

Interaction of Phloretin with the Human Red Cell Membrane and Membrane Lipids: Evidence from Infrared, Raman and ESR Spectroscopy

Guido Zimmer *, Herbert O. Günther **, and Hartmut Schmidt ***

* Gustav Embden-Zentrum der Biologischen Chemie Universität Frankfurt,
Theodor-Stern-Kai 7, D-6000 Frankfurt/M.

** Landesuntersuchungsamt für das Gesundheitswesen Südbayern, Augsburg

*** Institut für Physikalische Biochemie, Universität Frankfurt

Z. Naturforsch. 36 c, 586–592 (1981); received February 18, 1981

Membrane-Phloretin Interaction, Infrared Raman, ESR Spectroscopy

The transport inhibitor phloretin was bound to human red cell membrane and the concomitant structural changes were observed by spectroscopic methods. By the spin labeling method a decrease in fluidity of the membrane was found at 1 and 10 μM concentrations of the reagent. This result was obtained with the 2-(3-Carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy, and the 2-(14-Carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy lipid spin labels. Infrared spectroscopy of modified membranes revealed an intensity increase of the POO^- band at about 1250 cm^{-1} . Moreover, a shift of the peak at 1050 cm^{-1} to 1100 cm^{-1} was observed in the presence of phloretin. Raman spectroscopy of the membranes did not contradict the results found with infrared and ESR spectroscopy: In the phloretin modified membrane we observed a lack of the band at 1085 cm^{-1} , which leads to suggest that the POO^- and/or C-C regions are less fluid. Changes of the extracted red cell membrane lipids were less characteristic, and the results differed from those found in red cell membrane.

The way of interaction of transport inhibitors with their target site can be investigated by binding studies. In particular, such investigations carried out on one type of inhibitor interfering with different types of membrane transport may be rewarding and could generally improve our understanding of membrane structure-function relationship.

The transport inhibitor phloretin being an example of that kind, thus inhibits transport of sugar, urea, anions, and the Na^+K^+ pump [1]. According to Jennings *et al.* [2], phloretin appears to be distributed on both protein and lipid binding sites in the red cell membrane. Work on thin lipid membranes, carried out by Andersen *et al.* [3], has produced evidence that the effects of the reagent on diverse transport phenomena may be related to adsorption at the membrane lipid surface, whereby a dipole potential opposite to the preexisting one is created.

By our experiments on the red cell membrane and the extracted membrane lipids, using spectroscopic methods, we aimed at a better insight into possible rearrangements on the molecular level.

Materials and Methods

Phloridzin and phloretin were obtained from Carl Roth, Karlsruhe, FRG, as pure substances and used

Reprint requests to Dr. Guido Zimmer.

0341-0382/81/0700-0586 \$ 01.00/0

without further purification. Only buffer substances of the highest purity available were used.

Preparation of red cell membranes

Erythrocyte membranes were prepared from fresh red cell concentrates by the method of Dodge *et al.* [4]. After lysis with 15 mM sodium phosphate buffer for about 17 h the red cells were washed with 15 mM and, subsequently, with 10 mM sodium phosphate buffer pH 7.5. On completion of the washing procedure, the suspensions were frozen over night, centrifuged, then resuspended with water, and lyophilized.

Lipid extraction of the red cell membrane

The method used generally followed the prescription given by Dawson *et al.* [5]. 100 mg of red cell membrane were suspended with 2 ml of 0.9% NaCl, and homogenized by means of a Potter-Elvehjem homogenizer. 14 ml methanol and 28 ml chloroform were added, mixed thoroughly, and let stand at room temperature. Thereafter, the suspension was filtered with paper. 9 ml of 0.9% NaCl were subsequently added and mixed vigorously. The mixture was allowed to separate in the refrigerator for about 2 h at 4 °C. The lower phase was filtered or separated by a funnel, the upper phase was discarded. The lower phase was concentrated in a vacuum rotary device to about 3–5 ml or to dryness.



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

Infrared spectroscopic studies

Incubation of membranes with small amounts of inhibitor

10 mg of membrane were suspended with 0.25 ml 1% sodium dodecylsulfate by slowly stirring in a 10 ml beaker for 15 min at 22 °C. There was a ratio of sodium dodecylsulfate/protein of 0.5 mg/1.0 mg. Subsequently, 1.25 ml of 0.9% NaCl was added. The P_H was about 6.75. After homogenization in a 5 ml Potter Elvehjem homogenizer, 15 μ l of stock solution of phloretin in abs. ethanol or of the appropriate dilution was added. Thereafter, the suspension was slowly stirred for an incubation time of 30 min at 22 °C. Then the suspensions were frozen and lyophilized. After lyophilization each sample was dissolved with 2 ml H₂O and dialysed against H₂O with stirring for 90 min at 5 °C. Now 0.12 ml of the sample was taken and added to 200 mg of KBr (for spectroscopy, Merck) with 0.4 ml of H₂O. After drying in a vacuum desiccator spectra were recorded by microdiscs of 5 × 1 mm size with about 30 to 50 μ g substance (10–30 mg KBr) in a Beckman IR-10 spectrometer.

Gel filtration studies of membranes incubated with an excess of inhibitors

The incubation period was carried out as described above, phloretin or phloridzin were used at 2.5 mM final concentrations each.

Immediately after incubation period, the suspension were gelfiltered on Sephadex G-75 columns (450 × 12 mm), which were equilibrated with 0.5 M NaCl, 0.01 M K₂HPO₄, pH 8.0–8.4, in order to remove unbound or slightly bound inhibitor. Afterwards the lipoprotein peaks, recorded at 280 nm, were lyophilized. The lyophilizates were subsequently dissolved with 3 ml H₂O and dialysed for 6 h against H₂O/NH₃ pH 7–8 in order to eliminate the salt content of the samples. Thereafter, aliquots of the samples, containing about 0.5 mg of membrane were taken and added to 200 mg of KBr with 0.4 ml of water. Further procedure for infrared spectroscopy as delineated above in the experiments without gel filtration.

Incubation of the extracted membrane lipids

2.5 mg of the extracted membrane lipids (containing about 27% cholesterol) were incubated for 30 min

at 21 °C in 0.9% NaCl containing 0.01 M sodium phosphate, pH 6.75. The total volume was 0.5 ml in presence of the amounts of phloretin shown in Fig. 2a–c.

Thereafter, dialysis was carried out for 30 min against water of 5 °C. Aliquots of the samples were taken and prepared for infrared spectroscopy as described above.

Laser Raman spectroscopic studies

65 mg of red cell membrane were weighed and homogenized with 1.4 ml 0.9% NaCl for 15 min by means of a Potter-Elvehjem device. Thereafter, 10 μ l of a 100 mM solution of phloretin in ethanol was added and homogenization was continued for 15 min. Controls were treated similarly without the addition of phloretin.

The measurements were carried out at room temperature in a capillary cell. Spectra were measured by a Spex Ramalog 5 spectrometer equipped with a Coherent Radiation CR 2 argon and Cr 500 krypton laser. Depending on the fluorescence background the Raman scattering was excited at 514 nm. For all spectra the optical resolution was chosen to be 4 cm⁻¹. The spectra were detected by means of a conventional photon-counting system. In order to improve the signal-to-noise ratio they were accumulated several times and stored using a CAT 1000 (TMC) Data System (horizontal resolution 1.5 cm⁻¹ per channel). In most of the cases the high frequency noise and the broad fluorescence background of the spectra were suppressed by digital filtering processing via the Fourier transform of the total spectrum. The filtering functions were carefully chosen in order to avoid electronically caused artifacts and to hold the spectral broadening within acceptable limits. This method has been described previously [6].

Electron paramagnetic resonance spectroscopy

Investigations on red cell membrane

A suspension of red cell membrane in 0.9% NaCl, 10 mM sodium phosphate pH 7.0 containing about 0.5 mg protein/ml was centrifuged in an Eppendorf centrifuge. The supernatant was discarded and the precipitated membranes (0.5 mg protein) were resuspended with 100 μ l of 0.9% NaCl, 10 mM sodium phosphate pH 7. Thereafter, 1 μ l phloretin of the appropriate dilution was added and mixed vigor-

ously. Subsequently, 1 μ l of a 5 mM solution of the spin labels 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy (Nos. 618 and 616, Syva, Palo Alto, Cal., USA) were added. After vigorous mixing, spectra were taken in a Bruker B-MN, 155-45 S1 6 spectrometer, at 22 °C.

Investigations on extracted red cell membrane lipids

The extracted lipids were stored in 0.9% NaCl at -80 °C and at a concentration of 5.0 mg/ml NaCl. Amounts of 0.5 mg of lipid were centrifuged in an Eppendorf centrifuge, the supernatant was discarded and the lipid pellets were resuspended with 50 μ l 0.9% NaCl, 10 mM sodium phosphate pH 7.0. Thereafter, 1 μ l of the appropriate dilution of phloretin was added. After intense mixing, 1 μ l of a 5 mM solution of spin labels was added and, after subsequent mixing, spectra were taken in the Bruker spectrometer specified above.

Results

Infrared spectroscopy

A series of infrared spectroscopic results are presented in Figs. 1 and 2a-e. It is clearly revealed that in red cell membrane the intensity of the band at 1250 cm^{-1} is progressively increased with rising concentration of phloretin. On comparison with the region of 1050-1100 cm^{-1} , this effect becomes particularly evident. The band at 1250 cm^{-1} is ascribable to antisymmetric POO^- vibrations [7].

Another point which already becomes evident at the lowest concentration of phloretin (0.1 μ M) is that the band occurring at 1050 cm^{-1} in the control spectrum is shifted toward a broad maximum at about 1100 cm^{-1} . This wave number is ascribed to the POO^- symmetric vibrations [7].

In the extracted red cell membrane lipids, for comparison, there is only an increase in band intensities, but no shift in wave numbers. This increase in band intensities concerns the region of 1050-1100 cm^{-1} , and even occurs at the lowest concentration of phloretin used (Fig. 2a). Contrary to the results obtained in red cell membrane, a distinct shoulder in the control spectrum of the extracted is already found at 1100 cm^{-1} .

In further experiments, after loading the red cell membrane with high (millimolar) concentration of

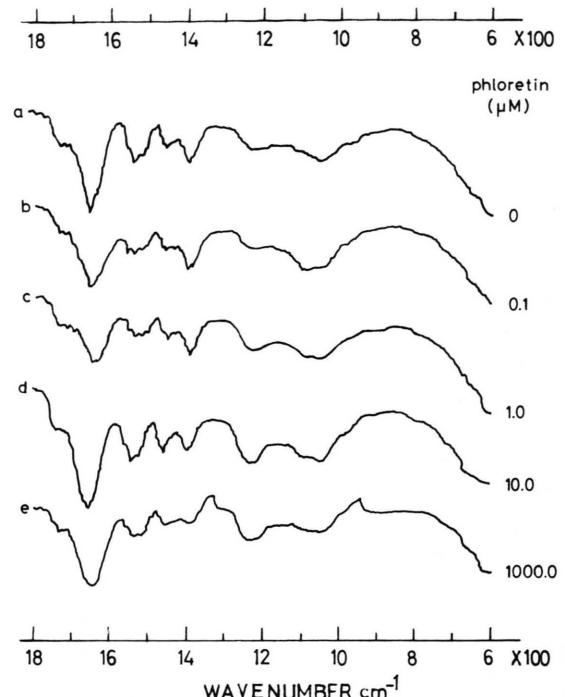


Fig. 1a-e. Infrared spectra of red cell membrane (KBr spectra) in presence of rising concentrations of phloretin.

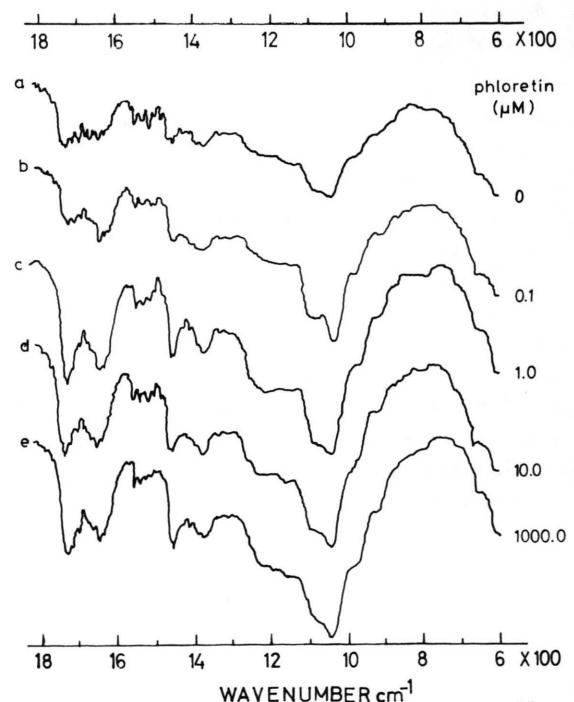


Fig. 2a-e. Infrared spectra of extracted red cell membrane lipids in presence of rising concentrations of phloretin.

phloretin or phloridzin, the unbound or loosely bound inhibitor was removed by gel filtration with Sephadex G-75, or G-100 (Fig. 3a). The lipoprotein peak was collected and, after dialysis, lyophilized. Thereafter, analysis by infrared spectroscopy was carried out. Representative results on comparing a control spectrum with those of membranes pre-treated either with phloretin or phloridzin are shown in Fig. 3b. These spectra reveal that both phloretin and phloridzin incubation shift the band from 1050 cm^{-1} to about 1100 cm^{-1} . Moreover, a distinct shoulder at about 1250 cm^{-1} is observed in the spectrum obtained from membranes in presence of phloretin, which is in accordance with the findings in red cell membrane, shown in Fig. 1a–e. In the preparation obtained in presence of phloridzin, this shoulder is remarkably weaker.

Raman spectroscopy

Somewhat different experimental conditions were used for Raman spectroscopic experiments (see Materials and Methods). No solubilization by sodium dodecylsulfate was carried out, and $700\text{ }\mu\text{M}$ concentration of phloretin was applied, at membrane concentrations of 65 mg/ml .

The results are shown in Fig. 4. Differences are clearly revealed in the region of $1050\text{--}1100\text{ cm}^{-1}$. As mentioned above, this region is ascribed to the POO^- symmetric stretching vibrations which overlap with the C-C-stretching bands. According to Lippert *et al.* [8] a distinct broad band at 1085 cm^{-1} indicates

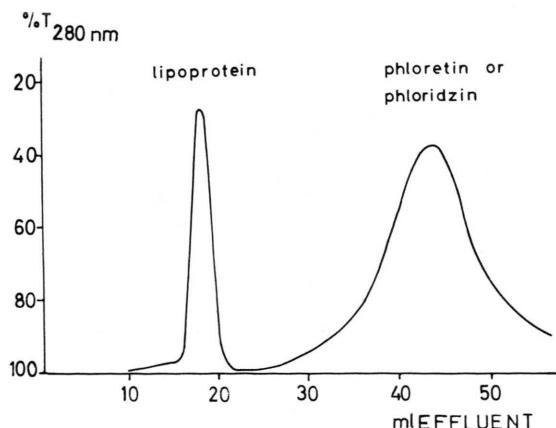


Fig. 3a. Gel filtration on Sephadex G-75 or G-100 of red cell membrane after incubation with 2.5 mM concentrations of phloretin or phloridzin. Separation of lipoprotein and inhibitor peaks.

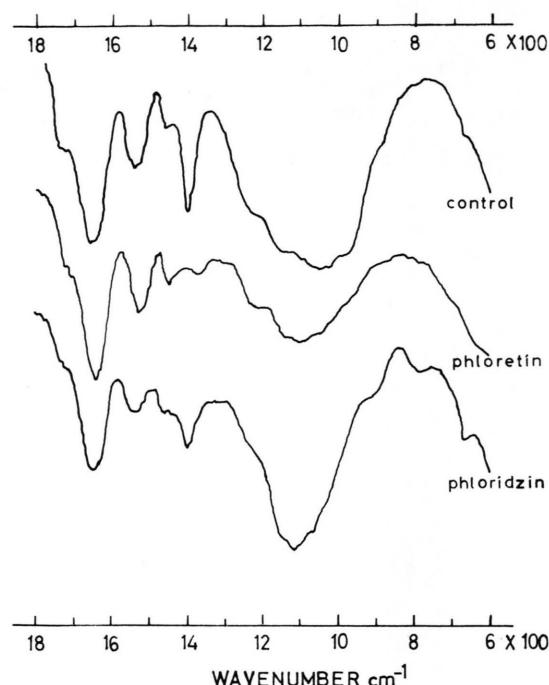


Fig. 3b. Infrared spectra of red cell membrane after gel filtration from top to bottom: control, phloretin loaded membrane, phloridzin loaded membrane.

Table I. ESR spectra of red cell membrane and extracted red cell membrane lipids in the presence of phloretin. Data were obtained with stearic acid spin label No. 618 (Svya). Parameter A/B was determined according to Butterfield *et al.* [10]. Order parameter s was calculated according to Gaffney [9].

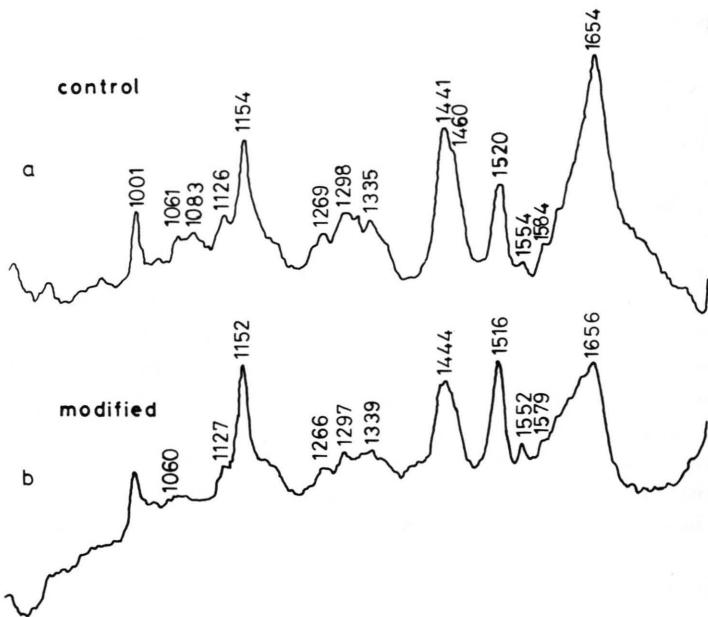
Red cell membrane

| Phloretin [μM] | Ratio A/B | Order parameter s | Number of exp. |
|--------------------------------|-------------------|---------------------|----------------|
| 0 | 1.131 ± 0.01 | 0.703 ± 0.001 | 6 |
| 0.1 | 1.149 ± 0.01 | 0.704 ± 0.001 | 7 |
| 1 | 1.141 ± 0.009 | 0.711 ± 0.003 | 6 |
| 10 | 1.122 ± 0.01 | 0.719 ± 0.003 | 3 |
| 100 | 1.121 ± 0.02 | 0.719 ± 0.009 | 3 |
| 1000 | 1.147 ± 0.007 | 0.735 ± 0.013 | 3 |

Extracted red cell membrane lipids

| Phloretin [μM] | Ratio A/B | Order parameter s | Number of exp. |
|--------------------------------|------------------|---------------------|----------------|
| 0 | 1.10 ± 0.009 | 0.699 ± 0.01 | 5 |
| 0.1 | 1.12 ± 0.02 | 0.692 ± 0.006 | 3 |
| 1.0 | 1.12 ± 0.01 | 0.696 ± 0.004 | 3 |
| 10 | 1.11 ± 0.02 | 0.694 ± 0.006 | 3 |
| 100 | 1.10 ± 0.01 | 0.691 ± 0.005 | 4 |
| 1000 | 1.16 ± 0.01 | 0.708 | 2 |

Fig. 4. Raman spectra of red cell membrane upper trace: control (a), lower trace: modified with phloretin (b), excitation 514 μm , numbers given are cm^{-1} . For production of the Figure, the spectra were reproduced with different preparations five times, and these spectra were again superimposed.



a fluid random orientation of the side chains. Thus, the complete lacking of this band in the samples treated with phloretin may indicate that the POO^- and/or C-C region of the membrane in presence of the inhibitor is less fluid (compare Fig. 4a and b).

We did not find any changes in the range of the C-H stretching vibrations at about 3000 cm^{-1} , and only slight differences between the control and the inhibitor-modified membrane in the region of the CH_2/CH_2 bending vibrations.

ESR spectroscopy

Further investigations were carried out on the membrane lipids, using ESR spectroscopy. Since experimentation with infrared as well as Raman spectroscopy had given indications of changes in the region of the lipid polar head groups, we made use of the spin label 2-(3-Carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinylloxyl (No. 618, Syva), the reporter group of which is located in the neighbourhood of this region. This method yielded results similar to those observed by infrared and Raman spectroscopy (Table I): In the presence of increasing amounts of the inhibitor phloretin there is less mobility in the polar/apolar interface region of the membrane, as indicated by the increase in order parameters [9]. In comparison, the extracted lipids

remain nearly unchanged. To some extent, the parameter A/B [10] gives the same information.

Spin label 616, carrying its nitroxide group at the apolar end of the stearic acid molecule, also shows

Table II. ESR spectra of red cell membrane and extracted red cell membrane lipids in the presence of phloretin. Data were obtained with stearic acid spin label No. 616 (Syva). The order parameter s was calculated as described in Table I.

Red cell membrane

| Phloretin [μM] | Order parameter s | Number of exp. |
|-----------------------------|---------------------|----------------|
| 0 | 0.251 ± 0.002 | 8 |
| 0.1 | 0.251 ± 0.002 | 3 |
| 1 | 0.250 ± 0.002 | 3 |
| 10 | 0.264 ± 0.003 | 8 |
| 100 | 0.256 ± 0.004 | 10 |
| 1000 | 0.252 ± 0.002 | 7 |

Extracted red cell membrane lipids

| Phloretin [μM] | Order parameter s | Number of exp. |
|-----------------------------|---------------------|----------------|
| 0 | 0.135 ± 0.002 | 6 |
| 0.1 | 0.134 ± 0.003 | 6 |
| 1 | 0.133 ± 0.002 | 5 |
| 10 | 0.133 ± 0.003 | 6 |
| 100 | 0.145 ± 0.004 | 5 |
| 1000 | 0.115 ± 0.004 | 4 |

an increase in order parameter at 10 μM concentrations of phloretin in red cell membrane. Again, the extracted membrane lipids do not yield a similar information (Table II). A certain perturbation of the apolar lipid region is, however, sensed by the spin label at 0.1 and 1 mM concentrations of phloretin.

Discussion

Up to now, only little is known about the structural change of cell membranes by binding of competitive transport inhibitors like phloretin and phloridzin.

Using infrared spectroscopy, we have found clear indications for an increase of intensities of symmetric (1100 cm^{-1}) and antisymmetric (1250 cm^{-1}) POO^- vibrations in the presence of phloretin. In previous investigations, it was also shown that L-sorbose, when bound to red cell membrane, increased – while D-glucose decreased – the intensities of antisymmetric POO^- vibrations [11]. These results suggest that the conformation of the membrane at the polar/apolar interface (in the region of the phospholipid phosphate groups) is involved in the competition of phloretin or sorbose with glucose for binding sites.

The increase of order parameter of spin label 618 in red cell membrane by phloretin (Table I), moreover, indicates a decrease in fluidity in the membrane interface region. These two phenomena, a decrease in fluidity of the red cell membrane interface and the change in configuration in the region of the lipid phosphate moieties are thus correlated events.

Furthermore, it was found that in red cell phloretin is about 80-fold more effective in inhibiting glucose transport than is phloridzin [12]. This is of interest, since both the molecules only differ in the glucose molecule attached to the phloretin moiety. The difference in inhibitory activities has not yet been clearly understood. In the present paper, it is shown that the decisive intensity increase of the antisymmetric POO^- vibrations, induced by phloretin, is not observed with phloridzin. On the contrary, the POO^- intensity at 1250 cm^{-1} is decreased in presence of phloridzin. Since the same event (decrease of intensity of the band at 1250 cm^{-1}) was observed with glucose when bound to red cell membrane [11], it is obvious, that the glucose moiety of phloridzin bound to the “glucalogue carrier” [13]

mainly prevents the effect of the phloretin part of the molecule on the region of phospholipid phosphate groups of the membrane. If the effect of phloretin on the membrane interface (lipid phosphate), however, is essential for competition in glucose transport, most of the membrane-bound phloridzin molecules thus become ineffective.

A change in red cell membrane conformation by glucose has been reported previously [14, 15]. From kinetic studies, Krupka [14] obtained evidence for opposite conformations of the red cell membrane induced by glucose and phloridzin, on the hand, and by phloretin, on the other hand. These results agree with our interpretation given above. Our observations are also comparable with the results of Jennings and Solomon [2]. These authors have obtained evidence for specific binding sites of phloretin at the red cell membrane, but not at the extracted membrane lipids. The results of Andersen *et al.* [3] who worked with thin lipid membranes revealing specific effects at the polar head groups by phloretin, however, may resemble those found with the integrated red cell membrane. The extracted membrane lipids behave differently.

It appears that phloretin reveals its clearcut effects in the integrated red cell membrane only. This implies that membrane lipid-protein interactions should be involved in the overall effect observed.

Concerning our results with Raman spectroscopy, we did not find a correlation of the decrease of band intensity at $1060-1100\text{ cm}^{-1}$ with the increases in small, neighboured bands, as observed by Lippert and Petricolas [16]. We attribute this to differences in the two system compared here. While Lippert *et al.* [16] investigated a clearly defined dipalmitoyl lecithin multilayer system, we worked with integrated, heterogeneous red cell membrane. Moreover, the distribution of membrane lipids in this membrane is heterogeneous [17].

In agreement with Goheen *et al.* [18] we also were unable to find concomitant intensity changes in the $1060-1100$ and the 2900 cm^{-1} regions, whereas in lipid model systems such effects were found [19]. Significant intensity changes in Raman spectra at around 2900 cm^{-1} were also seen as a temperature-dependent function of phospholipids [20]. These changes, however, may become smoothed down by contribution of protein [20]. This is an important point, since lipid-protein interaction in the red cell membrane may work in a similar way.

From the control Raman spectra we suggest that even in the absence of the inhibitory phloretin the red cell membrane is in a relatively rigid structure, similar to what was reported by Lippert *et al.* [8] (see also the values of 0.7 for order parameters in ESR spectroscopy, Table I). In presence of the inhibitor, however, the band at 1085 cm^{-1} disappears. This may be taken as an indication of a further

increase in rigidity (accordingly, the order parameter increases up to 0.735).

Acknowledgements

We thank Professor Ladislaus Lacko for his critical comments on the manuscript and discussion and Dr. Bernd Lammel for the use of his EPR spectrometer.

- [1] P. A. G. Fortes, in: *Membrane transport in red cells* (J. C. Ellory and V. L. Lew, eds.), pp. 175–195, Academic Press, New York 1977.
- [2] M. L. Jennings and A. K. Solomon, *J. Gen. Physiol.* **67**, 381–397 (1976).
- [3] O. S. Andersen, A. Finkelstein, I. Katz, and A. Cass, *J. Gen. Physiol.* **67**, 749–771 (1976).
- [4] J. T. Dodge, C. Mitchell, and K. J. Hanahan, *Arch. Biochim. Biophys. Acta* **382**, 51–57, (1975).
- [5] R. M. C. Dawson, N. Hemington, and D. B. Lindsay, *Biochem. J.* **77**, 226–230 (1960).
- [6] H. Schmidt and L. Bieker, *Arch. Biochim. Biophys.* **195**, 205–210 (1979).
- [7] H. Akutsu and Y. Kyogoku, *Chem. Phys. Lipids.* **14**, 113–122 (1975).
- [8] J. L. Lippert, L. E. Gorczyca, and G. Meiklejohn, *Biochim. Biophys. Acta* **382**, 51–57, (1975).
- [9] B. J. Gaffney, in: *Spin labeling. Theory and applications*, (L. J. Berliner, ed.), pp. 567–571, Academic Press, New York 1976.
- [10] D. A. Butterfield, D. B. Chesnut, A. D. Roses, and S. H. Appel, *Proc. Nat. Acad. Sci.* **71**, 909–913 (1974).
- [11] G. Zimmer, L. Lacko, and H. Günther, *J. Membrane Biol.* **9**, 305–318 (1972).
- [12] P. G. LeFevre, *Pharmacol. Rev.* **13**, 39–70 (1961).
- [13] V. E. Colombo and G. Semenza, *Biochim. Biophys. Acta* **288**, 145–152 (1972).
- [14] R. M. Krupka, *Biochemistry* **10**, 1143–1148 (1971).
- [15] G. Zimmer and L. Lacko, *FEBS Letters* **12**, 333–337 (1971).
- [16] J. L. Lippert and W. L. Peticolas, *Proc. Nat. Acad. Sci.* **68**, 1572–1576 (1971).
- [17] P. R. Cullis, *FEBS Letters* **68**, 173–176 (1976).
- [18] S. C. Goheen, T. H. Gilman, J. W. Kauffman, and J. E. Garvin, *Biochim. Biophys. Res. Commun.* **79**, 805–814 (1977).
- [19] K. G. Brown, W. L. Peticolas, and E. Brown, *Biochim. Biophys. Res. Commun.* **54**, 358–364 (1973).
- [20] W. Curatolo, S. P. Verma, J. D. Sakura, D. M. Small, G. G. Shipley, and D. F. H. Wallach, *Biochemistry* **17**, 1802–1807 (1978).