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A Relationship Between the Rate of Osmotic Hemolysis and Membrane Structure*

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Abstract. The rate of osmotic hemolysis was measured. A semilogarithmic plot of the decrease in intact cell concentration that took place during hypotonic shock was used to define an apparent rate of hemolysis, K. A relationship between the rate of hemolysis and the membrane fluidity was examined. The temperature dependence of K showed that K values were almost constant below 30°C and increased rapidly when the temperature was above 30°C. This was quite different from the temperature dependence of the membrane fluidity. The result of pH depedence of K also showed that there was no close correlation between the rate of hemolysis and the membrane fluidity. It was suggested that the interaction of hemoglobin with the erythrocyte membrane was involved in controlling the course of hemolysis.

Introduction

A considerable progress has been made in the area of biomembranes since the fluid mosaic model of biomembrane structure was proposed by Singer and Nicolson (1972). A variety of physical techniques have been applied to the study of the dynamics of biomembranes. The study of the erythrocyte membranes has been benefited from these new techniques. Of biomembranes, that of the human red blood cell is the most easily prepared free from contamination by other cellular structures. The organization of the erythrocyte membranes has been extensively studied and the molecular components were exceptionally well characterized (Branton et al., 1981).

We have studied lateral mobility of erythorcyte membrane proteins by the fluorescence photobleaching recovery technique (Chang et al., 1981) and rotational mobility of integral protein band 3 by the saturation transfer spectroscopy (Sakaki et al., 1982), and demonstrated that there was interaction between integral protein and cytoskeleton. This paper describes the early dynamics of osmotic hemolysis of the erythrocyte. It is different from the classical osmotic fragility which reflects the surface-to-volume ratio, intracellular osmotic pressure, and membrane permeability of red cell population (Valeri, 1973). The rate of osmotic hemolysis is a rather complicate parameter. Nevertheless, it is directly related to the process of the rupturing of membranes. Some researchers have suggested that the rate of osmotic hemolysis was closely associated with the fluidity of the lipid bilayer (Araki

* A part of the experiments in the present paper was done in the Department of Biophysics, Faculty of Science, Kyoto University.

and Rifkind, 1981). We reinvestigated this problem, and found that there were some large differences between the rate of hemolysis and the membrane fluidity according to their temperature and pH dependencies. It was suggested that the interaction of hemoglobin with the erythrocyte membrane could be involved in controlling the coures of hemolysis.

Abbreviations used in the present paper are as follows: DIDS, 4,4'-bis (isothiocyano)-2,2'-stilbenedisulfonate; DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenol; DPH, 1,6-diphenyl-1,3,5-hexatrien; ESR, electron spin resonance; PBS, phosphate-buffered saline; PC*, spin-labeled phosphatidylcholine.

Materials and Methods

Materials. Human blood samples were obtained from the Shanghai Red Cross Blood Center and used within 2 weeks. Triton X-100 was produced by the Shanghai Chemical Reagent's Factory. DNP was purchased from the Shanghai Chemical Reagent's Station. DNFB was the product of the Shanghai Xing Ta Chemical Plant. PC* was synthesized in Ohnishi Laboratory (Kyoto University, Japan). All other reagents were of analytical grade.

PBS was prepared from 155 mM NaCl/5 mM sodium phosphate, pH 7.5. The cells were washed three times, then resuspended in the same buffer. The cell concentrations were adjusted to approx. 5×10^8 cells/ml.

Rate of osmotic hemolysis. The measurement of the time course of percent hemolysis, $(1\text{-}C_t/C_o)\%$, after hypotonic shock is based on the change of intact cell concentration, where C_t is the cell concentration at time t; C_o is the initial cell concentration. In order to measure continuously during hemolysis the parameter of cell concentration instead of the release of hemoglobin was used. Pre-experiment showed that the contribution of hemoglobin to O.D. of cell suspension was very small (1.5%) at 660 nm (Zhang, 1984). According to the present calibration curve, it is easy to convert the absorbance of the suspension into cell concentration (Zhang, 1984).

40 μl of an isotonic erythrocyte suspension was rapidly mixed with 2 ml of hypotonic solution by an adjustable quantitative injector. In order to change the temperature of the suspension, the injector was placed in a thermostatic bath. A spectrometer (type 72, Shanghai Optical Instrument Factory) was modified to set up a magnetic stirrer at the bottom of the photometric cell holder and the circular water arround the cell was used. The O.D. electric signals of the suspension at 660 nm were through an A/D converter (Mountain Computer Inc.) fed to an Apple II plus microcomputer (RAM 48K) at 0.25 s intervals. The design for the measurement program was described previously (Zhang and Zhu 1985). The major functions of the program can be summarized as follows. (1) By the step signal during the erythrocyte mixing with hypotonic solution, the start time (t=0) is automatically set. (2) Zero set is automatically done. (3) All data points (50–60) are stored in the microcomputer and polynomial curve fittings are performed according to the least-squares method,

$$\log(C_t/C_o) = a_o+a_1t+a_2t^2+a_3t^3+a_4t^4$$

where $a_o, a_1 \cdots a_4$ are the coefficients of the polynome. (4) A printer (Epson MP–80) can print out both the mean rate of hemolysis during the period of 2–3 s and the plot of log (C_t/C_o) vs. time.

ESR spectra. The ESR spectra of spin labels were measured in Ohnishi Laboratory, Department of Biophysics, Kyoto University, when the author was engaged in research work there. The spin label (PC*) was phosphatidylcholine with 12-nitroxide stearic acid at the 2-position. Incorporation of PC* into the erythrocyte membranes was carried out according to the procedure of Tanaka and Ohnishi (1976). Briefly, first, the PC* vesicles were prepared by ultrasound. After incubation with PC* vesicles, the erythrocyte membranes were labeled with PC*. The ESR signals were recorded with a JEOL Model FE-2X spectrometer equipped with a variable temperature accessory.

pH dependence. The effect of pH on the rate of hemolysis was examined in the following buffers: NaAC/HAC (10 mM) for pH 3.8, 4.5 and 5.0; Na_2HPO_4/NaH_2PO_4 (5 mM) for pH 5.5, 6.0, 6.5, 7.0 and 7.5; Tris/HCl (10 mM) for pH 8.0, 8.5 and 9.0. All the buffers contained 155 mM NaCl. In each buffer, the concentration of the erythrocytes was approx. 5×10^8 cells/ml, and after incubation for 30 min at room temperature the rate of osmotic hemolysis was measured.

Osmotic fragility. The osmotic fragility was measured by a modification of the method of Maeda et al. (1977). This is a continuous measurement method instead of the classical multiple-tube method. This mehtod is not as time-consuming as old one, and is more accurate. We used the same equipment as that for mesauring the rate of hemolysis to process data and to print out the curve of osmotic fragility.

Results and Discussion

Fig. 1 shows a typical tracing of osmotic hemolysis of normal human erythrocytes in the time domain. Jay and Rowlands (1975) observed the process of hemolysis of individual erythrocyte by means of cinematography. They suggested that the classical hemolytic time was composed of three components: the swelling time, the potassium leakage time, and the hemoglobin leakage time. The theoretical analysis of hemolysis rate was further discussed (Anderson and Lovrien 1977). It was suggested that the overall course of hemolysis could be described in terms of two rate processes: the fast process with the rate constant K_1, and the slow process with the rate constant K_2. As shown in Fig. 1, a nearly linear relationship holds between $\log(C_t/C_o)$ and time for the region of 2 to 4 s. The slope of this region is defined as the apparent rate constant for hemolysis (Araki and Rifkind 1981). It is cor-

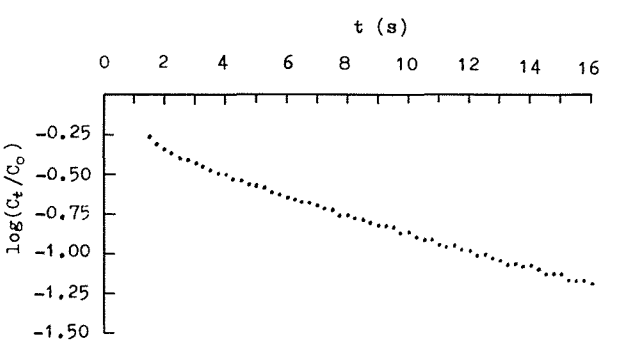

Fig. 1. Typical time course of osmotic hemolysis at 17°C in 31 mM NaCl/5 mM sodium phosphate, pH 7.5. The ordinate plots the change of cell concentration. The apparent rate constant of hemolysis between 2 and 3 s, K, was 0.090 s^{-1}.

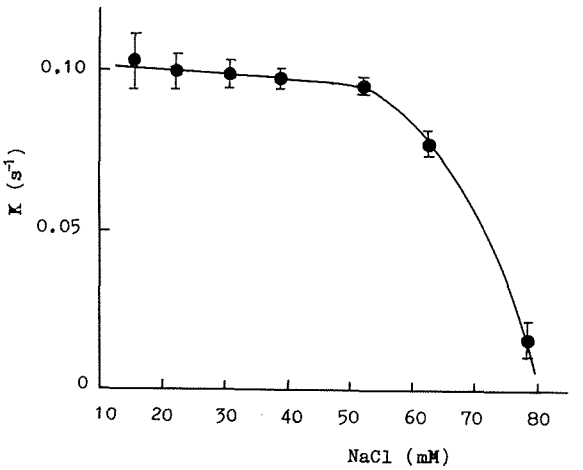

Fig. 2. The rate of hemolysis as a function of NaCl concentration at 17°C. All the solutions were buffered with 5 mM sodium phosphate, pH 7.5.

responding to K_1. The following curved part represents the presence of the K_2 process. In our experimental conditions, the goodness of fit for polynomical curve fitting was always above 99.9%. Using the isotonic solution the dead time of mixing cell with solution was obtained to be about 0.5–1 s, and the fluctuation of cell concentration was within ±2% until 30 s after mixing.

It is seen in Fig. 2, a plot of K vs. NaCl concentration, that there is a dramatic change of K value with the salt concentration when the salt concentration is above 50 mM, when the salt concentration is below 20 mM, the high rate of hemolysis causes a large measurement error. So we chose 31 mM NaCl/5 mM sodium phosphate (pH 7.5) as the standard salt concentration for hypotonic shock. The mean K value of 16 normal blood samples was $(0.096 \pm 0.017)\ s^{-1}$.

Table 1 shows the effect of membrane modifications on the K parameter. The relative rate in the Table is the rate relative to the control incubated with PBS. Triton X-100 is a non-ionic detergent which can be used to solubilize the integral protein and intercalate between lipid molecules due to the hydrophobic interaction (Helenius and Simons 1975). At a low concentration (0.02%) in which no cell lysis was observed, the rate of hemolysis

Table 1. Effect of membrane modification on the rate of hemolysis.

Reagent	Concentration	Relative rate[1]
Triton X-100[2]	0.02 (v/v%)	1.79
Procaine[3]	10 (mM)	1.08
Glutaraldehyde[2]	0.013 (v/v%)	0.79
DNP[4]	0.4 (mM)	0.84
DNFB[5]	5 (mM)	0.89

1) Relative rate is expressed as the ratio of K value of erythrocytes treated with reagent to that of control.
2) Erythrocytes were treated for 15 min at room temperature.
3) Erythrocytes were treated for 1 hr at 30°C.
4) Measurement was started after 1 min treatment at room temperature.
5) Erythrocytes were treated for 0.5 hr at 30°C.

was increased (Table 1). Glutaraldehyde has the effect of crosslinking between proteins, some sulfhydryl groups and amino groups of lipids (Steck, 1972). At 0.013% concentration it induced a decrease of K value. According to Araki and Rifkind (1981), Triton X-100 induced an increase of membrane fluidity, and glutaraldehyde decreased fluidity. Based on the ESR signal of spin label, with the addition of other chemical modifications they suggested a correlation between the changes in the rate of hemolysis and the changes in the fluidity of the lipid matrix; that is, the greater the fluidity the larger the K value. Thus, the rupturing of the cell membrane could be closely associated with the fluidity of the lipid bilayer.

To examine this relation, the temperature dependencies of K value and the membrane fluidity were investigated. We used the overall splitting value ($2T_{//}$) of the ESR spectrum as a parameter of the membrane fluidity. The ESR spectra of intact erythrocytes labeled with PC* were shown in Fig. 3. Fig. 4 illustrates the temperature dependence of the rate of hemolysis to compare with the ESR spectrum parameter of the membrane fluidity. The following properties can be obtained from the ESR spectra: (1) increasing the temperature from 5 to 42°C leads to a decrease in $2T_{//}$, that is, the mobility of the lipid molecule becomes faster when the temperature is increased; (2) there are two break points in the fluidity plot at 13°C and 26°C, respectively. The precise cause of break points is unclear, maybe due to protein-lipid binding (Bieri and Wallach 1976). In contrast to fluidity, the K values

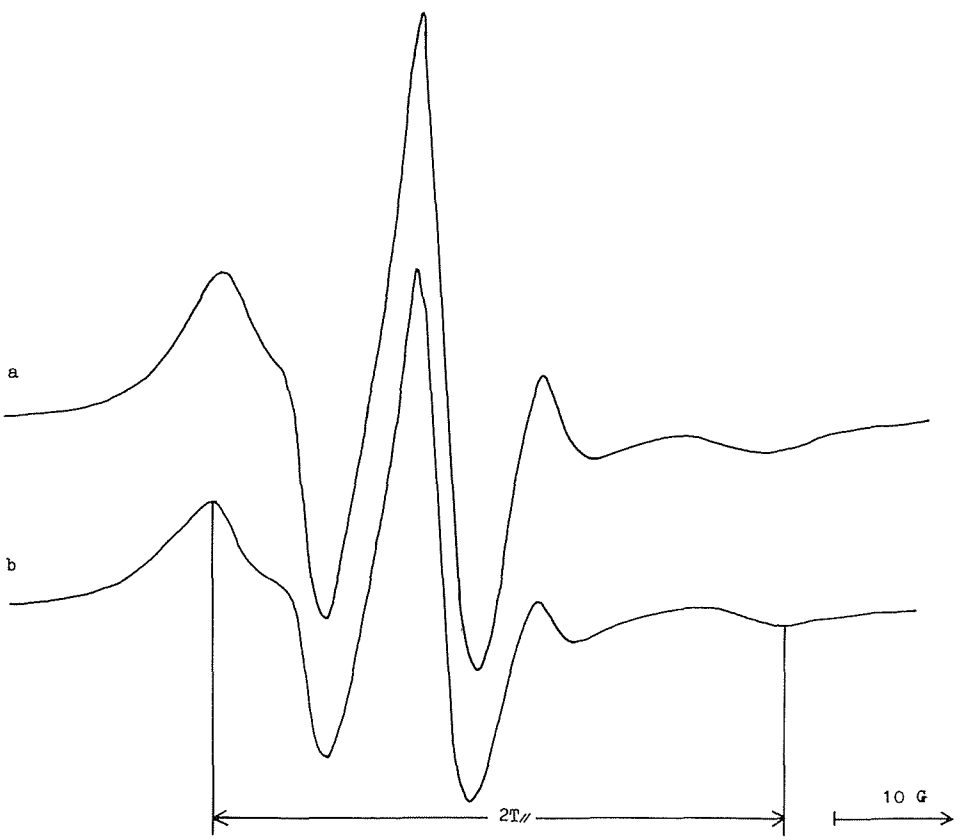

Fig. 3. ESR spectra of intact erythrocytes spin-labeled with PC*. The spectrum a and b were measured at 37°C and 29°C, respectively. $2T_{//}$ is a spectrum parameter called overall splitting value.

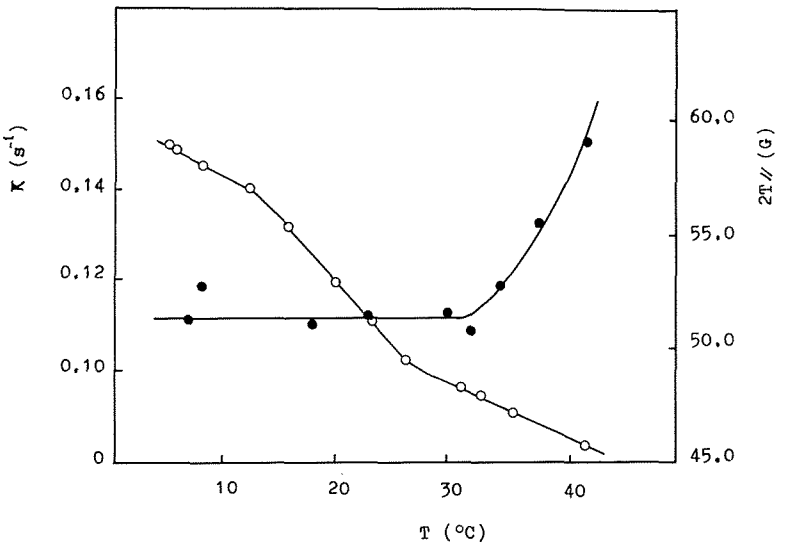

Fig. 4. Comparison of the temperature dependence of the rate of hemolysis (●) and the membrane fluidity (○). The overall splitting value, $2T_{//}$, of the ESR spectrum was chosen as a parameter of the membrane fluidity. For ESR spectra, the erythrocytes were spin-labeled with PC*.

were almost constant in the temperature region of 7°C to 30°C, and increased rapidly when the temperature was above 30°C. On the scale of semilogarithmic plot, a plot of the log K against 1/T gave a straight line, and the Arrhenius energy of activation was 5.3 kcal·mol^{-1}. The experimental results presented above show that the kinetics of hypotonic hemolysis has some connection with the membrane fluidity but there is rather a difference in their temperature dependencies. For the ESR spectra, the membrane fluidity is estimated at the molecular level. But, it can be thought that the whole hemolysis process is not only controlled by the bilayer fluidity but also by the other structural factors of the membrane.

As noted in the introduction, the rate of osmotic hemolysis and the osmotic fragility are two different parameters. Many lines of evidence were presented (Araki and Rifkind 1981). From the temperature dependence we also found their differences. The osmotic fragility parameters H_{50}, a salt concentration (%) at 50% hemolysis, were 0.4056 ± 0.002 and 0.3903 ± 0.0013 at 25°C and 40°C, respectively. To ocmpare with the temperature character of K value, increasing the temperature increases the rate of osmotic hemolysis but decreases the osmotic fragility. In study on early hemolytic course of burned rabbits the difference between above two parameters was also found. In this case at a certain time phase the K value was decreased but H_{50} was increased (unpublished data).

DNP is an anionic amphipatic drug. It can cause the biconcave erythrocytes to become crenated. An interpretation for the shape change has been proposed by Sheetz and Singer (1974). They proposed that DNP molecules intercalated mainly into the lipid in the exterior half of the bilayer, expand that layer relative to the cytoplasmic half, and thereby induced the cell to crenate. In our experiment DNP decreased the rate of hemolysis (see Table I).

Heubush et al. (1985) recently investigated the erythrocyte volume response to the salt concentration. They found that the osmotic volume response of the erythrocytes was nonlinear, and proposed that the spectrin-actin network provided the force against the osmo-

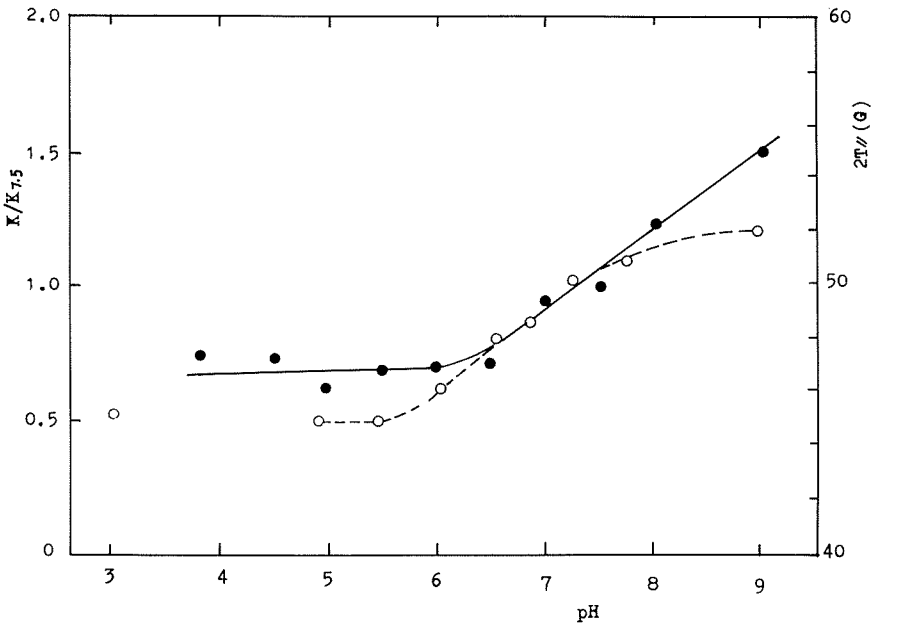

Fig. 5. pH dependence of the rate of hemolysis (●). The erythrocytes (5×10^8 cells/ml) were incubated in each buffer for 0.5 hr at room temperature. K and $K_{7.5}$ are the rates of hemolysis in experimental pH and in pH 7.5, respectively. The data of the over-all splitting value (○) are taken from Yamaguchi et al. (1982). The spin label was 12-nitroxide stearic acid. Temperature was 22°C.

tically induced volume change. Therefore, the mechanical restriction provided by the membrane cytoskeleton must be considered.

Fig. 5 shows the pH dependence of the rate of hemolysis at room temperature. The K values are about 70% of $K_{7.5}$ in the pH range of 3.8 to 6.0 and increase linearly in the pH about 6.5 to 9.0. Using fatty acid spin labels, Yamaguchi et al. (1982) measured the membrane fluidity of human erythrocytes in the pH range of 3.0 to 9.0. The dotted curve in Fig. 5 is one of their results. In the alkaline range $2T_{//}$ increased with the pH, that is, the membrane fluidity is decreased. But, the membranes remained in the high mobility state while the pH is in the acidic range as shown in Fig. 5. If we accept the proposition presented by Araki and Rifkind (1981), the K values in the acidic pH will increase because of the higher mobility. Together with the temperature dependence of the rate of hemolysis, the correlation between the rate of hemolysis and the membrane fluidity must be re-evaluated.

Jarolín and Mirčevová (1982) measured the degree of fluorescence polarization, P, both for fresh human erythrocytes and ATP-depleted erythrocytes stored for 3–4 weeks. The P values were 0.235 and 0.210, respectively. However, after rejuvenation of stored erythrocytes with glucose and inosin, in DPH-labeled membranes P rose again (0.232) almost to the original value as compared with the fresh membranes. These changes were probably due to variations of ATP level in the erythrocytes. The spin label ESR study also gave such conclusion that ATP-depletion increased the fluidity of intact erythrocyte membrane. It was suggested that the fluidity change was caused by protein-lipid interactions which depended on the metabolic state of the cells, particularly ATP levels (Kamada et al., 1983). When the cells were incubated in non-nutrient medium at 37°C, ATP level was depleted to

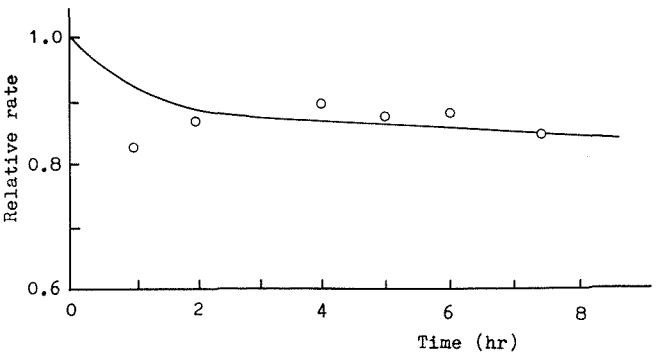

Fig. 6. Changes of the rate of hemolysis during incubation of erythrocytes in PBS at 37°C. The relative rate is the rate relative to the control before incubation. The cell concentration was approx. 5×10^8 cell/ml. K values were measured at 15°C.

a half of the original contents after 8 hr, and almost completely depleted after 24 hr (Palek and Liu, 1979). To investigate the effect of ATP level on the rate of hemolysis, we incubated the erythrocytes in PBS solution for various times at 37°C, and measured K values. On the basis of the correlation between the K value and the membrane fluidity, the rate of hemolysis would be increased while ATP level decreased. It was found, however, that in the storage of 1 to 8 hr the K values were almost constant, about 0.85 of the original value (Fig. 6). On the other hand, the erythrocytes stored for 33 days and 44 days were also measured. The K values were both 0.87 of the original one, similar to the result of incubation at 37°C. These results indicate that ATP level, thereby the membrane fluidity, has some effect on the K value, but there is no close correlation between them.

By the light-scattering measurement (Salhany et al., 1980), the monomolecular lipid layers measurement (Szundi et al., 1980), and the saturation transfer ESR spectroscopy (Cassoly, 1982), several laboratories provided evidence for the hypothesis that hemoglobins in erythrocytes were interacted with the membrane. Two classes of binding sites on the erythrocyte membrane were identified. Presumably band 3 is the one site with high-affinity and each erythrocyte binds 1×10^6 hemoglobin tetramers. The second class of binding sites (4×10^6 sites/cell) may be glycophorin and some kinds of polar head groups of phospholipid with a very much low affinity.

We have investigated the effect of trichloroethylene on the permeability of membrane (to be published). At high concentrations, trichloroethylene can break up liposome and release the contents inside liposome. Over a range of trichloroethylene: phospholipid molar ratios 5:1 to 25:1, the drug caused obvious release of hemoglobins which had been encapsulated in phospholipid liposomes. Hemoglobin release reached a maximum at molar ratio 15:1. Even if trichloroethylene concentration in suspension was more increased no more hemoglobin could be released. The maximum release fraction was about 80%. This result also suggested that there was interaction between hemoglobin and membrane. The binding between the hemoglobin and membrane is closely related to pH and ionic strength of the medium. The affinity of hemoglobin to membrane at pH 6.8 is far lower then at pH 6.0 (Shaklai et al., 1977). The isoelectric point of hemoglobin is 6.8. Thus, as pH is lower than 6.8, the amounts of positive charge of hemoglobin are increased, and therefore the interaction between hemoglobin and membarne is increased. The result of pH dependence of K value (Fig. 5) could be explained partly as the following: the interaction between hemoglobin and membrane is rather strong at low pH, thus the rate of hemoly-

sis is decreased; when pH is above 6.8, the rate of hemolysis is increased along with the decrease of the binding effect. In the light of these results it is reasonable that hemoglobins are not all free inside the erythrocyte, and some of them may be involved in controlling the course of hemolysis and probably some other membrane-related events.

DIDS is an inhibitor of the anion transport of the erythrocyte. After binding of DIDS to externally exposed portions of band 3, the conformational change of band 3 can affect the affinity of protein binding sites on the cytoplasmic side of membrane (Salhany et al., 1980). We used a similar inhibitor DNFB (Zaki et al., 1975) to observe the effect of band 3 on the rate of hemolysis and found that K value was decreased as DNFB was added (Table 1). This result may come from the interaction between hemoglobin and band 3 or the change in cytoskeleton structure through band 3. The molecular basis remains to be established with certainty.

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References

Anderson, P.C. and R.E. Lovrien (1977) Human red cell hemolysis rate in the subsecond to seconds range. An analysis. *Biophys. J.* 20: 181–191.

Araki, K. and J.M. Rifkind (1981) The rate of osmotic hemolysis. A relationship with membrane bilayer fluidity. *Biochim. Biophys. Acta* 645: 81–90.

Bieri, V.G. and D.F.H. Wallach (1976) Lipid-protein relationship in erythrocyte membranes revealed by paramagnetic quenching of protein fluorescence. *Biochim. Biophys. Acta* 443: 198–205.

Branton, D., C.M. Cohen and J. Tyler (1981) Interaction of cytoskeletal proteins on the human erythrocyte membrane. *Cell* 24: 24–32.

Cassoly, R. (1982) Interaction of hemolgobin with the red blood cell membrane. *Biochim. Biophys. Acta* 689: 203–209.

Chang, C.-h., H. Takeuchi, T. Ito, K. Machida and S. Ohnishi (1981) Lateral mobility of erythrocyte membrane proteins studied by the fluorescence photobleaching recovery technique. *J. Biochem.* 90: 997–1004.

Helenius, A. and K. Simons (1975) Solubilization of membranes by detergents. *Biochim. Biophys. Acta* 415: 29–79.

Heubusch, P., C.Y. Jung and F.A. Green (1985) The osmotic response of human erythrocytes and the membrane cytoskeleton. *J. Cell Physiol.* 122: 266–272.

Jarolín, P. and L. Mirčevová (1982) Changes in fluidity of erythrocyte membranes after storage of erythrocytes and regeneration of cellular ATP level. *Biochim. Biophys. Acta* 688: 460–464.

Jay, A.W.L. and S. Rowlands (1975) The stages of osmotic haemolysis. *J. Physiol.* 252: 817–832.

Kamada, T., S. Setoyama, Y. Chuman and S. Otsuji (1983) Metabolic dependence of the fluidity of intact erythrocyte membrane. *Biochem. Biophys. Res. Commun.* 116: 547–554.

Maeda, N., K. Aono, M. Sekiya, T. Suda and T. Shiga (1977) A computerized method for the determination of the osmotic fragility curve of erythrocytes. *Anal. Biochem.* 83: 149–161.

Sakaki, T., A. Tsuji, C.-h. Chang and S. Ohnishi (1982) Rotational mobility of an erythrocyte membrane integral protein band 3 in dimyristoylphosphatidylcohline reconstituted vesicles and effect of binding of cytoskeletal peripheral proteins. *Biochemistry* 21: 2366–2372.

Salhany, J.M., K.A. Cordes and E.D. Gainse (1980) Light-scattering measurements of hemoglobin binding to the erythrocyte membrane. Evendence for transmembrane effects related to a disulfonic stilbene binding to band 3. *Biochemistry* 19: 1447–1454.

Shaklai, N., J. Yguerabide and H.M. Raneny (1977) Classification and localization of hemoglobin binding sites on the red blood cell membrane. *Biochemistry* 16: 5593–5597.

Sheetz, M.P. and S.J. Singer (1974) Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte inetractions. *Proc. Natl. Acad. Sci. USA* 71: 4457–4461.

Singer, S.J. and G.L. Nicolson (1972) The fluid mosaic model of the structure of cell membranes. *Science* 175: 720–731.

Szundi, I., J.G. Szelenyi, J.H. Breuer and A. Berczi (1980) Interactions of haemoglobin with erythrocyte membrane phospholipids in monomolecular lipid layers. *Biochim. Biophys. Acta* 595: 41–46.

Tanaka, K.-I. and S.-I. Ohnishi (1976) Heterogeneity in the fluidity of intact erythrocyte membrane and its homogenization upon hemolysis. *Biochim. Biophys. Acta* 426: 218–231.

Valeri, C.R. (1973) Hemolysis and suspension stability of the blood. In *Physiological basis of medical practice*. Edited by J.R. Brobeck, pp. 4–57–4–61. The williams and Wilkins Company, Baltimor.

Yamaguchi, T., M. Koga, Y. Fujita and E. Kimoto (1982) Effect of pH on membrane fluidity of human erythrocytes. *J. Biochem.* 91: 1299–1304.

Zaki, L., H. Fasold, B. Schuhmann and H. Passow (1975) Chemical modification of membrane proteins in rleation to inhibition of anion exchange in human red blood cells. *J. Cell Physiol.* 86: 471–494.

Zhang Z.-h. (1984) Studies on the rate of osmotic hemolysis. *Fudan J.* (*Natural Sci.*) 23: 1–5 (In Chinese).

Zhang Z.-h. and Y.-y. Zhu (1985) Effects of pH on the hemolysis rate of human erythrocytes. *Fudan J.* (*Natural Sci.*) 24: 401–405 (In Chinese).

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