J. Membrane Biol. 101, 67–72 (1988)

The Journal of
Membrane Biology

Thermal Instability of Red Blood Cell Membrane Bilayers: Temperature Dependence of Hemolysis

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Summary. Rates of human red blood cell hemolysis were measured as a function of temperature. Three distinct temperature intervals for hemolysis were noted: a) At temperatures equal to or less than 37°C no hemolysis was observed for the duration of the incubation (30 hr). b) For temperatures exceeding 45°C hemolysis rates are rapid and are accompanied by gross changes in cellular morphology. The activation energy for hemolysis is 80 kcal/mole; this value is characteristic of protein denaturation and enzyme inactivation suggesting that these processes contribute to hemolysis at these high temperatures. c) Between 38 and 45°C the energy of activation is 29 kcal/mole, indicating that a fundamentally different process than protein inactivation is responsible for hemolysis at these relatively low temperatures. A mechanism based on the concept of the critical bilayer assembly temperature of cell membranes (N.L. Gershfeld, *Biophys. J.* **50**:457–461, 1986) accounts for hemolysis at these relatively mild temperatures: The unilamellar state of the membrane is stable at 37°C, but is transformed to a multibilayer when the temperature is raised; hemolysis results because formation of the multibilayer requires exposing lipid-free areas of the erythrocyte surface. An analysis of the activation energy for hemolysis is presented that is consistent with the proposed unilamellar-multibilayer transformation.

Key Words hemolysis · membrane · erythrocyte · pyrexia · phospholipid · phase transition · unilamellar · temperature

Introduction

When mammalian blood is heated above normal body temperature spontaneous hemolysis of the red blood cells occurs. Thus, for rabbit and for human erythrocytes even the small temperature increases associated with pyrexia are sufficient to induce hemolysis (Karle, 1968, 1969). Thermally induced hemolysis has been thought to involve three types of processes: a) inactivation of vital enzymes and denaturation of structural protein, b) formation of lytic agents in plasma, and c) melting of membrane lipids.

These processes however, seem irrelevant to the underlying mechanism for hemolysis induced by fever. While a body temperature of 42°C in mammals may be lethal, in erythrocytes glycolysis (Lamparelli, Rosina & Petrini-Arisi, 1965; Karle, 1969), and ATPase activity (Jackson, et al., 1973) appear nearly optimal. Moreover, spectrin structure as monitored by shear elasticity (Waugh, 1987), is normal at temperatures below 45°C (Rakow & Hochmuth, 1975; Waugh & Evans, 1979). Thus, enzyme inactivation and deterioration of structural protein appear unlikely to cause hemolysis with the relatively minor increases in temperature associated with pyrexia. It is only with the application of more extreme temperatures (>45°C) that enzyme inactivation is noted (Lamparelli et al., 1965; Jackson et al., 1973), and where spectrin structure is irreversibly altered (Rakow & Hochmuth, 1975). At these high temperatures the cells undergo obvious morphological transformations and rapid rates of hemolysis (Ham et al., 1948; Ponder, 1971) probably reflecting a general breakdown of the cell.

Formation of lytic agents in plasma under the mild condition of pyrexia also seems unlikely to contribute to hemolysis. If these compounds are produced by heating plasma then they should be present in the blood of burn patients, but their plasma does not cause hemolysis (Shen, Ham & Fleming, 1943). Nor does intravenous injection into dogs of plasma separated from heated dog whole blood cause hemolysis (Ham et al., 1948).

The third proposed hemolytic mechanism—structural transformation of the membrane lipid—has usually been attributed to melting of membrane phospholipids as manifested by the gel-liquid crystal transition (Chapman, 1967). However, the gel-liquid crystal transition temperature for erythrocyte lipids is far below 37°C (Gottleib & Eanes, 1974), and therefore the melting process cannot possibly contribute to hemolysis at temperatures above 37°C.

Although lipid melting cannot account for ther-

mally induced hemolysis, recent studies indicate that a transformation of the membrane bilayer occurs when the normal ambient temperature of the cell is exceeded (Gershfeld, 1986). According to this mechanism the growth temperature of the cell is a critical point where optimal membrane bilayer stability occurs. The properties exhibited by membrane bilayers in this critical state can explain why small increases above normal body temperature lead to hemolysis. A general outline of the critical properties of the bilayer and their consequences for erythrocyte membrane stability follows.

From studies utilizing total membrane lipid extracts of bacteria, and of human red blood cells dispersed in water it has been deduced that membrane bilayer assembly is a spontaneous process that occurs at a critical temperature T^*, the growth temperature of the cell. T^* is generally higher than the membrane's gel-liquid crystal transition temperature, and is primarily a function of the lipid composition in the metabolic pool forming the membrane (Gershfeld, 1986). These deductions are based on thermodynamic studies of aqueous dispersions of artificial phospholipids in which it was demonstrated that unilamellar lipid vesicles form only at T^* (Gershfeld & Tajima, 1979; Ginsberg & Gershfeld, 1985; Tajima & Gershfeld, 1985; Gershfeld, Stevens & Nossal, 1986). At temperatures above and below T^* nonunilamellar structures form; for the phospholipids that have been examined the nonunilamellar structures are multibilayers (Gershfeld et al., 1986).

It is implicit to this concept of bilayer assembly that when the temperature deviates from T^* the membrane bilayer must shift to another state, presumably multibilayer. Thus, for cells whose membranes normally consist of a unilamellar structure at T^*, when temperatures are altered a portion of the membrane will form the multibilayer state. Accordingly, for cells with a fixed surface area and in the absence of further membrane lipid synthesis, regions of the membrane must be deficient in lipid, and become leaky. How this is manifested in cells will depend on the organism. For poikilotherms which adjust the composition of their membrane lipids to accommodate to altered ambient temperatures (Marr & Ingraham, 1962), it has been suggested that the unilamellar-multibilayer transformation triggers the cells to synthesize new lipid consistent with the new growth temperature (Gershfeld, 1986). However, for most homeotherms which cannot accommodate to the new temperature by synthesizing new lipid, cell death must ultimately ensue. For erythrocytes, we believe hemolysis is the manifestation of the unilamellar-multibilayer transformation.

Preliminary kinetic studies of the unilamellar to multibilayer transformation with artificial phospholipids suggest that the processes are rapid when temperatures exceed T^* (Ginsberg & Gershfeld, 1986), but that supercooling of the unilamellar state may occur for temperatures below T^* (Tajima & Gershfeld, *unpublished*). However, these processes have not been evaluated in biological membranes. In the following experiments we have attempted to obtain the kinetics of these bilayer transformations in cell membranes by measuring hemolysis rates in human erythrocytes at temperatures near 37°C. Thus, for the modest increases in temperature usually encountered with pyrexia, hemolysis would be expected to occur as the critical temperature ($T^* = 37$°C) is exceeded. Moreover, the temperature dependence of the hemolysis rate should be characteristic of the activation energy of the bilayer to multibilayer transformation. When $T < T^*$ the unilamellar state is expected to supercool, and therefore no hemolysis is anticipated at temperatures below 37°C. In our study we examined hemolysis for the temperature interval of 4 to 50°C. The results are consistent with the properties of the unilamellar critical state: at or below T^*, the temperature of bilayer assembly, the membrane is stable; but for even small increases in temperature above T^* hemolysis occurs. An analysis of the energetics of the unilamellar-multibilayer transformation is presented and is shown to be consistent with the measured activation energy for hemolysis.

Materials and Methods

One pint of fresh whole blood containing a standard amount of ACD was obtained from a single donor; 5-ml samples of the blood were transferred under sterile conditions into sterile 15-ml polystyrene conical tubes (Falcon 2099) and capped. The samples were stored overnight at 4°C. After overnight storage tubes were transferred to constant temperature baths at 4, 20, 37, 40, 45 and 50°C. Tubes were withdrawn at timed intervals and centrifuged for 5 min at 1500 × g to separate the plasma. The plasma was slightly turbid and therefore was recentrifuged for 10 min at 10,000 × g yielding clear preparations of plasma. This last step was necessary to attain high degrees of reproducibility which amounted to ±0.1% hemolysis for incubations up to 30 hr. For incubations longer than 30 hr, especially for temperatures of 37°C and higher, variability became significantly greater. Our studies were limited to 30 hr of incubation. Hemoglobin content of the plasma was obtained by Drabkin's method (Drabkin & Austin, 1932).

The morphology of the packed cells was examined using a differential interference contrast optical microscope.

Hemolysis has generally been treated as a first-order kinetic process, i.e., the rate of cell destruction is proportional to the number of cells present (Ponder, 1971). Thus $-dN/dt = kN$ where $-dN$ is the number of red blood cells that have hemolyzed in the period dt, N is the number of cells present at time t, and k

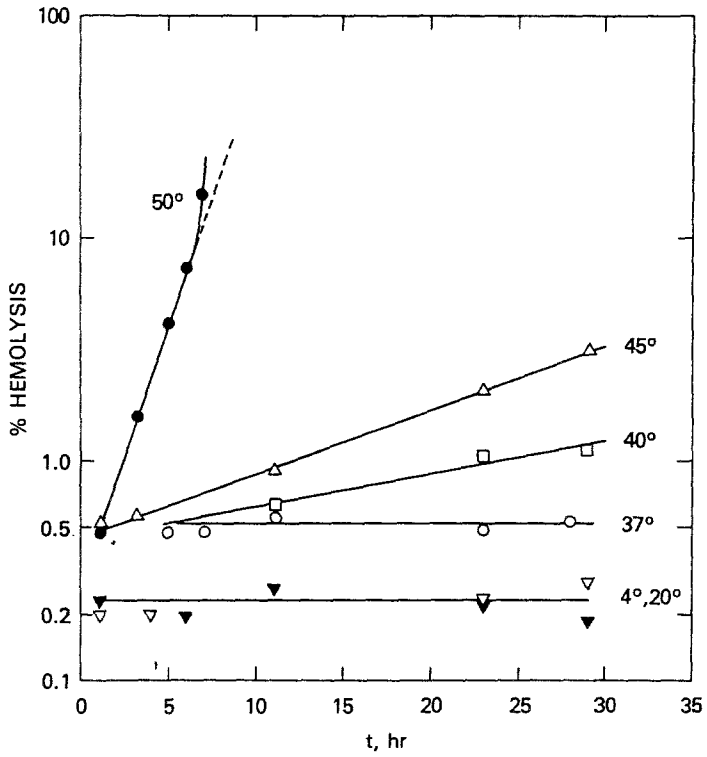

Fig. 1. Hemolysis rates of human red blood cells as a function of temperature, plotted as log (% hemolysis) *vs.* time according to Eq. (1)

is the rate constant. Since each cell contains the same amount of hemoglobin, the form of this equation may be converted to hemoglobin concentrations using the relation $dN = k'dc$, where dc is the concentration of hemoglobin in plasma that has accumulated in the interval dt. The integrated form of the rate equation then becomes

$$-\ln(c/c^\circ) = kt \qquad (1)$$

where c is the concentration of hemoglobin in the plasma at time t, and c° is the equivalent concentration in the cells at $t = 0$. Thus c/c° is the fraction of cells that have hemolyzed at any time t. This first-order rate equation will be used to obtain the rate constant k for hemolysis as a function of temperature.

The application of this kinetic analysis is complicated by the age distribution of the cells in any given whole blood sample; the distribution is generally manifested in a sigmoidal shaped % hemolysis *vs.* time curve which cannot be fitted by a single first-order rate equation (Ponder, 1971). Our studies were restricted to relatively short incubation times (30 hr) where first-order kinetics was generally observed. This time limit was also set by the period in which the cells incubated at 37°C were able to remain hemolysis-free. The kinetic data will indicate that the same subset of cells was involved for each temperature examined.

Results

Figure 1 is a semilogarithmic plot of the percent hemolysis in whole blood as a function of time for incubation temperatures that vary from 4 to 50°C. The cells incubated at 4 and 20°C show very little hemolysis (0.2%) over the entire interval. At 37°C the blood initially shows the same low % hemolysis, but over a period of about 3 hr the amount increases slightly to about 0.5%, and remains constant at that level for the duration of the experiment. This initial increase in hemolysis, with similar rates, is also observed at 40 and 45°C. Further hemolysis at these temperatures obeys the first-order rate law of Eq. (1), as shown in Fig. 1.

The cells incubated at 50°C obey the first-order rate law for hemolysis for the first 6 hr of the experiment, as seen in Fig. 1. For longer times higher than expected rates of hemolysis are observed, perhaps an indication of hemolysis from another subset of the cell population. For the data of Fig. 1 it is reasonable to assume that the same subset of the cell population is hemolyzing between 38 and 50°C because the kinetic data are all first-order, and extrapolate at $t = 1$ hr to the same starting point, 0.5% hemolysis.

Assessment of changes in cell morphology with temperature was only qualitative because of the variability in the appearance of the multiple slide preparations of the same batch of cells. After 29 hr of incubation at temperatures between 4 and 37°C the biconcave disk was the predominant state, but

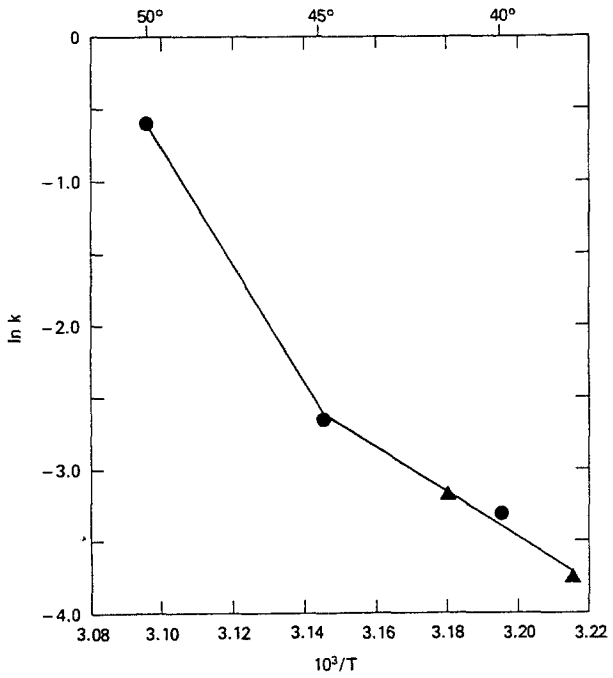

Fig. 2. Arrhenius plot of the first-order rate constants k, (hr^{-1}), for hemolysis as a function of temperature. ●, k calculated from data in Fig. 1. ▲, k calculated from data of Karle (1969). Energies of activation E^* were obtained from slopes according to Arrhenius relation: 38°C < T < 45°C, E^* = 29 kcal/mole; at 50°C minimum estimate for E^* is 80 kcal/mole (*see* text)

increasing numbers of crenated cells form with increasing temperatures.

For the temperatures of 40 and 45°C biconcave disks are still observed, but crenated cells are predominant. Red cell ghosts are also observed at these temperatures, especially at 45°C. The cells incubated at 50°C are strikingly different than those incubated at lower temperatures. Even after incubating only 30 min, many ghosts and cell fragments are present; biconcave disks were not seen.

From the slopes of the lines given in Fig. 1,k, the rate constant for hemolysis, was calculated for each of the indicated temperatures. The activation energy E^* may be obtained from the temperature dependence of the rate constant for hemolysis using the Arrhenius relation $k = A \exp(-E^*/kT)$. Accordingly, a semilog plot of k *vs.* $1/T$ is given in Fig. 2. Rate constants were also calculated for the hemolysis data presented by Karle (1969); to maintain consistency with our results we used only the data where incubation times were less than 30 hr. Figure 2 indicates that between 38 and 45°C the rate constants fall on a single line with an activation energy of 29 kcal/mole. The rate constant at 50°C is about four times higher than the value required to fall on this line. We estimate the activation energy at 50°C to be 80 kcal/mole from the slope of the line drawn between 45 and 50°C recognizing that it is a minimum estimate for E^*. The significance of these activation energies will be considered in the Discussion with respect to distinguishing among possible mechanisms for hemolysis.

Discussion

In agreement with previous studies (Karle, 1968, 1969) thermally induced spontaneous hemolysis of human red blood cells has been demonstrated whenever temperatures are elevated above normal body temperature. At temperatures above 45°C an activation energy for hemolysis of approximately 80 kcal/mole is obtained (Fig. 2). This high value is consistent with activation energies for protein denaturation (Eyring & Stearn, 1939), and with measured calorimetric heats of denaturation in erythrocyte ghosts (Jackson et al., 1973). The reported inactivation of glycolysis (Lamparelli et al., 1965), loss of ATPase activity (Jackson et al., 1973), and spectrin denaturation (Rakow & Hochmuth, 1975) at temperatures exceeding 45°C offer further evidence that the hemolysis observed at these elevated temperatures is due in large measure to thermal denaturation of membrane protein. The gross morphological changes observed for the erythrocytes at 50°C are also consistent with our hemolysis data for this temperature.

In the temperature interval of 38 to 45°C, E^* for hemolysis is 29 kcal/mole, significantly less than expected for protein denaturation. Thus, the hemolytic process in this temperature interval appears to be fundamentally different than the processes occurring above 45°C where protein denaturation and enzyme inactivation are evident. Indeed, the evidence indicates that all vital enzymatic systems still function at temperatures near 37°C. What is most striking about this phenomenon is that even a one-degree elevation above the normal body temperature is sufficient to initiate hemolysis (Karle, 1969).

The unilamellar-multibilayer transition (Gershfeld, 1986) provides an explanation for this phenomenon. Accordingly, when the erythrocyte membrane is assembled the lipid bilayer formed is unilamellar. This bilayer is a critical state in that it forms only at the critical (growth) temperature of the cell T^* (37°C). Upon elevation of the temperature the unilamellar state transforms to a new equilibrium state consisting of multibilayers, thereby exposing cell surface regions which are devoid of lipid; the cell becomes leaky and hemolysis results. The energetics of this transformation is illustrated

in the potential energy diagram of Fig. 3(*A*). The internal energy of unilamellar (*Eu*) and multibilayer (*Em*) states are almost identical (LeNeveu et al., 1977) and the activation energy for the transformation (E^*) is presumed to be our measured value for hemolysis of 29 kcal/mole.

To demonstrate that the unilamellar-multibilayer transformation is a reasonable model for thermally induced hemolysis, we estimate E^* for $T > T^*$ from the following energetically equivalent two-step process: i) lipid from the unilamellar state dissolves into the surrounding aqueous phase, leaving a hole in the membrane, followed by ii) assembly or reaggregation to form the multibilayer state. Because these are sequential processes, the overall rate is determined by the process with the highest activation energy; this value cannot be greater than 29 kcal/mole. In Fig. 3(*B*) E_d^* for the first process (dissolution from the unilamellar state) is the slow step. Although no independent measurements for E_d^* are available, the energetics of the second process (reaggregation), and E_a^* can be estimated. From the potential energy diagram in Fig. 3(*B*), $E^* \cong (Es - Eu) + E_a^*$, where *Es* is the internal energy of the lipid in solution, and E_a^* is the activation energy for assembly of multibilayer from dissolved lipid. The first term, $(Es - Eu)$ is the heat of solution of the lipid, and is estimated to be about 15 to 20 kcal/mole (Gershfeld, et al., 1986). The second term, E_a^* the activation energy for assembly of dissolved lipid into multibilayers, consists of the activation energy for diffusion of the lipid to the site of multibilayer aggregation and the activation energy of association into multibilayers. On the basis of micelle relaxation studies which indicate that micelle formation is largely diffusion controlled (Aniansson et al., 1976), we assume that multibilayer aggregation is also dominated by diffusion of the lipid to the site of aggregation. We estimate the activation energy for diffusion in water is a minimum of 5 kcal/mole (Glasstone, Laidler & Eyring, 1941). Thus, for temperatures greater than T^*, E^* is approximately 20 to 25 kcal/mole, in sensible agreement with the experimental value of 29 kcal/mole obtained for hemolysis.

For temperatures below T^*, the theory predicts that the unilamellar-multibilayer transformation and hence hemolysis would also occur. However, supercooling of the unilamellar state below T^* has been observed in artificial phospholipid dispersions (Tajima & Gershfeld, *unpublished*); the unilamellar structure formed at T^* is maintained when $T < T^*$. We therefore anticipated that the erythrocytes would not hemolyze at temperatures below 37°C due to supercooling of the critical bilayer state.

In summary, we have demonstrated that thermally induced hemolysis of human red blood cells at temperatures near 37°C conforms to the theory that the maximum stability of the unilamellar state in membranes occurs at a critical temperature, the growth temperature of the cell (Gershfeld, 1986). In principle, the critical point occurs as a singularity in temperature where even minor deviations from the critical temperature T^* should lead to a transformation of the bilayer to the nonunilamellar state. In biological systems temperatures are not normally controlled to the rigorous conditions necessary for maintaining critical state, but usually fluctuate around the normal body temperature. The reason that the membranes are able to maintain the critical bilayer state (i.e., the unilamellar state) under the conditions of fluctuating body temperatures is the slow process entailed in transforming the bilayer to the multibilayer state when temperatures are raised, and supercooling of the state when temperatures are lowered. Implicit to this mechanism is the possibility that a minimum hole size is required to form in the membrane before hemolysis can occur; this would account for the apparent delay encountered before hemolysis is detected. The theory, however, does not account for the sigmoidal shape of the hemolysis curve, which has been attributed to an age distribution in the cell population (Ponder, 1971; Martin et al., 1975). Since the lipid composition of the erythrocyte membrane is not significantly altered with age (Phillips, Dodge & How, 1969), no

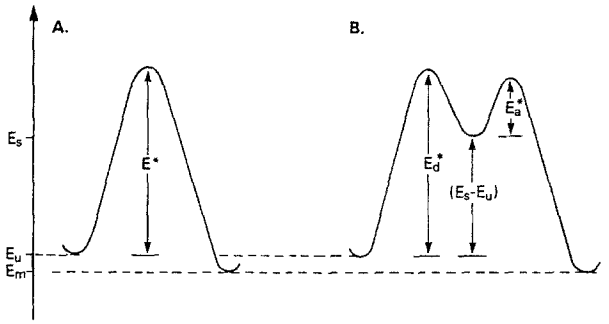

Fig. 3. Potential energy diagram illustrating: (*A*) E^* for transition from unilamellar to multibilayer state in membrane. *Eu* and *Em* are the internal energies of the unilamellar and multilamellar states. According to LeNeveu et al. (1977) $Eu \cong Em$. (*B*) Evaluation of E^* from energetically equivalent processes in which lipid dissolves from unilamellar state as T increases above T^* leaving hole in bilayer; this step is followed by aggregation of lipid in solution forming multibilayer state. $(Es - Eu)$ is approximately the heat of solution of the lipid in water (Gershfeld et al., 1986). E_d^* is the activation energy for dissolution of the unilamellar state. $E_d^* \cong E_a^* + (E_s - E_u)$ where E_a^* is the activation energy for process of multibilayer aggregation from lipid in solution; it is dominated by lipid diffusion in water. *See* text for a more detailed description of analysis

explicit delay mechanism due to the age of the cell is entailed in the lipid transformation theory. Our results suggest instead that the sigmoidal shape of the hemolysis curve may involve age-dependent changes in membrane protein.

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Received 29 June 1987; revised 29 September 1987