic acid residue. To explore which of these two possible modifications caused inactivation, we replaced glycinamide (NH_2CH_2CO-NH_2) with taurine ($NH_2CH_2CH_2SO_3H$) in the amidation procedure. As a result, the negatively charged sialic acid carboxyl groups were replaced by negatively charged sulfonic acid groups, but the substituent side chain remained similar, although not identical, in size to glycinamide.

Glycophorin amidated with taurine was found to contain about 32 taurine residues per molecule by amino analysis (Table IV), on the basis of 2 phenylalanine residues per glycophorin molecule (Tomita & Marchesi, 1975). Amidation with taurine destroyed the ability of glycophorin to inhibit ^{3}H-labeled EMC virus attachment as effectively as did amidation with glycinamide, in that the percent virus attachment was as high as the level found in the absence of glycophorin (Table II). This suggests that the negative charge on the sialic acid residue was not, itself, the only factor for interacting with EMC virus but that the size of the arm carrying the charge was also important. Presumably the sialic acid residue has to be of the correct size to fit into the receptor binding site on the virus.

Effect of Periodate Oxidation–Borohydride Reduction on the Biological Properies of Glycophorin. Suttajit and Winzler (1971) showed that periodate oxidation–borohyride reduction of glycophorin specifically attacked the three-carbon-long, polyhydroxy side chain in the sialic acid residues (Figure 1). Treatment of glycophorin with periodate at a molar concentration equal to that of the sialic acid present, followed by borohyride reduction, removed carbon atom 9 and its hydroxyl. When double the amount of periodate was used, both carbon atoms 8 and 9 and associated hydroxyl groups were removed from most of the sialic acid residues present. The same authors also showed that these conditions resulted in no changes in other carbohydrates or in any of the amino acids in the glycophorin polypeptide chain. This treatment decreased the ability of glycophorin to inhibit hemagglutination by influenza virus (Suttajit & Winzler, 1971) or by MN antiserum (Ebert et al., 1972).

We treated glycophorin with a range of periodate concentrations from zero to four times the molar ratio of sialic acid before reduction with borohydride. We confirmed that molar ratios of periodate to sialic acid of 1 or 2 curtailed the ability of glycophorin to inhibit influenza virus or MN antiserum hemagglutination; in contrast, we found that EMC virus hemagglutination was not affected, being inhibited at the same levels as by the untreated glycophorin (Table I). However, when the molar ratios of periodate to sialic acid was increased to 3 or 4, the ability of glycophorin to inhibit EMC virus hemagglutination was reduced. These higher periodate concentrations could conceivably have caused damage to amino acids or other carbohydrates in glycophorin but this possibility was not investigated.

Table V: Hemagglutination and Attachment of ^{3}H-Labeled EMC Virus to Erythrocytes Containing Different Sialic Acid Species

erythrocyte	sialic acid species[a]	HA titre	% virus attachment
human	NeuNAc	2560	78.4
bovine	NeuNGc	<40	1.8
equine	NeuNGc	1920	63.6

[a] Abbreviations: Neu5Ac = *N*-acetylneuraminic acid; Neu5Gc = *N*-glycolylneuraminic acid.

We also examined the ability of periodate-modified glycophorin to cause EMC virus aggregation as measured by analysis on sucrose gradients. We found that glycophorin modified by a periodate to sialic acid ratio of 1:1 caused about 31% of the virus to pellet to the bottom of a sucrose gradient or was about 40% as effective as untreated glycophorin (Table III); the same periodate-treated glycophorin reduced the size of the virus peak in a sucrose gradient (Figure 2c) but not as effectively as untreated glycophorin (Figure 2a,b). Virus mixed with glycophorin treated with a periodate to sialic acid ratio of 3:1 behaved like virus in the absence of glycophorin when examined on sucrose gradients (Figure 2c) and on the basis of the amount of radioactivity in the pellet (Table III); i.e., the ability of glycophorin to interact with EMC virus was destroyed.

Effect of Amino Group Substituents in Sialic Acid on EMC Virus Receptor Properties. We reported previously that EMC virus does not agglutinate bovine red cells (Angel & Burness, 1977), an observation we confirmed again (Table V). We have now also shown that less than 2% of a ^{3}H-labeled EMC virus preparation became bound to bovine erythrocytes, whereas close to 80% attached to human cells (Table V). *N*-Acetylneuraminic acid is apparently the only sialic acid species present in human erythrocytes (Klenk et al., 1955), while the predominant sialic acid in bovine erythrocytes is *N*-glycolylneuraminic acid (Fukuda et al., 1982). To examine the possibility that EMC virus does not interact with bovine erythrocytes because it cannot recognize receptors containing *N*-glycolylneuraminic acid, we measured attachment to equine erythrocytes, the sialic acid of which is 99% *N*-glycolyl derivative (Reuter et al., 1988). We found that EMC virus hemagglutination of these cells was about 75% and attachment about 80% of that to human erythrocytes (Table V), demonstrating that EMC virus recognizes *N*-glycolylneuraminic acid and suggesting that failure of the virus to bind to bovine cells must be due to other factors.

Discussion

In the study reported here, we examined whether the role of sialic acid in the attachment of viruses to red cells is *direct* being part of the binding site of the receptor, or *indirect*, for instance, interacting with positively charged amino acids to hold the receptor in the correct configuration for virus recognition.

It was predicted that if sialic acid has a structural, indirect role, blocking positive charges on basic amino acid residues or negative charges on sialic acid residues should destroy receptor activity. We show in this report that blocking negative charges in sialic acid residues in glycophorin by amidation did indeed destroy its ability to serve as a receptor for EMC virus.

However, blocking positive charges on lysine or arginine residues in glycophorin did not destroy its receptor activity toward EMC virus (or influenza virus). Therefore, whatever sialic acid negative charges interact with, it is not with these basic amino acids. It must be added though that we did not exclude interaction with histidine positive charges in the study. Nevertheless, as a result of these findings, we now favor the view that sialic acid has a direct role in binding EMC virus to its receptors.

In the second part of the study we explored which groups within sialic acid itself are important for virus–receptor recognition. We examined the role of the carboxyl group at position 1, the nature of the substituent on the amino group in position 5, and the polyhydroxy side chain containing carbon atoms 7–9 (Figure 1).

We have shown previously that attachment of EMC virus to receptors on red cells or the binding of glycophorin to EMC virus–Sepharose is inhibited by NaCl, suggesting that the attachment involves weak ionic bonds (Pardoe & Burness, 1981; Allaway & Burness, 1987). The results presented here are consistent with an important role for the carboxyl group in sialic acid, this negatively charged group possibly interacting with positive charges in the receptor binding site on the virus. However, more than simple negative charges are required. We showed that virus does not interact with glycophorin in which carboxyl groups are replaced by negatively charged sulfonic acid groups (Table II) or with bovine red cells that contain as much sialic acid as human red cells (Table V). We presume that the size of the sialic acid residue and the way it is presented to the virus are important for recognition to occur and for binding to take place.

The structure proposed by Wiley and Skehel (1987) for the complex of sialyl lactose with influenza virus hemagglutinin, as deduced by X-ray crystallography, shows sialic acid playing a direct role, in this case, sitting in the receptor binding pocket on the hemagglutinin. Bonds between the polyhydroxy side chain in sialic acid with amino acids in the pocket could be envisaged, but whether the sialic acid carboxyl group interacted with structures in the pocket was uncertain. The results we describe in the present report confirm a role for the polyhydroxy side chain but suggest no role for the carboxyl group of sialic acid in its interaction with influenza virus.

In contrast, although we have clearly established a role for the sialic acid carboxyl group in EMC virus attachment, the requirement for the polyhydroxy side chain is less certain. Glycophorin oxidized with periodate then reduced with borohydride under conditions that should have removed carbon atoms 8 and 9 together with associated hydroxyl groups from sialic acid residues still inhibited EMC virus hemagglutination as efficiently as untreated glycophorin (Table I). However, glycophorin treated under less vigorous conditions, which were selected to remove just carbon atom 9 and its hydroxyl, did not cause aggregation of virus as effectively as untreated controls (Figure 2). We did not confirm what modifications were brought about by the periodate–borohydride treatment. Nevertheless, the changes that ensued destroyed the ability of glycophorin to inhibit influenza virus hemagglutination (Table I) so presumably must have been similar to those reported by Suttajit and Winzler (1971) who obtained essentially the same result as we did for influenza virus.

We next examined the effect of two different substituents on the amino group in sialic acid (Figure 1) by examining attachment to cells containing *N*-acetyl- and *N*-glycolylneuraminic acids, respectively. We found that EMC virus hemagglutination or attachment to cells containing *N*-acetylneuraminic acid was, at the most, only marginally higher if at all (Table V), suggesting that this region of the sialic acid moiety was not as important as other regions in the attachment process. Furthermore, since the glycolyl group is slightly larger than the acetyl group, it would appear that the receptor binding site on the virus is large enough to accommodate these differences.

Our results suggest that the secondary and tertiary structure of glycophorin A is not maintained by the interaction of sialic acids with basic amino acid residues. The implication is that attachment of any virus to glycophorin A that is inhibited by neuraminidase is through direct binding to sialic acid moieties rather than to a binding site created by interaction of sialic acid with positive charges in another part of the receptor. The implication of a direct role for sialic acid in binding viruses in addition to EMC virus is consistent with the X-ray crystallographic data of Wiley and Skehel (1987) for influenza virus described above and with the results of Paul et al. (1989) showing that sialic acid is the minimum determinant for reovirus attachment.

The direct binding of EMC, influenza, and reoviruses to sialic acid residues could indicate lack of specificity in the process. However, we have shown in this report that EMC and influenza viruses require different groups in sialic acid for attachment. Furthermore, reovirus appears unable to attach to 9-*O*-acetylated forms of sialic acid (Pacitti & Gentsch, 1987), unlike EMC virus (M. P. Baldeh, I. U. Pardoe, and A. T. H. Burness, unpublished results).

Our results have additional implications. Blocking lysine or arginine residues in glycophorin A with acetic anhdride or with butanedione, respectively, had no deleterious effect on the binding of the red cell receptor to EMC virus. This suggests that neither amino acid was part of the site on glycophorin A directly involved in EMC virus binding.

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References

Allaway, G. P., & Burness, A. T. H. (1986) *J. Virol. 59*, 768–770.

Allaway, G. P., & Burness, A. T. H. (1987) *J. Gen. Virol. 68*, 1849–1856.

Angel, M. A., & Burness, A. T. H. (1977) *Virology 83*, 428–432.

Blaskovic, D., & Styk, B. (1976) *Methods Virol. 1*, 163–235.

Burness, A. T. H. (1981) in *Virus Receptors Part 2, Receptors and Recognition, Series B* (Lonberg-Holm, K., & Philipson, L., Eds.) pp 63–84, Chapman and Hall Ltd., London.

Burness, A. T. H., Fox, S. M., & Pardoe, I. U. (1974) *J. Gen. Virol. 23*, 225–236.

Eaton, B. T., & Crameri, G. S. (1989) *J. Gen. Virol. 70*, 3347–3353.

Ebert, W., Metz, J., & Roelcke, D. (1972) *Eur. J. Biochem. 27*, 470–472.

Enegren, B. J., & Burness, A. T. H. (1977) *Nature 268*, 536–537.

Fraenkel-Conrat, H. (1957) *Methods Enzymol. 4*, 247–269.

Fukuda, K., Kawashima, I., Tomita, M., & Hamada, A. (1982) *Biochim. Biophys. Acta 717*, 278–288.

Fukuda, M., Lauffenburger, M., Sasaki, H., Rogers, M. E., & Dell, A. (1987) *J. Biol. Chem. 262*, 11952–11957.
Furthmayr, H. (1978) *J. Supramol. Struct. 9*, 79–95.
Hamaguchi, H., & Cleve, H. (1972) *Biochim. Biophys. Acta 278*, 271–280.
Hoare, D. G., & Koshland, D. E. (1967) *J. Biol. Chem. 242*, 2447–2453.
Kathan, R. H., Winzler, R. J., & Johnson, C. A. (1961) *J. Exp. Med. 113*, 37–45.
Klenk, E., Faillard, H., & Lempfrid, H. (1955) *Hoppe-Seyler's Z. Physiol. Chem. 301*, 235–246.
Lange, L. G., Riordan, J. F., & Vallee, B. L. (1974) *Biochemistry 13*, 4361–4370.
Lisowska, E., & Duk, M. (1975) *Eur. J. Biochem. 54*, 469–474.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Marchesi, V. T., & Andrews, E. P. (1971) *Science 174*, 1247–1248.
Pacitti, A. F., & Gentsch, J. R. (1987) *J. Virol. 61*, 1407–1415.
Pardoe, I. U., & Burness, A. T. H. (1981) *J. Gen. Virol. 57*, 239–243.
Paul, R. W., & Lee, P. W. K. (1987) *Virology 159*, 94–101.
Paul, R. W., Choi, A. H., & Lee, P. W. K. (1989) *Virology 172*, 382–385.
Reuter, G., Stoll, S. Kamerling, J. P. Vliegenthart, J. F. G., & Schauer, R. (1988) in *Sialic Acids 1988, Procedings of the Japanese–German Symposium on Sialic Acids* (Schauer, R., & Yamakawa, T., Eds.) pp 88–89, Kieler Verlag Wissenschaft + Bildung, Kiel.
Ryzewski, C. N., & Pietruszko, R. (1977) *Arch. Biochem. Biophys. 183*, 73–82.
Schauer, R. (1987) *Methods Enzymol. 138*, 132–161.
Suttajit, M., & Winzler, R. J. (1971) *J. Biol. Chem. 246*, 3398–3404.
Tomita, M., & Marchesi, V. T. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2964–2968.
Warren, L. (1959) *J. Biol. Chem. 234*, 1971–1975.
Wiley, D. C., & Skehel, J. J. (1987) *Annu. Rev. Biochem. 56*, 365–394.
Yankeelov, J. A. (1972) *Methods Enzymol. 25*, 566–579.

Electrostatic Contributions to the Binding of Myosin and Myosin–MgADP to F-Actin in Solution†

Stefan Highsmith
Department of Biochemistry, School of Dentistry, University of the Pacific, San Francisco, California 94115
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ABSTRACT: The ionic strength dependence of skeletal myosin subfragment 1 (S1) binding to unregulated F-actin was measured in solutions containing from 0 to 0.50 M added lithium acetate (LiOAc) in the absence and presence of MgADP. The data were analyzed by using a theory based on an ion interaction model that is rigorous for high ionic strength solutions [Pitzer, K. S. (1973) *J. Phys. Chem. 77*, 268–277] in order to obtain values for K, the equilibrium association constant when the ionic strength is zero, and for $|z_M z_A|$, the absolute value of the product of the net electric charges of the actin binding site on myosin (z_M) and the myosin binding site on actin (z_A). The presence of MgADP reduced K by a factor of 10, as expected, and reduced $|z_M z_A|$ by about 1 esu^2. Because the presence of MgADP is not likely to change the net charge of the myosin binding site on actin, these data are consistent with a model in which MgADP binding to S1 reduces its affinity for actin by a mechanism that reduces the net electric charge of the acting binding site on S1. The value of $|z_M z_A|$ in the absence of ADP was 8.1 ± 0.9 esu^2, which, if one uses integer values, suggests that z_M and z_A are in the 8+ to 1+ esu and 1− to 8− esu ranges, respectively. ADP binding then reduces z_M to the 7+ to 0.88+ esu range.

In the absence of regulatory proteins, the interactions at the interface of actin and myosin are determined primarily by the actin binding site on myosin, which in turn is controlled by the structure of the ligand that occupies the distant myosin nucleotide binding site. Chemomechanical energy transduction by muscle comprises the details of the mechanism of this control, which are not well understood. One facet of the transduction mechanism consists of the changes in the affinity of myosin for actin that are caused by changes in the ligand bound at the myosin nucleotide binding site. The standard free energy change for myosin subfragment 1 (S1)[1] binding to F-actin in solution at 25 °C and 140 mM ionic strength is increased 3–4-fold as S1-bound MgATP is hydrolyzed and then dissociates from the acto–S1 complex during the ATP hydrolysis cycle (Lymn & Taylor, 1971). In muscle, mechanical force is generated by the acto–S1–nucleotide complex as it forms and stabilizes. This coupling of increased binding energy and force generation makes the details of the bound nucleotide-induced structural changes at the actin binding site on S1 as they relate to actin binding of interest. Two broad approaches have been used to investigate the acto–S1 interface and its modification by S1-bound nucleotides. One is the

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[1] Abbreviations: S1, myosin subfragment 1; OAc, acetate; z_M, net electric charge of the actin binding site on myosin; z_A, net electric charge of the S1 binding site on actin; K_{app}, apparent association constant; K, association constant in zero ionic strength; I, ionic strength; a, activity; m, molarity; c, concentration; γ, activity coefficient; b, α, $\beta(0)$, $\beta(1)$, and C, constants in the Pitzer (1977) treatment of ions in solution.

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