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THE RATE OF OSMOTIC HEMOLYSIS

A RELATIONSHIP WITH MEMBRANE BILAYER FLUIDITY

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A first-order semilogarithmic plot of the decrease in turbidity that takes place during hemolysis is used to define an apparent rate of hemolysis. The effect on this rate of hemolysis of various membrane modifications is studied. Triton X-100, ethanol and chlorpromazine, which dissolve into the membrane, all increase the rate of hemolysis, even though the same concentration of ethanol and chlorpromazine has been shown to decrease the osmotic fragility. Glutaraldehyde, azodicarboxylic acid-bisdimethylamide (diamide) and intracellular Ca^{2+} are used to produce cross-links on membrane proteins. All of these reagents decrease cell deformability but have different effects on the rate of hemolysis, with Ca^{2+} increasing, glutaraldehyde decreasing and diamide producing almost no effect on the rate. These modifications are also found to alter the ESR specra of the stearic acid spin-label, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl, which probes mobility in the hydrophobic core of the lipid bilayer. A correlation between the effect of membrane modification on bilayer fluidity and the rate of hemolysis suggests that the rate-limiting process which determines the rate of hemolysis involves rupturing of the bilayer.

Introduction

The erythrocyte must pass through many small capillaries in the course of its 120–130 day life span [1]. The viability of the erythrocyte therefore depends on its ability to undergo drastic deformations without rupturing of the cell membrane. These properties of the erythrocyte are determined by the excess surface area to volume ratio and by the elastic properties of the cell membrane which enable it to undergo these deformations without lysis [2].

The osmotic fragility, where hemolysis is studied as a function of salt concentration, has been used extensively as a measure of the viability of the erythrocyte [3]. There have been relatively few studies investigating the rate of osmotic hemolysis [4–6], and it has generally been assumed that the rate provides information similar to that of the fragility curve.

In this paper we take a closer look at the rate of osmotic hemolysis and the factors which determine this rate constant. The effect on the rate of hemolysis of various membrane modifications is investigated. By comparing the observed effect on the rate with previously reported effects on the osmotic fragility, it is shown that the rate actually measures a different process than the osmotic fragility curve. The fragility curve is primarily a function of the initial surface area to volume ratio [7]. On the other hand, the rate is shown to be determined by the rupturing of the erythrocyte membrane, which occurs after the cells are already spherical. The effects of different mem-

brane modifications on the rate of lysis together with spin label results are used to further elucidate the membrane rupture process.

Experimental

Materials

Chlorpromazine hydrochloride, azodicarboxylic acid-bisdimethylamide (diamide), glutaraldehyde, and fatty acid-free bovine serum albumin were obtained from Sigma (St. Louis, MO). Ionophore A 23187 was a generous gift from Dr. R.L. Hamill, Eli Lilly and Company (Indianapolis, IN). Spin-label 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (I(1, 14)) was purchased from Syva (Palo Alto, Ca).

Blood samples were obtained from healthy adult males whose hematological indices were confirmed to be normal. After removing plasma from freshly drawn heparinized blood, erythrocytes were further washed two or three times by isotonic barbital buffer (145 mM NaCl/2.4 mM sodium barbital, pH 7.4).

Membrane modification was performed on washed erythrocytes at a 2% hematocrit (approx. $3 \cdot 10^8$ cells/ml). Unless otherwise specified, the isotonic barbital buffer (145 mM NaCl/2.4 mM sodium barbital, pH 7.4) was used as an incubation medium. Detailed conditions for each modification are described in the results section.

Rate of osmotic hemolysis

The time course of hemolysis was measured in hypotonic solution (47.6 mM NaCl/2.4 mM sodium barbital, pH 7.4) at 30°C. Unless otherwise specified, 50 μl of an isotonic erythrocyte suspension (2% hematocrit or approx. $3 \cdot 10^8$ cells/ml) was mixed with 2 ml of hypotonic solution by the rapid mixing device of the Aminco anaerobic cell accessory, and the change in turbidity at 690 nm was recorded on a Cary 14 spectrophotometer and simultaneously stored in a digital memory unit at 0.3 s intervals for a period of 5 min. 690 nm was used because hemoglobin does not absorb in this wavelength region. We ruled out scattering artifacts by showing that the same rate constant can be obtained with a cell suspension diluted 4-fold.

The zero time point was determined from the absorbance at 690 nm of the same erythrocyte suspension mixed with 2 ml of isotonic solution. In the hypotonic solution used for these experiments more than 95% of the total decrease in absorbance found for complete hemolysis in water is reached within 5 min, after which time no additional change in absorbance is detected.

The use of the decrease in turbidity to follow hemolysis is complicated by the significant though smaller decrease in turbidity due to swelling of the cell [4]. Because of the ease with which it is possible to obtain extremely accurate results for the change in turbidity, this method has been used extensively to follow both the osmotic fragility as well as the time course of hemolysis [4,8–11]. In our experiments (see below), it was necessary to detect reliably what are frequently small changes in the rate of hemolysis. We have therefore also used the decrease in absorbance, i.e., turbidity, to follow hemolysis.

The apparent rate constant for hemolysis was defined as the steepest slope of the first-order semilogarithmic plot of the decrease in absorbance versus time. The steepest linear region is usually observed between 2 and 5 s, and about 40% of the decrease in absorbance occurs within this time (Fig. 1).

Throughout this paper we refer to this apparent rate constant, which we found could be determined reproducibly to within 1%, as the rate of hemolysis. Although the rate constant determined in this way is not necessarily identical to the rate at which hemoglobin leaks out of the cell, it should be related to this process (see below), and changes in our rate constant should reflect changes in the hemolysis reaction.

SDS-polyacrylamide gel electrophoresis

After incubation the erythrocytes were quickly washed twice by isotonic buffer at 4°C. The erythrocytes were then lysed by 20 vols. 10 mM Tris buffer (pH 7.4). The membranes were separated by centrifugation and further washed by additional Tris buffer.

SDS-electrophoresis of these membranes were run on 5% acrylamide gels according to the method of Fairbanks [12]. No sulfhydryl-reducing agent was used for solubilizing the membranes nor for electrophoresis.

Electron spin resonance (ESR)

Incorporation of spin-label I(1, 14) into the erythrocyte was carried out according to Adams et al.

[13]. Equal volumes of washed erythrocytes and 5% fatty acid-free bovine serum albumin solution containing spin-label were gently mixed at room temperature for 4-6 h. Excess spin-label was then removed by washing four to five times with isotonic buffer. The supernatant from the final wash showed no observable ESR signal. Spin-labeled erythrocytes were then subjected to membrane modification as described before. After the modification erythrocytes were washed once quickly with isotonic buffer and resuspended in isotonic buffer (hematocrit 50%). The ESR spectra of the spin-labeled erythrocytes were then immediately recorded on a Jeolco JES-ME-IX spectrometer with a variable temperature accessory, used to maintain the sample temperature at 30°C.

Results

The time course of osmotic hemolysis

Fig. 1 is a normalized semilogarithmic plot of a typical curve obtained when 50 μl of an isotonic erythrocyte suspension of 2% hematocrit (approx. $3 \cdot 10^8$ cells/ml) is mixed with 2 ml of hypotonic solution, resulting in a final solution of 47.6 mM NaCl/2.4 mM sodium barbital, at pH 7.4.

This curve shows many of the features previously reported by Anderson and Lovrien [6]. The linear region of our semilogarithmic plot measures what we define as our rate of hemolysis and is related to the k_1 of Anderson and Lovrien [6]. This process measures the initial rupturing of the erythrocyte and the leakage of its hemoglobin. The curvature of the semilogarithmic plot is indicative of the slower k_2 process also observed by these investigators.

Extrapolation of the linear region back to zero percent decrease in turbidity indicates a lag which is of similar magnitude to that previously reported [14, 6]. This lag is attributed to the preleakage steps which primarily involve the entry of water required to convert the biconcave discocyte to a spherical cell. Essentially no hemoglobin leakage was detected by Anderson and Lovrien [6] during this time period. The decrease in turbidity which we observe (Fig. 1) may be attributed to the decrease in turbidity which takes place when the cells swell [4].

In order to determine to what extent the swelling of the cell may also effect the linear region of the semilogarithmic plot used to determine the rate of

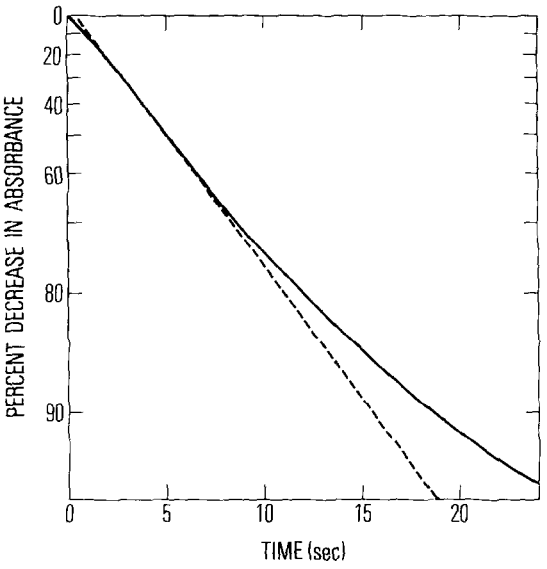

Fig. 1. Typical time course of hemolysis at 30°C in 47.6 mM NaCl buffered by 2.4 mM sodium barbital (pH 7.4). Dotted line is the steepest slope from which the apparent rate of hemolysis is calculated.

hemolysis, we compared the rates of hemolysis in 47.6 mM NaCl/2.4 mM sodium barbital (pH 7.4) of cells initially in isotonic media (145 mM NaCl/2.4 mM sodium barbital, pH 7.4) with an aliquot of the same cells in 100 mM NaCl/2.4 mM sodium barbital (pH 7.4). In the lower NaCl concentration the cells are already swollen and almost spherical, but no hemolysis takes place. The rates of hemolysis were $(1.70 \pm 0.01 \cdot 10^{-1}$ s^{-1} and $(1.72 \pm 0.01) \cdot 10^{-1}$ s^{-1}, respectively. The excellent agreement of these two sets of rate constants indicates that the swelling of the cell does not significantly contribute to the rate of hemolysis obtained from the slope of the semilogarithmic plot.

These results also suggest that the distribution of cell sizes present in a normal blood sample and their different percent volume increase on hemolysis [15] do not directly produce a distribution of rates of hemolysis. This expectation is also consistent with our previously reported finding that there is no significant difference in the rates of hemolysis for cells separated by centrifugation [16], even though the more dense cells are more spherical and swell to a lesser extent. There may nevertheless be a distribution of rates for the rupturing of erythrocytes, which

need not parallel the distribution of cell sizes.

The slower phase of hemolysis (curvature in Fig. 1 and k_2 process of Anderson and Lovrien [6]) is much slower than the more rapid process which determines our rate of hemolysis and will not have much of an effect on the rate of hemolysis calculated from the linear region of Fig. 1.

These rates of hemolysis are therefore a reliable indicator of the process associated with the initial rupturing of the cell which coincides with the rapid leakage of hemoglobin, even though the actual rates calculated from turbidity may be different from those calculated from the release of hemoglobin [6].

Effect of membrane-soluble compounds on the rate of osmotic hemolysis

The erythrocyte membrane was perturbed with Triton X-100, ethanol, and chlorpromazine. These lipid-soluble compounds do not chemically react with any of the membrane components at the low concentrations used.

Triton X-100 is a nonionic detergent which intercalates between lipid molecules and/or binds to integral proteins [17]. At a relatively high concentration of approx. 0.1 v/v% it actually solubilizes the membrane [18,19] and produces hemolysis.

We have investigated the effect of Triton X-100 over a range of concentrations where no lysis is observed and no protein solubilization is observed [19]. As shown in Table I, significant increases in rate are observed at 0.005 v/v%, with larger effects at higher concentrations.

Chlorpromazine and aliphatic alcohols are among a large number of anesthetics and tranquilizers of which the interactions with erythrocyte membranes have been extensively studied [20]. These molecules alter the morphology of the cell with crenated echinocytes formed by anionic and non-ionized compounds, while positive compounds form cup shaped stomatocytes [21,22,23]. Both types of changes in morphology decrease the osmotic fragility or the hypotonicity of the salt concentration necessary to lyse the cells [24–27].

The concentration required to produce the maximum decrease in fragility for any compound varies with the different compounds studied. However, this concentration is generally associated with the number of molecules bound to the erythrocyte and the appearance of the morphological change [28,23]. At higher concentrations of these compounds an increase in the osmotic fragility takes place [23,26,27] together with changes in the binding properties [23, 29] and additional morphological changes [22].

Ethanol is one of the least reactive of this group of molecules and even the highest concentration used in our study (Table I) is expected to have only a small effect on the osmotic fragility [28,20]. The increase in rate of hemolysis found for both 1% and 2% ethanol must therefore be associated with the effects produced at low concentrations of these molecules where they decrease the osmotic fragility.

TABLE I

DEPENDENCE OF THE RATE OF OSMOTIC HEMOLYSIS ON THE CONCENTRATION OF MEMBRANE-MODIFYING REAGENTS

'Relative rate' is the rate relative to the control incubated with isotonic buffer. Hct, hematocrit.

Reagent	Concentration	Relative rate
Triton X-100 *	0.005	1.10
(v/v%)	0.01	1.47
	0.02	2.0
Ethanol *	0.5	1.00
(v/v%)	1.0	1.02
	2.0	1.10
Chlorpromazine *	0.01	1.04
(mM)	0.05	1.25
	0.1	1.34
Diamide **	1.0	1.02
(mM)	5.0	1.03
Glutaraldehyde *	0.0025	0.99
(v/v%)	0.005	0.84
	0.010	0.72
	0.015	0.69
Ca^{2+}(+ ionophore) ***	0.0 + 1	1.01
(mM + μg/ml)	2.0 + 0	1.00
	0.5 + 1	1.15
	2.0 + 1	1.24
	5.0 + 1	1.33

* Erythrocytes were incubated at room temperature for 15 min (Hct 2%).

** Erythrocytes were incubated at 37°C for 60 min (Hct 2%).

*** Erythrocytes were incubated at 37°C for 30 min (Hct 2%).

Chlorpromazine is effective in protecting against hemolysis at a much lower concentration than ethanol [28]. The 0.01 mM concentration in the presence of a 2% hematocrit or $3 \cdot 10^8$ cells/ml (Table I) corresponds to a concentration of about $2 \cdot 10^7$ molecules per cell. This concentration will convert 20–30% of the cells to stomatocytes [23] and is above the 0.008 mM concentration necessary to produce a 50% decrease in relative hemolysis but below the concentration required for a maximal decrease in osmotic fragility [27]. The highest concentration used (0.1 mM) corresponds to about $2 \cdot 10^8$ molecules per cell and is just past the point where the maximal resistance to osmotic fragility is observed [27] and essentially all the cells are stomatocytes [23]. All three concentrations of chlorpromazine were found to increase the rate of hemolysis with a larger effect at higher concentrations.

From the concentration studies with individual blood samples standard conditions for the modification of the membrane by each compound were established. The magnitude of the change in rate and the failure to observe any hemolysis were used as the criteria for establishing the standard conditions for each compound. In Table II we show the mean ±S.D. of relative rates for a number of different subjects at these standard conditions. In all cases these membrane-soluble compounds increase the rate of hemolysis.

TABLE II

EFFECT OF MEMBRANE MODIFICATIONS ON THE RATE OF OSMOTIC HEMOLYSIS

'Relative rate' is the rate relative to the isotonic buffer control for each subject. See Table I for the incubation conditions for each chemical modification.

Reagent	Concn.	Number of subjects	Relative rate (mean ±S.D.)
Triton X-100	0.01 v/v%	4	1.61 ± 0.18
Ethanol	2.0 v/v%	5	1.09 ± 0.05
Chlorpromazine	0.1 mM	5	1.36 ± 0.11
Diamide	5.0 mM	5	1.04 ± 0.02
Glutaraldehyde	0.01 v/v%	5	0.75 ± 0.08
Ca^{2+} (+ ionophore)	2.0 mM	6	1.22 ± 0.06

Effect of protein cross-linking on the rate of osmotic hemolysis

To study the role of proteins, especially spectrin, in determining the rate of hemolysis, membrane proteins were modified directly by cross-linking. Cross-linking was produced by incubation with diamide, glutaraldehyde, or Ca^{2+} in the presence of ionophore. In Table I the effect of various concentrations of these reagents on the relative rate is shown. Table II shows the means ±S.D. of the relative rates for these cross-linking reagents at a standared set of conditions. In all cases the existence of cross-linked high molecular weight compounds was confirmed, at the standard conditions of Table II, by the appearance of a band near the top of the gel (Fig. 2) when the protein composition of the washed membranes was analyzed by SDS-polyacrylamide gel electrophoresis.

The sulfhydryl-oxidizing reagent, diamide, has been shown to cross-link exclusively spectrin (band I and II, Fig. 2) by formation of disulfide bonds [30]. Incubation with this reagent provides only a slight increase in the rate of hemolysis (Tables I and II), even though the electrophoresis shows the presence

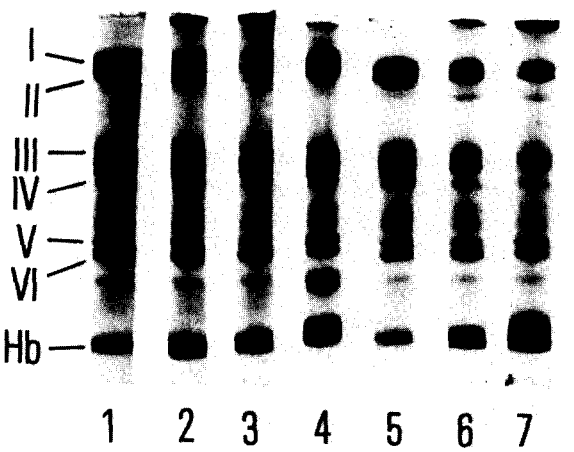

Fig. 2. Gel electrophoresis of membrane proteins. Erythrocytes were modified by incubation with various agents which induce cross-linking. The cells were hemolyzed in 10 mM Tris (pH 7.4) and washed to remove most of the hemoglobin. The membranes were then dissolved in sodium dodecyl sulfate. Bands are numbered according to the notation of Fairbanks et al. [13]. The band near the tops of gels 2, 3, 4, 6, 7 is due to cross-linking. 1, control; 2, 1 mM diamide; 3, 5 mM diamide; 4, 0.01 v/v% glutaraldehyde; 5, 5 mM $CaCl_2$, no ionophore; 6, 2 mM $CaCl_2$ plus 2 μg/ml ionophore A23187; 7, 5 mM $CaCl_2$ plus 2 μg/ml ionophore A23187.

of appreciable amounts of cross-linked compounds at the top of the gel, and a decrease of band I and II relative to band III (Fig. 2).

Glutaraldehyde is known to cross-link non-specifically between amino groups and possibly some sulfhydryl groups [31]. The cross-linking involves spectrin as well as other proteins and some lipids. Incubation with glutaraldehyde decreases the rate of hemolysis significantly even at very low concentrations (Table I). At a higher concentration it completely prevents hemolysis. The existance of high molecular weight compounds is also shown at the top of the gel for glutaradehyde modification, even though it is not as extensive as for diamide treated cells (Fig. 2).

In the presence of the divalent cation-selective ionophore A-23187 [32] an increase in the intracellular Ca^{2+} concentration takes place. Under these conditions a number of effects on the erythrocyte membrane have been reported, including the induction of an enzymatic system which produces cross-linking of membrane proteins, especially spectrin [33].

Intracellular Ca^{2+} also influences sodium and potassium transport [34], resulting in a decrease of intracellular K^+ and the cellular volume, and thereby of the osmotic fragility [35]. Only partial hemolysis of Ca^{2+} treated cells was observed in the 47.6 mM NaCl hypotonic solution, and rates of hemolysis could not be compared with that of the control. This problem was solved by using an isotonic solution composed at 123 mM KCl and 22 mM NaCl (10 mM Tris, pH 7.4) for the incubation medium. In this KCl/NaCl mixture, which is similar to that found in the cell, the osmotic fragility of Ca^{2+}-treated cells is nearly the same as that of the control. Under these conditions an increase in the rate of hemolysis is observed relative to that of the control (Tables I and II), in addition to the cross-linking (Fig. 2).

Incubation with Ca^{2+}, without ionophore, does not result in any increase in intracellular Ca^{2+}. In this case no high molecular weight cross-linked compounds are detected (Fig. 2) and no change in the rate of hemolysis is observed (Table I).

Effect of membrane modification on the fluidity of the lipid matrix

In the course of hemolysis, the erythrocyte membrane bilayer is ruptured. Since the stability of this bilayer can be reflected in its fluidity, a relationship may exist between fluidity and rates of hemolysis.

TABLE III

EFFECT OF MEMBRANE MODIFICATION ON THE RELATIVE ESR SIGNAL RATIO OF SPIN-LABEL I (1, 14)

This ratio, $(h_0/h_{-1})_{rel}$, is relative to the ESR signal ratio of the same spin-labeled cells which were subsequently incubated with isotonic buffer. See Table I for the incubation conditions for each chemical modification.

Reagent	Concn.	Number of subjects	$(h_0/h_{-1})_{rel}$ (mean ±S.D.)
Triton X-100	0.01 v/v%)	3	0.96 ± 0.01
Ethanol	2.0 v/v%	2	0.99 ± 0.01
Chlorpromazine	0.1 mM	3	0.91 ± 0.01
Diamide	5.0 mM	3	1.05 ± 0.04
Glutaraldehyde	0.01 v/v%	2	1.11 ± 0.05
Ca^{2+} (+ ionophore)	2.0 mM	3	0.95 ± 0.02

In recent years techniques have been developed to look at the fluidity of membranes by incorporation of fluorescent or spin-labeled probes into membranes [17]. We have used the stearic acid spin-label I(1, 14) for this purpose. The nitroxide radical in this spin-label is located near the end of the fatty acid chain, and therefore reflects mobility in the internal hydrophobic core of the lipid bilayer.

The fluidity was estimated by an empirical parameter, the ratio of signal height, h_0/h_{-1}, where h_0 corresponds to the height of the center band and h_{-1} corresponds to the height of the high field band. This parameter has been shown to reflect the fluidity of the lipid matrix [36] and is more sensitive to small changes in fluidity than the conventional order parameter.

The changes in these parameters for different membrane modifications are shown in Table III. The ratio of signal height is expressed relative to that of the unmodified spin-labeled control, $(h_0/h_{-1})_{rel}$ with an increase in this parameter indicating a decrease in fluidity.

A significant increase in fluidity is found for the cells treated by intracellular Ca^{2+}, Triton X-100 and chlorpromazine, while a significant decrease in fluidity is found for the cells treated by diamide and glutaraldehyde. These results coincide with former reports which indicate that ethanol [37], intracellular

Ca^{2+} [13] and Triton X-100 [17] increase the fluidity of the bilayer.

Previous workers [38] have failed to detect a change in fluidity due to chlorpromazine by stearic acid spin-labels which probe the hydrophobic portion of the bilayer. Our ability to detect a difference may be due to the fact that the parameter h_0/h_{-1} used by us to detect changes in fluidity is more sensitive to small changes in fluidity than the order parameter [36] used by these other investigators.

The results of Table III, together with those of Table II, suggest a relationship between the rates of hemolysis and lipid fluidity. In order to quantitate the relationship between these two properties of the cell, the rate of osmotic hemolysis of membrane-modified spin-labeled cells was measured on the same samples used to obtain the ESR spectra. Fig. 3 shows a good correlation between the changes in the rate of hemolysis and the changes in the fluidity of the lipid matrix for the various different chemical modifications.

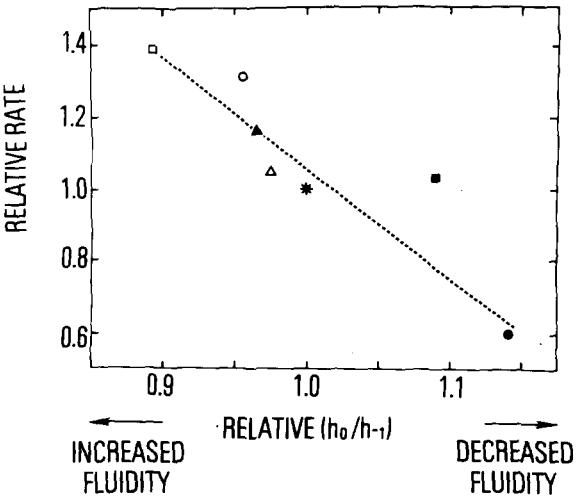

Fig. 3. Comparison of the change in rate of osmotic hemolysis and the altered environment of the stearic acid spin-label I(1, 14) incorporated into the membrane bilayer for different membrane modifications. The determination of the rate of hemolysis and the ESR spectrum of the spin-labeled membrane were both performed on aliquots of the same sample. All modifications were made on the same day that the blood was drawn. The conditions for each chemical modification are the same as given in Table I. □, chlorpromazine; ○, Triton X-100; △, ethanol; ▲, Ca^{2+} plus ionophore; ■, diamide; ●, glutaraldehyde. The signal intensity ratio and the rate are both expressed relative to those of a control run (*).

Diamide is the only modification for which the correlation is relatively poor; it shows a decrease in fluidity even though there is a small increase in the rate of hemolysis. It is quite possible that diamide alters the fluidity in a way which does not affect the rate of hemolysis.

Discussion

Osmotic hemolysis has generally been studied by investigating the osmotic fragility which measures the salt dependence of hemolysis. A comparison of the rate of hemolysis for various modifications (Tables I and II) with previously published osmotic fragility data indicates that the change in the rate does not necessarily parallel changes in the fragility.

Thus, chlorpromazine, which increases the rate of hemolysis, has been shown to decrease the osmotic fragility at the concentrations of Table I [24,25,27]. A similar effect on the fragility has also been observed for ethanol [20], which also increases the rate of hemolysis. On the other hand, intracellular Ca^{2+} produced by incubation with 0.5 to 5 mM Ca^{2+} and ionophore, which increases the rate of hemolysis, does not appreciably change the osmotic fragility when a decrease in intracellular potassium ion is prevented by incubation in 123 mM KCl and 22 mM NaCl (see above).

The same dichotomy between the osmotic fragility and the rate of osmotic hemolysis has been observed when these two parameters are measured on blood samples from individuals of different ages [16] without any chemical modification of the membrane. In this case increasing age increases the osmotic fragility but decreases the rate of osmotic hemolysis. Furthermore, no change in the rate of osmotic hemolysis was observed when washed cells were incubated at 4°C for several days even though the osmotic fragility does increase during this same period of time [39].

These results indicate that the rate of osmotic hemolysis and the osmotic fragility measure two different steps associated with the hemolysis reaction.

The osmotic fragility is a thermodynamically controlled process resulting from the entry of water. Since the erythrocyte membrane cannot tolerate stretching [40] water entry occurs mainly during the swelling process. The fragility is, therefore, mainly determined by the ratio of the initial volume and the

critical volume (V_i/V_c). This relationship between fragility and the ratio V_i/V_c has previously been demonstrated in anesthetics and tranquilizers like chlorpromazine and ethanol. Thus, the decrease in osmotic fragility at low concentrations of these compounds has been related to the increase in the membrane surface, i.e., the critical volume, which takes place without altering the initial cell volume in isotonic solutions [25–27,41,42].

On the other hand, the rate of hemolysis is expected to be determined by the slowest step in the hemolysis reaction. The similarity between the rate of hemolysis for cells in isotonic solution and those preswollen in 100 mM NaCl (see above) indicates that the swelling of the cell from a biconcave discocyte to a sphere does not determine the rate of hemolysis. This is consistent with the observation that permeability obtained by the classical hemolysis time method [5] gives a smaller value than that obtained by direct measurement of the volume change during swelling or shrinking without hemolysis [43].

The results of Jay and Rowlands [14] on the hemolysis of individual cells further substantiate this point. These investigators were able to measure directly both the time required for swelling and the time required for lysis. For permeants such as glycerol which enter the cell relatively slower there is a correlation between these two times, even though the hemolysis time is slower than the swelling time. For water the swelling time is more than an order of magnitude shorter and there is no longer any correlation between the swelling time and the lysis time. The rate of hemolysis must therefore measure the cell rupture process which follows the swelling process, and can change independently of the osmotic fragility.

The effect of different membrane modifications on the rate (Tables I and II) makes it possible to delineate further the rupture process which has been shown to be the rate limiting step in the hemolysis reaction.

The ESR spin-label studies (Table III and Fig. 3) demonstrate that the fluidity of the lipid bilayer correlates with the rate of hemolysis. The significance of this correlation is particularly striking in view of the fact that we are looking at six different membrane modifications, each of which alter the membrane in a somewhat different manner. Some of these modifications, such as ethanol [37] and Triton X-100, [17] simply dissolve into the lipid bilayer, thereby increasing fluidity, while the effect of intracellular Ca^{2+} on the fluidity is not thought to be due to any direct effect of Ca^{2+} on the bilayer, but to be instead the indirect result of the Ca^{2+}-induced protein cross-linking [13], which decreases the amount of phospholipids associated with protein.

We have also observed [44] that an increase in the cholesterol : phospholipid ratio, which has been shown to decrease the fluidity of the bilayer [45], also decreases the rate of hemolysis. This effect is of particular interest because the erythrocyte membrane cholesterol is thought to interact directly only with other lipids, and not with the membrane proteins [46].

The correlation involving these different modifications therefore indicates that the rupturing of the cellular membrane is closely associated with the fluidity of the lipid bilayer. Since fluidity is expected to be a measure of the strength of the intermolecular interactions within the bilayer, the relationship between fluidity and rates of hemolysis indicates that the rate limiting step in the hemolysis reaction involves the rupturing of the bilayer [47].

The dynamic nature of the membrane bilayer [48] makes it impossible to distinguish between a phenomenon originating in the lipid region and one originating at the lipid-protein interface of the bilayer. However, our results on chemical modifications involving cross-links (Tables II and III) seem to indicate that the spectrin network [49] does not play a major role in determining the rate of hemolysis. Thus, glutaraldehyde, diamide and intracellular Ca^{2+} are shown in Fig. 2 to produce cross-links, which involve spectrin [31,30,33]; nevertheless, they have very different effects on the rates of hemolysis (Table II), with Ca^{2+} increasing the rate, glutaraldehyde decreasing the rate, and diamide having almost no effect on the rate.

These results are particularly interesting in view of the fact that glutaraldehyde, intracellular Ca^{2+} and diamide all decrease erythrocyte membrane deformability [50–52]. Thus it appears as though the membrane-rupturing process associated with the rate of hemolysis is unrelated to the membrane-deformability process.

This hypothesis seems to be supported by recent results on the mechanical properties of the erythro-

cyte membrane [53]. Thus the elastic modulus for deformation at constant area, associated with membrane deformability, has been found to be several orders of magnitude smaller than the elastic modulus of the membrane during area expansion, which should be associated with the stretching of the membrane prior to rupturing [40,54,55]. The spectrin network, which has been linked to membrane deformability [40,50,56], may enable the membrane to bend during deformation and return to its original shape without fragmentation. On the other hand, this network may offer little resistance to the approximately 2–3% stretching [57] necessary for lysis. Instead the large force required to stretch and rupture the membrane may originate predominantly in the bilayer [47,58] as suggested by the correlation between fluidity and rates of hemolysis.

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