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Conformational alterations within the glycocalyx of erythrocyte membranes studied by spin labelling

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The structure of the glycocalyx of the membrane of human erythrocytes and spectrin-depleted vesicles was studied under various conditions by two spin-labelling approaches: (i) covalently labelling sialic acid residues of the glycocalyx and (ii) incorporation of a charged hydrophobic spin probe, CAT 16, being sensitive to alterations on the membrane surface into the lipid phase. Although cell electrophoretic measurements which were performed, additionally, indicated an erection of the glycocalyx upon decreasing the ionic strength of the suspension medium a more restricted mobility of spin-labelled sialic acid residues was found, in this case probably due to electrostatic interactions. The enhanced mobility of the spin probe CAT 16 at low ionic strength as well as in the case of neuraminidase-treated cells could be caused by reduced steric and electrostatic interaction with glycoproteins and glycolipids. La^{3+} adsorption and virus attachment on the human erythrocyte membrane were accompanied with a reduced mobility of sugar headgroups of the surface coat. No indication of cluster formation or lateral segregation of glycophorin molecules was found upon virus binding. After denaturation of the spectrin cytoskeleton of intact erythrocytes, increased mobility of spin-labelled sialic acid residues was observed.

Introduction

The biological significance of the glycocalyx of eucaryotic cells in vital processes, e.g., cell-cell recognition, aggregation (or agglutination) and fusion, virus infection, is well established [1–3]. It is supposed that, besides initiation of important biological phenomena after specific binding of molecules, for instance hormones, the glycocalyx is a main component in regulation of morphogenesis and growth [2]. Malignant transformation of cells is accompanied by alterations of the cell surface, especially of carbohydrates of oligosaccharide chains of glycolipids and glycoproteins [4,5]. The role of transmembrane glycoproteins in maintaining as well as inducing the cell shape of human erythrocytes is still under discussion [7–9].

The molecular structure and dynamics of the glycocalyx, its alteration in response to vital events (see above) and to the physicochemical environment, its involvement in transmembrane signalling [7,10] are known to only a small extent. Cell electrophoresis offers an integral insight in structural transition of the glycocalyx [11,12]. In addition to electron microscopic studies [13], spectroscopic methods seem to be exceptionally suita-

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ble for a systematic understanding of the membrane surface coat on a molecular level. Electron paramagnetic resonance (EPR) has been widely applied to study the molecular behaviour of biomembranes using spin labels [14]. The spin labelling approach for studying the glycocalyx of plasma membranes was applied in two different ways. (A) Charged hydrophobic probes incorporated into the lipid phase were proposed to be sensitive to events on the membrane surface due to exposure of the N-O group to the surrounding aqueous phase [15–17]. (B) Another possibility to characterize cell-surface phenomena was presented by the covalent labelling of sialic acid residues of glycoproteins [10,18–20] and glycolipids [18,21,22].

In the present paper we demonstrate the potency of these EPR spin-labelling approaches in studying the molecular structure of the glycocalyx under those conditions which are relevant to cell-cell recognition, adhesion and fusion. We investigated the influence of ionic strength and surface charge as well as the state of the cytoskeleton on the structure of the surface coat by (a) covalent labelling of the sialic acid residues of the glycocalyx and (b) by incorporating a charged spin probe, being sensitive to alterations directly on the outer membrane surface, into the erythrocyte membrane [17]. The parallel approach of EPR and cell electrophoresis which was performed additionally should provide a more comprehensive insight into glycocalyx structure. Furthermore, the influence of La^{3+}-promoting cell-cell aggregation [23], as well as virus binding, on the mobility of cell-surface constituents was examined using human erythrocytes as a model system.

Material and Methods

If not stated otherwise, the following suspension media for erythrocytes, ghosts and vesicles were used:
buffer 1: 150 mM NaCl/5.8 mM phosphate buffer (pH 7.4);
buffer 2: 5.7 mM NaCl/262 mM sucrose/5.8 mM phosphate buffer (pH 7.4);
buffer 3: 150 mM NaCl/20 mM sodium acetate buffer (pH 5.2).

Erythrocyte preparation. Human erythrocytes of several donors were used (blood-bank, Berlin-Lichtenberg). All experiments were performed not more than 48 h after blood sampling. Removal of plasma and buffy coat (at $500 \times g$, 5 min) was followed by three washings with 5 vol. of the desired suspension medium at $2000 \times g$ (10 min). Erythrocyte ghosts were prepared according to the procedure of Dogde et al. [24].

Spectrin-depleted erythrocyte vesicles. Spectrin-depleted vesicles were obtained by in vitro aging of human erythrocytes as described by Lutz et al. [25]. Erythrocytes were washed three times and, subsequently, incubated in 150 mM NaCl/10 mM glycylglycine (pH 7.4) under sterile conditions (500 U/ml penicillin G, VEB Jenapharm) at 37°C for 48 h. The hematocrit was adjusted to 20%. The erythrocyte suspension was placed into a dialysis bag which was suspended in an excess of medium. During incubation the pH was checked carefully and readjusted. Thereafter, the supernatant of the suspension ($2000 \times g$, 10 min) was washed four times in buffer 2 at 15000 rpm (Janetzki K-24 centrifuge, rotor 6×26) for 30 min (4°C). The cell sediment was washed three times in buffer 1 at $2000 \times g$ (10 min).

Liposome preparation. DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, Fluka) was used without further purification. The samples gave a single spot upon thin-layer chromatography. DPPC was added to buffer 1 or buffer 3 in a final concentration of 10 mM. Vesicles were prepared by sonication with a Branson sonifier for 10 min at 40 W.

Spin labelling. CAT 16. A thin film of the spin label 1-oxyl-2,2,6,6-tetramethyl-4-dimethylaminopiperidinecetyl bromide (CAT 16, Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia) was prepared on a glass wall. Thereafter, the erythrocyte suspension (hematocrit 50%) was added and shaken for about 15 min at room temperature. Subsequently, three washings in the desired suspension medium followed ($2000 \times g$). The label-to-phospholipid ratio was in the order of 1 : 30 to 1 : 50. In the case of DPPC vesicles, the label CAT 16 was added prior to sonication (final concentration 0.1 mM). EPR spectra of these samples gave no evidence of the formation of free radicals due to sonication.

Spin labelling of sialic acid residues. Sialic acid residues of glycoproteins as well as glycolipids of

erythrocyte ghosts were covalently labelled with the spin label Tempamine (2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl, Reanal, Budapest) following the procedure of Feix and Butterfield [18]. Labelling was performed in 150 mM NaCl/5.8 mM phosphate buffer (pH 8.0) by oxidizing the C-7, C-8 and C-9 vicinal hydroxyl groups of sialic acid residues of ghost membranes (5 mg protein/ml) to a terminal aldehyde in the presence of 1 mM $NaIO_4$ (10 min at 4°C). The periodate-activated sample was spin-labelled overnight by reductive amination with 2 vol. 1 mM Tempamine and 2 vol. $NaBH_3CN$ at 4°C. The negative charge of the sialic acid residues is preserved. After incubation the sample was washed six times with buffer 1. No EPR signal could be detected in the supernatant. Spectrin-depleted vesicles of aged erythrocytes were labelled in the same way.

Neuraminidase treatment. Prior to the aging procedure of erythrocytes and spin labelling with CAT 16, surface charge removal was performed with neuraminidase (5.5 U/mg from Atrobacter, Staatl. Inst. Immunpräp. Nährmed., Berlin-Weißensee, or from *Vibrio cholera*, Serva) as described previously [26]. The enzyme treatment took 1 h at 37°C under continuous stirring in 150 mM NaCl/5.7 mM KCl/5 mM glucose/5.8 mM phosphate buffer (pH 7.4). The actual enzyme efficiency was checked by electrophoretic mobility measurements. The enzyme treatment was stopped by four washings in ice-cold buffer 1.

Virus treatment. After spin labelling, erythrocytes as well as ghosts were resuspended in buffer 3 or buffer 1. In the case of intact cells the hematocrit was 2.5%. The number of ghosts corresponded to the number of cells at a hematocrit of 2.5%. Subsequently, influenza virus A_0PR8, which was kindly delivered from the Institut für Angewandte Virologie, Berlin-Schöneweide, was added to a final concentration of 40 μg virus protein/ml ($2.3 \cdot 10^4$ HAU/mg virus protein). After 10 min incubation the samples were centrifuged and the pellets were used for EPR measurements. All steps were performed at 4°C.

La^{3+}-treatment. After spin labelling the samples were incubated in the presence of 0.5 mM $LaCl_3$ for 5 min at room temperature prior to EPR measurements.

Electrophoretic mobility measurements. The electrohoretic mobility of intact erythrocytes, ghosts and spectrin-depleted vesicles was measured in isotonic NaCl/sucrose media (0.3 mM $NaHCO_3$ (pH 7.4)) at 20°C by means of a cytopherometer (Opton, F.R.G.).

EPR measurements. EPR spectra were recorded on a Varian E-3 and an ESR 231 spectrometer (Centre for Scientific Instrumentation, Academy of Sciences of the G.D.R.) with variable temperature equipment. Flat quartz cells for aqueous solutions were used. The temperature was measured by a small thermistor inserted into the sample cell (accuracy ± 0.2°C).

Evaluation of EPR spectra. For the spin label CAT 16 in the low-temperature region the external peak distance, $2A'_z$, was used as a relative measure of mobility of the probe [17]. At 37°C with fast label, an apparent correlation time τ_{CAT} (τ in ns) according to:

$$\tau_{CAT} = 6.65 \cdot 10^{-10} \times \Delta H_{+1}[G] \times \left[(I_{+1}/I_{-1})^{1/2} - 1 \right] \quad (1)$$

was estimated, similar to our previous report [17] with ΔH_{+1} the linewidth of the low-field component and I_{+1} and I_{-1} the heights of the low- and high-field lines, respectively. In the case of spin-labelled sialic acid residues, we calculated for better comparison [18] the following correlation times (τ in ns) [27]:

$$\tau_B = -5.9 \cdot 10^{-10} \times \Delta H_0[G] \times \left[(I_0/I_{+1})^{1/2} - (I_0/I_{-1})^{1/2} \right] \quad (2)$$

$$\tau_C = 6.65 \cdot 10^{-10} \times \Delta H_0[G] \times \left[(I_0/I_{+1})^{1/2} + (I_0/I_{-1})^{1/2} - 2 \right] \quad (3)$$

ΔH_0 and I_0 are the linewidth and the amplitude of the midfield line, respectively. Furthermore, according to Schreier et al. [14], we used $\Delta\tau = \tau_C - \tau_B$ as a relative measure of order of the motion of the sialic acid residues.

Statistics. If not stated otherwise, we used the two-tailed *t*-test for paired observations at a significance level of $\alpha = 0.05$.

Results

Characterization of the Tempamine binding site on sialic acids

The EPR spectrum of spin-labelled sialic acid residues on human erythrocyte membranes is shown in Fig. 1. The shape of the spectra suggests a rapid isotropic rotation of the covalently attached spin label. This was found for the whole temperature range measured (5 to 50°C). No immobilized component, especially at low temperature, was detected. However, the differences between the correlation times τ_B and τ_C ($\Delta\tau$) indicated at least a small anisotropy of the label motion which was more pronounced at low temperatures (Fig. 2). The temperature-dependence of τ_B and τ_C and their difference are presented in Fig. 2. Plotting these values in Arrhenius coordinates, a straight line over the whole temperature range was observed (not shown).

The isotropic hyperfine splitting, a_{iso}, of the covalently attached spin label was within the experimental error, similar to a_{iso} of the free label in aqua dest. (16.8 G). No indication of Heisenberg spin exchange was found, in accordance with the results of Feix and Butterfield [18]. Comparing the correlation times of the covalently attached spin label (at about 20°C) it could be stated that our values $\tau_B \approx 1.1$ ns, are similar to those reported previously by Feix and Butterfield [18] (0.84 ns for ghost membranes) and Lee and Grant [20] (1.03 ns for glycophorin incorporated into DPPC vesicles). Small differences could arise from sample preparation, e.g., all labelling and EPR measuring steps were performed by Feix and Butterfield at low ionic strength (0.5 mM sodium phosphate buffer (pH 8.0)).

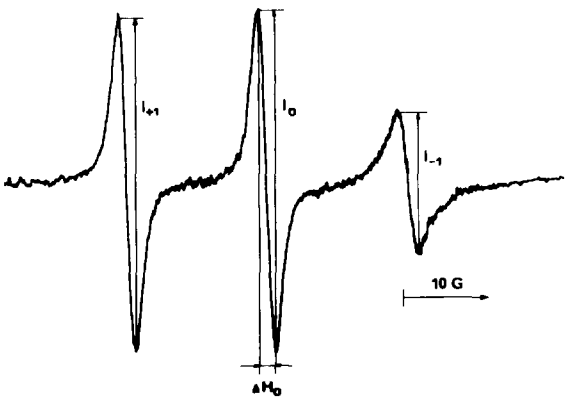

Fig. 1. EPR spectrum of covalently spin-labelled (Tempamine) sialic acid residues of glycolipids and glycoproteins of human erythrocyte membranes (37°C, buffer 1 (pH 7.4)). The parameters used for calculation of correlation times are shown.

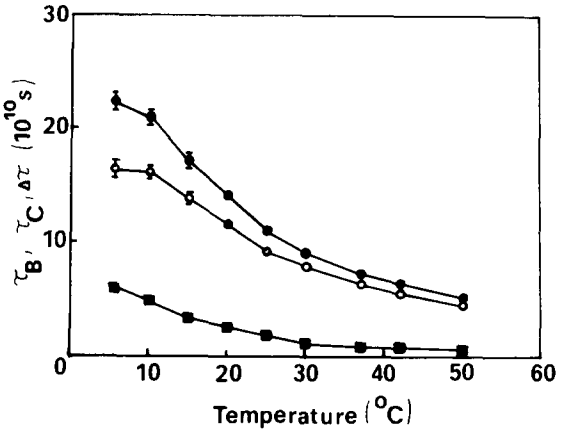

Fig. 2. Temperature dependence of the correlation times τ_B (○) and τ_C (●) and their difference $\Delta\tau$ (■) of spin-labelled sialic acid residues of the glycocalyx of human erythrocytes (buffer 1 (pH 7.4)). The standard error of the estimate is given.

After incubation of spin-labelled ghosts at 50°C for 10 min (buffer 1), lower values of τ_B, τ_C and $\Delta\tau$ were measured at 20°C in comparison to the control (Table I) The shape of the spectra as well as τ_B, τ_C, $\Delta\tau$ and a_{iso} of covalently labelled sialic acid residues of spectrin-depleted erythrocyte vesicles were not significantly different in buffer 1 (cf. Table I).

EPR spectra of membranes labelled with CAT 16

Typical EPR spectra of intact erythrocytes labelled with the positively charged spin probe CAT 16 at different temperatures are shown in Fig. 3. The lineshape of the EPR spectra is similar to those from the corresponding probe CAT 12 with a shorter alkyl chain in erythrocytes [17,28] and *E scherichia coli* membranes [29], respectively. Because of the complex shape of the EPR spectra at room temperature [17,28], spectra were recorded at 5°C and 37°C in order to approximate better the slow and fast motional lineshapes, respectively. Although no anisotropic motion of such a choline label was reported by Hubbel et al. [28], we are well aware that anisotropic motion takes

TABLE I

RELATIVE CORRELATION TIMES AND DIFFERENCES

Relative correlation time, τ_B/τ_{Bcon}, and relative difference, $\Delta\tau/\Delta\tau_{con}$, of correlation times τ_B and τ_C of spin-labelled sialic acid residues of the glycocalyx of human erythrocytes and spectrin-depleted erythrocyte vesicles under various conditions at 20°C (pH 7.4), τ_{Bcon} and $\Delta\tau_{con}$ refer to erythrocytes without further treatment (buffer 1). The standard error of the estimate is given. s indicates significant differences in comparison to the control. Each value represents at least four independent measurements.

Sample	Modification/ suspension medium	τ_B/τ_{Bcon}	$\Delta\tau/\Delta\tau_{con}$
Erythrocyte membrane	control/buffer 1	1.00 ± 0.01	1.00 ± 0.02
Erythrocyte membrane	buffer 2	1.24 ± 0.04 s	1.18 ± 0.05 s
Erythrocyte membrane	incubation at 50°C for 10 min/buffer 1	0.85 ± 0.02 s	0.72 ± 0.03 s
Erythrocyte membrane	addition of influenza virus (40 μg virus protein/ml) buffer 1	1.37 ± 0.03 s	1.13 ± 0.02 s
Erythrocyte membrane	addition of $LaCl_3$ (0.5 mM)/buffer 1	1.14 ± 0.02 s	1.17 ± 0.15 s
Spectrin-depleted vesicles	buffer 1	1.14 ± 0.07	1.03 ± 0.10
Spectrin-depleted vesicles	buffer 2	1.33 ± 0.03 s	1.29 ± 0.05 s

place to a certain extent. Nevertheless, because the shape of the spectra did not differ significantly from those of isotropic motion, the apparent correlation time, τ_{CAT}, was calculated for spectra measured at 37°C. As shown previously [17], the CAT 16 probe is localized exclusively on the outer surface of the human erythrocyte membrane. The absence of spectral parts of free rotating label molecules indicated a strong anchoring of the long cetyl residue in the lipid bilayer.

Membrane spectra of CAT 16 similar to those of erythrocyte membranes were recorded in case of DPPC vesicles and virus membranes. The comparison of the values of the external peak distance, $2A'_z$, as well as their temperature dependence, are shown in Fig. 4. The $2A'_z$ of CAT 16 incorporated into influenza virus membranes was about 2 G

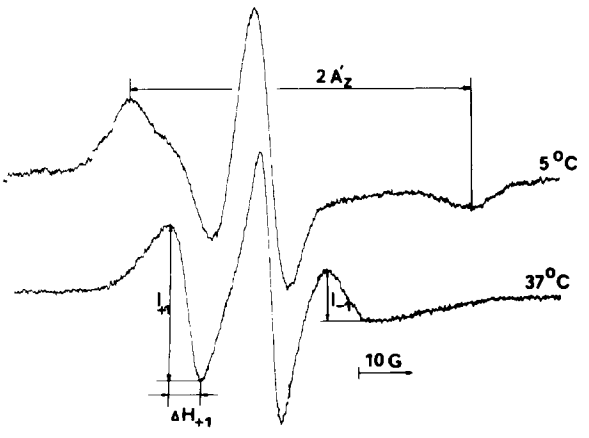

Fig. 3. EPR spectra of the charged cationic spin probe CAT 16 incorporated into intact human erythrocyte membranes at different temperatures (buffer 1 (pH 7.4)). The spectral parameters used for evaluation are indicated.

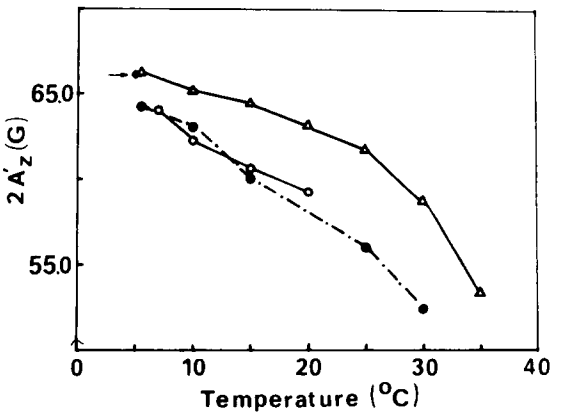

Fig. 4. External peak distance, $2A'_z$, of the spin label CAT 16 incorporated into various membranes as a function of temperature. ○, human erythrocyte membranes (buffer 1 (pH 7.4)); ●, △, DPPC vesicle membranes in buffer 1 (pH 7.4) ○ and in buffer 3 (pH 5.2) (△); ◆ (arrow), influenza virus membrane (buffer 1 (pH 7.4)).

TABLE II

EXTERNAL PEAK DISTANCES AND RELATIVE CORRELATION TIMES WITH CAT 16

Difference of the external peak distance, $2A'_z$ (at 5°C) ($\Delta 2A'_z = 2A'_z - 2A'_{zcon}$), and the relative correlation time τ_{CAT}/τ_{CATcon} (at 37°C) of the spin label CAT 16 incorporated into the human erythrocyte membrane. $2A'_{zcon}$ and τ_{CATcon} refer to erythrocytes without further treatment (buffer 1 (pH 7.4) or buffer 2 (pH 5.2)). The standard error of the estimate is given. s indicates significant differences in comparison to the control. Each value represents at least four independent measurements.

Sample	Modification/ suspension medium	$\Delta 2A'_z$ (G)	τ_{CAT}/τ_{CATcon}
Erythrocyte membrane	control/buffer 1	0 ±0.14	1.00±0.04
Erythrocyte membrane	buffer 2	−0.75±0.10 s	0.93±0.02 s
Erythrocyte membrane	neuraminidase treatment/buffer 1	−0.74 ±0.14	0.93±0.04
Erythrocyte membrane	neuraminidase treatment/buffer 2	−0.64 ±0.15 s	0.97±0.02
Erythrocyte membrane	addition of influenza virus (40 μg virus protein/ml) buffer 1	0.76 ±0.06 s	1.19±0.05 s
Erythrocyte membrane	addition of influenza virus (40 μg virus protein/ml) buffer 3	0.96 ±0.22 s	1.20±0.03 s

larger in comparison to those of human erythrocyte and DPPC vesicle membranes (buffer 1, pH 7.4, 5°C), indicating a more restricted motion in virus membranes. Upon decreasing the pH to 5.2 (buffer 3) in the case of DPPC vesicles $2A'_z$ increased significantly. This can be accounted for, at least to a certain extent, by a loss of the neutral character of DPPC. This is in accordance with the observed enhancement of the phase-transition temperature of DPPC upon lowering the pH, suggesting that this lipid behaves as a negatively charged lipid [30,31]. However, such a strong pH dependence was not observed with virus and erythrocyte membranes ($2A'_z$ was unaffected between pH 7.4 and 5.2).

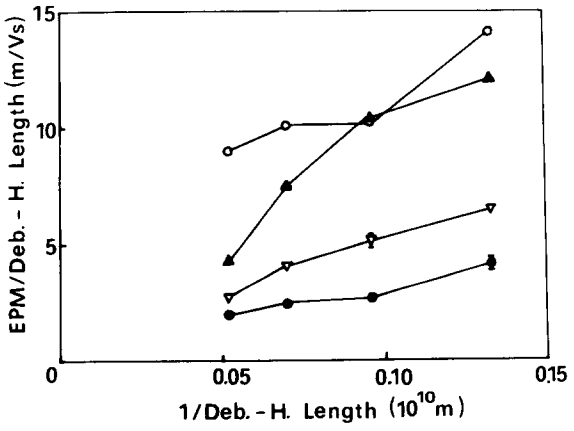

Fig. 5. The ratio of the electrophoretic mobility (EPM) and the Debye-Hückel length of intact human erythrocytes (○), neuraminidase-treated erythrocytes (●), spectrin-depleted vesicles (▲) and neuraminidase-treated spectrin-depleted vesicles of in vitro aged erythrocytes (▽) at 20°C. The standard error of the estimate is given.

Conclusions similar to those on the basis of $2A'_z$ could be drawn by evaluating the correlation time, τ_{CAT}: 3.1 ns for human erythrocyte membranes (37°C), 4.4 ns for influenza virus (37°C), 2.1 ns (pH 5.2) and 3.4 ns (pH 7.4) for DPPC vesicles at 40°C.

Influence of ionic strength and surface charge reduction on the molecular structure of the membrane surface

EPR measurements. In contrast to media of physiological ionic concentrations (buffer 1), the correlation time, τ_B, as well as the qualitative measure of anisotropy, $\Delta\tau$, increased significantly when the NaCl concentration was 5.7 mM (buffer 2) (Table I). This effect was independent of the

presence of sucrose. In these and all other experiments, the behaviour of τ_C was similar to that of τ_B.

The isotropic hyperfine splitting, a_{iso}, remained constant. Furthermore, no indication was found of spin-spin interactions. Similar results were obtained with spin-labelled spectrin-depleted vesicles, for which the effect of low ionic concentration seems to be more pronounced than in intact erythrocytes (Table I).

A significant decrease in the external peak distance, $2A'_z$, of CAT 16 membrane spectra (5°C) was found by transferring intact erythrocytes into a suspension medium of low NaCl concentration (buffer 2). After charge reduction of the glycocalyx by neuraminidase treatment, a similar significant decrease in $2A'_z$ was observed at high (buffer 1) and low (buffer 2) NaCl concentration (Table II). Although not significant, the values of τ_{CAT} at 37°C reflected the same tendency as $2A'_z$ (Table II). Electrophoretic mobility measurements showed that the charge was reduced to about 40%.

Electrophoretic mobility measurements. The results of electrophoretic measurements of untreated and neuraminidase-treated human erythrocytes and spectrin-depleted vesicles are presented in Fig. 5 as the ratio of mobility and Debye-Hückel length against the inverse of the Debye-Hückel length. From this plot one may infer parameters of the charge distribution within the cell-surface coat [32–34]. In the case of an uncharged glycocalyx (charges located only directly on the membrane surface) and no viscous interaction between glycocalyx and liquid flow (classical model) a straight line would be obtained. The electrophoretic mobility behaviour of neuraminidase-treated and untreated human erythrocytes was similar to that reported elsewhere [32,33]. At high ionic strength the electrophoretic mobility of spectrin-depleted vesicles was comparable to that of intact erythrocytes, which is in agreement with results of Müller et al. [35], who found that the sialic acid content per membrane surface area of these vesicles is similar to that of fresh cells. However, these authors did not measure the electrophoretic mobility at low ionic concentration. Remarkably, we found a much stronger dependence of the electrophoretic mobility of spectrin-depleted vesicles on ionic strength than with intact erythrocytes. Switching the vesicles between media of high and low ionic concentration it was proved that the electrophoretic mobility measured was reversible. Comparing neuraminidase-treated vesicles and erythrocytes, the electrophoretic mobility as a function of ionic strength was similar, whereas at high NaCl concentration (150 mM) the electrophoretic mobility of vesicles was significantly higher, in contrast to previous results [35].

No differences in the electrophoretic mobility behaviour of control and artifically aged human erythrocytes (in both cases of untreated and neuraminidase-treated cells) were observed (not shown), suggesting that no loss of sialic acids and portions of glycophorin due to proteolytic activity took place during the aging procedure. Furthermore, the electrophoretic mobility of Dodge ghosts did not differ significantly from that of intact cells [36].

All results presented were independent of the neuraminidase used.

Influence of virus attachment on the molecular structure of the membrane surface

It is well known that cell-virus and cell-cell fusion as well as cell lysis of erythrocytes is caused by the influenza virus only at a pH of about 5.2, while aggregation is observed over a broad pH range, including pH 7.4 [37–39]. Therefore, we investigated the influence of virus binding at the cell surface on the mobility of the spin label CAT 16 at pH 5.2 and 7.4. Cell-cell aggregation and fusion as well as haemolysis were carefully checked [40] and were found to be in accordance with data reported previously [38,39]. At both pH values were observed a significant increase in $2A'_z$ and τ_{CAT} (Table II), respectively, suggesting a more hampered motion in the presence of the influenza virus. The half-linewidth of the low field at 5°C was unaffected. No pH-dependent changes were observed. A similar result was obtained in the case of covalently spin-labelled sialic acid residues of the glycocalyx, which serve as the receptor site for influenza virus [37]. A significant enhancement of τ_B as well as of $\Delta\tau$ was found in the presence of the virus (Table I; measurements were performed only at pH 7.4, buffer 1). Likewise, a_{iso} remained unaffected and spin-spin interactions were not observed.

Influence of La^{3+} on the mobility of sialic acid residues

An intense cell-cell aggregation of human erythrocytes was observed after addition of $LaCl_3$ [23]. A significant decrease in the mobility of sialic acid residues was ascertained in the presence of $LaCl_3$ (0.5 mM final concentration, buffer 1). The relative measure of anisotropy $\Delta\tau$ increased, but not significantly.

Discussion

Mobility of sialic acid residues of the glycocalyx

Under our conditions, similar to those of Feix and Butterfield [18], about 40% of sialic acid residues were covalently labelled and 70% of the label fraction was bound to the integral sialoglycoproteins (most prominently glycophorin A) [10,18]. The remaining label was attached to glycolipids [10,18]. About $4 \cdot 10^5$ molecules of glycophorin A, $7 \cdot 10^4$ of glycophorin B and $3.5 \cdot 10^4$ of glycophorin C are present at the membrane surface of an erythrocyte [41]. The N-terminal segment of glycophorin which is exposed to the outer membrane surface consists of about 64 amino acids to which 16 oligosaccharides are linked [42]. These are terminated by sialic acids. Keeping in mind that 32 negative charges are localized at the headgroup of a glycophorin molecule [43,44], it can be estimated that about 8 sialic acid residues per molecule glycophorin are labelled. Our data showed that under all experimental conditions the oligosaccharide chains were characterized by a considerable freedom of motion in a hydrophilic environment, which is in agreement with previous studies [3,19–22]. The isotropic hyperfine splitting, which is also a measure of the polarity, was similar to that of the free label in water. This supports the assumption that the dielectric constant within the glycocalyx is similar to that of water due to the small volume occupied by glycolipids as well as by glycoproteins within the surface coat [32].

Upon decreasing the temperature, a reduction of the mobility and an enhancement of anisotropy were observed. At low temperatures an aggregation of intramembranous particles within the human erythrocyte membrane was observed [45,46]. The major intrinsic membrane proteins, band 3 and glycophorin, are the constituents of these intramembranous profiles [47–49]. The temperature-dependent intramembranous particle aggregation as the reason for the observed mobility behaviour of the bound label is contradicted by the following: (i) a_{iso} remained constant; an enhanced charge-charge interaction should be accompanied by an increase in a_{iso} [22]. (ii) No evidence of spin-spin interactions was discovered. (iii) The absence of any detectable immobilized component argued against a strong intramolecular immobilization of the oligosaccharide headgroups.

It is assumed that also other membrane constituents influence the temperature-dependent oligosaccharide headgroup mobility. Although it has been stated that the dynamic state of terminal sugars is very weakly correlated with the properties of the bilayer membrane itself [22], it can be expected that the independent degrees of freedom of sugar chains are superimposed upon the motion of the hydrophobic membrane-inserted portion of the protein [2]. Furthermore, there is experimental evidence for a transmembrane linkage between glycophorin and the cytoskeleton of the erythrocyte membrane [6,7,10], which offers a mechanism of information transfer through the membrane. Such a linkage is also in agreement with our results. After denaturation of spectrin at 50°C [50] an enhanced mobility of sialic acid residues was established, suggesting that the connection between both proteins was disturbed. Taking into account that no differences between the label mobility of intact erythrocytes and vesicles which lacked spectrin were found, it is concluded that the motion was determined by different membrane components and properties.

Phospholipid headgroup environment reflected by CAT 16 mobility

The N-O group of the CAT 16 label is located in the vicinity of phospholipid headgroups [15,17,28]. The tempoyl residue of CAT 16 is able to rotate about the C-N bond, but in membranes this motion is restricted by steric as well as electrostatic interactions [28], for instance, with negatively charged sialic acids [29]. Since a pH-dependent alteration of the erythrocyte membrane fluidity was found [51] we explained the lack of a pH-dependent signal to CAT 16 in virus and

erythrocyte membranes by an interaction between glycocalyx constituents and the label. The absence of any significant change in electrophoretic mobility of intact erythrocytes over a broad pH range has been reported [22]. In contrast, the signal of CAT 16 incorporated into DPPC vesicle membranes exhibited a strong pH-dependence.

A comparison of EPR spectra showed that sialic acid residues were characterized by a higher mobility, $\tau_{CAT} \approx 1.2$ ns, than CAT 16, $\tau_{CAT} \approx 3.1$ ns, using Eqn. 1. The mobility of CAT 16 in membranes was much more influenced by the lipid fluidity than was the motion of sialic acid residues. This was obvious from the temperature dependence of the shape of the spectra as well as of the parameters ($2A'_z$, τ_{CAT}) evaluated. $2A'_z$ showed a strong temperature dependence at the phase transition of DPPC membranes (Fig. 4) [30,31]. Also, the observed shift of the phase transition to higher temperatures upon lowering the pH [30,31] was felt by the label. Furthermore, the difference of $2A'_z$ between virus and erythrocyte membranes is consistent with the highly restricted phospholipid headgroup environment of the viral envelope surface detected by ^{31}P-NMR [52].

Structural alterations of the glycocalyx

Due to the charged nature of the surface coat, some influence of the ionic strength of the surrounding medium on the oligosaccharide conformation and mobility should be expected. Decreasing the ionic strength we found an increased correlation time as well as enhanced anisotropy of labelled sialic acid residues on erythrocyte membranes. A reversible intramembranous particle aggregation at low ionic concentrations has been described [46,49,53]. Furthermore, raising the concentration of glycophorin within the membrane a more restricted motion of labelled sialic acid residues was detected which could be caused by a viscosity effect or by attractive forces [20,22]. However, for electrostatic reasons, aggregation of intramembranous particle, or at least, of charged glycophorin molecules, should be not expected at low ionic concentrations [54]. From our results we have also no evidence for an aggregation of glycophorin molecules at a low NaCl concentration for the same reasons mentioned above (i–iii).

An alternative explanation of our results could be the following. Using a more sophisticated model for charge distribution within the glycocalyx, Donath and Pastushenko [31,32] concluded from the electrophoretic mobility behaviour of human erythrocytes (similar to that shown in Fig. 5) an increase of the glycocalyx thickness from about 5.5 nm at physiological ionic concentrations to 12 nm at low ionic concentrations due to increased repulsion of charges. Such an increase in thickness is energetically favoured at low ionic strength [11]. An increase in thickness which is accompanied by a decreased segment intensity does not necessarily have result in a higher label mobility. Electrostatic interaction (repulsion) can restrict mobility and introduce some ordering. This can be illustrated measuring the thickness of the glycocalyx in Debye-Hückel units. At low NaCl concentrations (buffer 2) the relative thickness of the glycocalyx is about 30% smaller than in physiological NaCl concentrations (buffer 1). From this estimation, it follows that the interaction at low ionic strength is much higher, even taking into account the greater distance between the fixed charges. It is proposed that the physicochemical conditions of the glycocalyx determine the preferred positions of the completely fixed charges by having a certain degree of flexibility. Rüppel et al. [44] proposed two conformations of the sugar-containing headgroups: (a) a flat conformation where the headgroups form a two-dimensional layer spread on the membrane surface, and (b) a three-dimensional conformation in which the headgroups are oriented into the aqueous phase.

Similar conclusions can be drawn from electrophoretic mobility and EPR measurements on spectrin-depleted vesicles. However, the dependence of electrophoretic mobility behaviour on ionic strength was stronger in that case. This cannot be explained by (1) an irreversible loss or alteration of conformation of sialic acid residues, on (2) by an ionic-strength-dependent transversal distribution of charged phospholipids. (The differences of the electrophoretic mobility between neuraminidase-treated erythrocytes and vesicles cannot account for such an effect.)

Taking into account that the electrophoretic mobility behaviour of aged erythrocytes was similar to that of fresh cells, we suppose that, besides electrostatic forces, other components are also in-

volved in determining the structure of the surface coat. Also, EPR measurements indicated a more hindered motion of sialic acid residues of spectrin-depleted vesicles at low NaCl concentration in comparison to intact erythrocytes.

The less restricted motion of CAT 16 at low ionic strength is consistent with the idea of a glycocalyx which is erected into the aqueous phase, decreasing steric as well as electrostatic interactions with this label. Rüppel et al. [44] calculated that in the three-dimensional conformation 80 lipids interact with the protein headgroup, whereas in the two-dimensional structure 400 lipids are affected by the headgroup. Assuming $5 \cdot 10^5$ copies of glycophorin [41] and about $3 \cdot 10^8$ phospholipid and cholesterol molecules [55] in the outer leaflet per erythrocyte, it can be estimated that about $2 \cdot 10^8$ molecules of the lipid phase are influenced by glycophorin in the flat conformation. The enhanced mobility of CAT 16 in the case of neuraminidase-treated cells is rather caused by reduced electrostatic interaction between the positive choline group and the negative sialic acid than altered gylcocalyx thickness, which seems to be not influenced by this treatment [34].

The specific protein-lipid interaction of glycophorin [56] and the effect of glycophorin on permeability [37] as well as on flip-flop behaviour of phospholipids of vesicle membranes are well documented. It is reasonable to assume that conformational changes of the external part of this molecule, creating any macromolecular stress (e.g., vertical displacement within the membrane) can influence, at least locally, these properties, offering possible mechanisms for information transfer across the membrane as well as for interface recognition. The biological significance is obvious.

La^{3+} adsorption and virus attachment

Although it was supposed that La^{3+} is adsorbed in the glycocalyx near the membrane surface, inducing an additional binding between glycolipids and phospholipid headgroups [23,59], the CAT 16 label did not indicate any significant mobility change directly on the membrane surface [17]. However, the altered mobility of labelled sialic acid residues by La^{3+} confirms that the intense cell-cell aggregation [23] is accompanied by structural changes within the surface coat [59,60].

In contrast to ionic strength variation, the virus attachment on the cell surface is a local event, suggesting that the effect on the binding site was more pronounced, as concluded from the spectra measured. The motion of CAT 16 was restricted by virus attachment at both pH levels (5.2 and 7.4) since the $2A'_z$ and τ_{CAT} were significantly higher. Since no influence on the fluidity of the hydrophobic part of the lipid phase was found after virus binding [40,61], these alterations were caused by a direct interaction between components of the virus membrane with CAT 16 or by an indirect effect of the virus via glycophorin-CAT 16 interaction. Also, the sugar headgroup motion of glycophorin molecules was more hindered by virus attachment. A similar effect on glycophorin incorporated into membranes was reported after addition of the lectin wheat-germ agglutinin [20,22]. The authors suggested a clustering of glycophorin molecules. We found in the case of erythrocyte membranes no experimental evidence either for those clusters (also points i–iii mentioned above were valid) or for a lateral segregation creating protein-free lipid areas at contact areas. We rather assume that virus binding fixes the sialoglycoproteins in their position with respect to the membrane surface and induces conformational changes.

The question arises as to whether the binding of the virus on the outer membrane leaflet could be communicated to the cytoskeleton via a glycophorin-spectrin linkage. In a forthcoming paper we will present some experimental indications for such a connection, probably important for initiating endocytosis of enveloped viruses by target cells.

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