Biochimica et Biophysica Acta, 980 (1989) 191–201
Elsevier

BBA 74374

Relationship of hyperthermia-induced hemolysis of human erythrocytes to the thermal denaturation of membrane proteins

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(Received 17 August 1988)
(Revised manuscript received 22 November 1988)

Key words: Erythrocyte; Hemolysis; Thermal denaturation; Hyperthermia; Membrane protein; DSC

Hemolysis of human erythrocytes as a function of time of exposure to 47.4–54.5 °C was measured and correlated to thermal transitions in the membranes of intact erythrocytes as determined by differential scanning calorimetry (DSC). Curves of hemoglobin leakage (a measure of hemolysis) as a function of time have a shoulder region exhibiting no leakage, indicative of the ability to accumulate sublethal damage (i.e., damage not sufficient to cause lysis), followed by a region of leakage approximating pseudo-first-order kinetics. Inverse leakage rates (D_2) of 330–21 min were obtained from 47.4–54.5 °C, respectively. A relatively high activation energy of 304 ± 22 kJ/mol was obtained for leakage, eliminating the involvement of metabolic processes but implicating a transition as the rate-limiting step. Membrane protein involvement was suggested by the very low rate (10^{-2} of the rate from erythrocytes) and low activation energy (50 ± 49 kJ/mol) of hemoglobin leakage from liposomes containing no membrane protein. A model was developed that predicts a transition temperature (T_m) for the critical target (rate-limiting step) of 60 °C when measured at a scan rate of 1 K/min. DSC scans were obtained from intact erythrocytes and a procedure developed to fit and remove the transition for hemoglobin denaturation which dominated the scan. Three transitions remained (transitions A, B, and C) with T_m values of 50.0, 56.8, and 63.8 °C, respectively. These correspond to, but occur at slightly different temperatures than, the A, B, and C transitions of isolated erythrocyte membranes in the same salt solution (T_m = 49.5, 53–58, and 65.5 °C, respectively). In addition, the relative enthalpies of the three transitions differ between isolated membranes and erythrocytes, suggestive of membrane alterations occurring during isolation. Thus, all analyses were conducted on DSC scans of intact erythrocytes. The B transition is very broad and probably consists of several transitions. An inflection, which is seen as a distinct peak (transition B3) in fourth-derivative curves, occurs at 60.8 °C and correlates well with the predicted T_m of the critical target. Ethanol (2.2%) lowers the T_m of B3 by 4.0–4.5 K, close to the shift of 3.3 K predicted from its effect on hemolysis. Glycerol (10%) has very little effect on both hemolysis and the T_m of B3, but it stabilizes spectrin (ΔT_m = 1.5 K) against thermal denaturation. Thus, we propose a model for hyperthermic hemolysis in which the major denaturation of spectrin (T_m = 50 °C), while possibly necessary for lysis, is not sufficient, and that the rate-limiting step is a transition at 60 °C, possibly due to the denaturation of an additional membrane protein.

Introduction

Erythrocytes undergo repeated cycles of deformation during circulation in the body. Thus, in addition to deformability, they must have the capacity to resist hemolysis and fragmentation which is referred to as stability. The molecular basis of stability can be studied by utilizing procedures which alter stability. Exposure of erythrocytes to hyperthermia, or supraoptimal temperature, induces a decrease in stability with resulting hemolysis and fragmentation. The basic morphological changes occurring in heated erythrocytes were described long ago [1,2]. These include sphering, bud formation, fragmentation, and hemolysis. Lloyd et al. [3] observed a slightly different series of changes: sphering, hemolysis, crenation, and total destruction. Each of these steps follows a strict time-temperature relation, i.e., the fraction of erythrocytes undergoing each morphological

Abbreviations: PBS, phosphate-buffered saline (pH 7.4); TCTFE, 1,1,2-trichloro-1,2,2-trifluoroethane.

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change increased with increasing temperature and time of exposure. A 10 min exposure at temperatures of 45, 60, 63 and 67°C, respectively, was required for each event. Moussa et al. [4] measured survival, defined as the fraction of non-hemolyzed cells, microscopically at temperatures from 44 to 60°C. They obtained curves of survival vs. time of exposure which also obeyed a time-temperature relation and could be fit by two models: a two-step kinetic model and a statistical model based on a population of cells having a normal distribution of susceptibility to thermal damage. They found an activation energy of 60 kcal/mol for the first step in the kinetic model.

The morphology of the fragmentation process has been studied in considerable detail by Coakley et al. [5,6]. Erythrocytes develop regularly spaced strings of vesicles when heated to 51–54°C and exposed to shear stress. These appear to arise from the growth of Rayleigh instabilities when the structure maintaining the form of the erythrocyte, presumably the membrane skeleton [7], is weakened. The hemolysis of erythrocytes occurs at lower temperatures in the presence of thermal shear flow on microscope slides, suggesting the importance of mechanical stress of some sort in the final stage of hemolysis [6].

The molecular basis of thermal hemolysis is unknown, but studies of several hereditary erythrocyte membrane disorders illustrate the importance of the membrane skeleton for stability against osmotic, shear, and thermal stress [8]. Erythrocytes from some patients suffering from hereditary elliptocytosis (HE) are sensitive to shear-induced fragmentation and also contain no band 4.1 protein [9]. Other HE subjects have been reported to have defective spectrin molecules [8]. Hereditary pyropoikilocytosis (HPP) is a severe anemia characterized by erythrocyte fragility and abnormal sensitivity to heat [10]. HPP erythrocytes fragment at lower than normal temperatures and contain a spectrin that appears to denature about 5 K lower than normal spectrin. Thus, thermal damage, possibly denaturation, to membrane skeletal proteins may play a role in hyperthermia-induced hemolysis.

The thermal properties of erythrocyte membrane ghosts have been studied by differential scanning calorimetry (DSC). Four major transitions (A–D) have been detected [11]. The A transition ($T_m = 49°C$) is due exclusively, or almost exclusively, to the denaturation of spectrin [12], several other components of the membrane skeleton (bands 2.1, 4.1, and 4.2) and the cytoplasmic portion of band 3 are involved in the B transition ($T_m \cong 55°C$), and the transmembrane portion of band 3 denatures during the C transition ($T_m \cong 65°C$) [13,14]. The cytoplasmic portion of band 3 denatures during the B transition; however, the structural changes occurring in bands 2.1, 4.1, and 4.2 have not been determined.

The denaturation of spectrin has been suggested to play a role in high-temperature induced alterations in the deformability of erythrocytes since there is an initial increase in deformability at 48–55°C, close to the denaturation temperature of spectrin ($T_m = 49°C$) [15]. Denaturation of membrane proteins might also be expected to be important in fragmentation and hemolysis. However, these processes are dependent on both temperature and time of exposure. Thus, a more quantitative comparison of the extent of denaturation of any specific membrane protein is necessary to demonstrate a correlation with any physical or functional alteration of the erythrocyte.

All previous measurements of the thermal transitions of the erythrocyte membrane have been conducted on isolated ghosts. These transitions, particularly the B transition, are sensitive to the salt concentration and pH of the suspending solution and possibly other environmental conditions [16]. Thus, the temperatures of these transitions in the intact erythrocyte are not known precisely, however they must be determined before an accurate correlation can be made to hemolysis.

In this paper we compare hemolysis to the thermal transitions of intact erythrocytes. A model is described which allows the prediction of the denaturation temperature and DSC profile of the critical target for hemolysis. Procedures were developed to measure the membrane transitions in intact erythrocytes to eliminate artifacts due to membrane isolation. We demonstrate that the denaturation of spectrin, while possibly necessary, is not sufficient for hemolysis.

Materials and Methods

Erythrocyte preparation and hemolysis

Human erythrocytes were purified from freshly drawn human blood by washing three times in a 10-fold excess of phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). The buffy coat and the top of the pellet was removed after each centrifugation by aspiration to remove all white blood cells. Erythrocytes were used on the day of isolation since there is a slight increase in the rate of hemolysis at 47–54°C after 2 to 3 days storage at 4°C. Membrane ghosts were isolated by the method of Steck et al. [17] and used the same day.

Temperature-induced hemolysis was measured by suspending the erythrocyte pellet to a hematocrit of 3% in PBS, adding 4 ml of this suspension to each of a number of small test tubes, and placing these in a waterbath at the desired temperature (47.4 to 53.5°C). The test tubes were removed at fixed time intervals, cooled for 30 s in water at room temperature, and centrifuged at $700 \times g$ for 6 min to pellet the unlysed cells. The supernatant was drawn off and ten drops of 15% Triton X-100 added to dissolve any membrane

ghosts. This last step was necessary to eliminate light scattering from the ghosts which was significant at high levels of lysis. The hemoglobin released was determined from the absorbance at 543 nm and the hemoglobin retained was calculated as a fraction of total hemoglobin. Rates of leakage were determined by a least-squares analysis of the linear region of the lysis curves on a semi-log plot.

The effect of ethanol and glycerol on hemolysis was determined by adding a solution of ethanol or glycerol in PBS at twice the concentration desired to an equal volume of erythrocytes in PBS at a 6% hematocrit and gently mixing. The suspension were immediately heated in a waterbath and leakage measured as described above.

Liposomes

Liposomes containing hemoglobin were prepared essentially as described by Hunt et al. [18]. Lipids were isolated from membrane ghosts by chloroform/methanol extraction using the method of Bligh and Dyer [19]. The erythrocyte lipids (65 mg), dissolved in 10 ml of a mixture of 1,1,2-trichloro-1,2,2-trifluoroethane (TCTFE)/diethyl ether (1 : 1.92, by vol.), were added to 10 ml of a solution of hemoglobin in 5 mM KH_2PO_4 adjusted to pH 7.4 and shaken violently for 2 min. The cytosol (almost pure hemoglobin), freshly isolated from human erythrocytes by hypotonic lysis and centrifugation at $48000 \times g$ for 30 min to remove the membrane ghosts, was used as a source for hemoglobin. The volatile TCTFE and ether were removed under vacuum in a rotary evaporator, and the resulting liposomes washed twice by centrifugation at $10000 \times g$ for 15 min and resuspended in PBS. By light microscopy these liposomes have a maximum diameter of 1–2 μm, and they have previously been shown to be single bilayers and capable of supporting oxygen transport in rats [18]. Hemolysis was measured at 45–60 °C as described above for intact erythrocytes.

Differential scanning calorimetry (DSC) and critical target analysis

DSC scans were obtained with a Microcal-2 scanning calorimeter (1.21 ml sample cells) interfaced to a DEC Pro 380 computer. The sample and reference cells of the DSC were cooled to 0 °C and the degassed erythrocyte or membrane suspension in PBS and the reference solution (PBS) added. The scan was started (1 K/min scan rate) when the sample and reference equilibrated at 0 °C (approximately 40 min after addition of cells). The scan was obtained to 100–102 °C, the sample cooled back to 0 °C, and a second scan (the rescan) obtained which was used to correct for the non-linear baseline.

The procedure and assumptions employed for predicting the shape and location of the transition of the critical target for temperature inactivation of Ca^{2+}-uptake and ATPase activity of the Ca^{2+}-ATPase of sarcoplasmic reticulum [20] and for the hyperthermic killing of Chinese hamster lung cells [21] have been previously described. The prediction of the inactivation or transition temperature of the critical target for hemolysis is similar. If hemoglobin leakage is caused by the irreversible inactivation of a critical target (i.e., if the rate-limiting step for hemoglobin leakage is the inactivation of a single target), and if the rate of leakage is proportional to the rate of inactivation, then it is possible to predict the temperature dependence of inactivation (fraction inactivated vs. temperature) and the shape of the transition profile as determined by DSC or any similar method of measuring the relative amounts of native and inactivated target. The basic model is that the critical target is inactivated in a single step ($N \xrightarrow{k} I$) with a rate constant k. From standard radiation target theory, the rate constant for hemolysis obtained from the linear region of a semi-log plot of hemoglobin retained vs. time must be the same as the rate constant for inactivation of the critical target [22]. An approach similar to radiation target theory is applicable to temperature-induced hemolysis if the rate-limiting transition is stochiastic (i.e., random and discrete). This is true for protein denaturation as well as for other conformational changes at the molecular level.

Hemolysis follows pseudo-first-order kinetics and obeys the Arrhenius relation (see Results). Thus, if the above assumptions are valid, similar relations hold for inactivation or denaturation of the critical target and

$$\frac{df_{I}(t)}{dt} = k(T)[1 - f_{I}(t)] \tag{1}$$

$$k(T) = e^{A - E_A/RT} \tag{2}$$

where f_I is the fraction of the critical target inactivated or denatured, k the rate constant of inactivation, and E_A the activation energy. The fraction denatured $f_I[T(t)]$ as a function of time while temperature is scanned at a constant rate, which is needed to predict the DSC profile, is found by replacing k in Eqn. 1 by the Arrhenius relationship (Eqn. 2) and letting $T = T_0 + \nu t$ where ν is the scan rate and T_0 the starting temperature. The resulting equation

$$\frac{df_{I}[T(t)]}{dt} = e^{A - E_A/R(T_0 + \nu t)}(1 - f_{I}[T(t)]) \tag{3}$$

can be solved by numerical integration. The excess specific heat is given by

$$C_p^{ex} = \Delta H_{cal}\frac{df_{I}[T(t)]}{dT} \tag{4}$$

where ΔH_{cal} is the calorimetric enthalpy associated with the transition $N \rightarrow I$. Eqn. 4 was used to predict the shape and transition temperature of the DSC profile of

the critical target for hemolysis. It is not possible to predict the magnitude of C_p^{ex} since ΔH_{cal} cannot be determined from rates of hemoglobin leakage alone. The transition in the critical target which is rate-limiting can only be detected by DSC if the value of ΔH_{cal} is sufficiently large. This model was also used to fit the transition for hemoglobin denaturation by employing an iterative procedure to obtain the values of E_A and A giving the best fit to the DSC profile as previously described [20].

Results

Hemolysis

Curves of hemolysis, plotted as fractional hemoglobin retained vs. time of incubation at constant temperatures from 47.4 to 54.5°C for human erythrocytes in PBS, are given in Fig. 1. These curves have a rather long shoulder region, characterized by D_q (the time at which the linear portion extrapolates to a fraction hemoglobin retained of unity). This is indicate of the ability to accumulate substantial sublethal damage before lysis and release of hemoglobin. At times greater than D_q, the log of hemoglobin retained decreases linearly, indicative of pseudo-first-order kinetics. The slope of this region is defined as the rate constant of lysis (k). The value of k increases while D_q decreases with increasing temperature of exposure. These parameters are given in Table I. In addition, D_o, the time to reach a value of hemoglobin retained of $1/e$ on the linear region ($D_o = 1/k$), and n, the extrapolation number (intercept of the ordinate), are also given. The rate at which hemolysis occurs is more easily visualized from D_o than from k.

The extent of lysis was measured immediately after cooling the erythrocyte suspension. Thus, there was no further expression of damage and the lysis measured was that occurring during exposure to the elevated temperature.

TABLE I

The rate constant of hemoglobin leakage k, the inverse of k (D_o, time on the linear portion of the leakage curve for $1-1/e$ of the hemoglobin to leak out), the length of the shoulder region (D_q), and the extrapolation number (n) derived from curves of hemoglobin leakage as a function of time at temperatures of 47.4–54.5°C.

T(°C)	$k(s^{-1}) \times 10^4$	D_o(min)	D_q(min)	n
47.4	0.502 ± 0.021	332	397	3.3
48.5	0.962 ± 0.017	173	267	4.7
49.4	1.52 ± 0.10	110	176	4.9
50.5	1.95 ± 0.08	85.4	143	5.4
51.6	2.51 ± 0.08	66.4	77.7	3.2
52.6	4.28 ± 0.19	38.9	61.1	4.8
53.5	3.83 ± 0.32	43.5	30.1	2.0
54.5	8.02 ± 0.18	20.8	46.0	9.2
			$n_{avg} = 4.7 \pm 2.0$	

The value of n (approx. 5) is defined as the number of critical targets per cell that must be inactivated for lysis and release of hemoglobin. However, the definition of what constitutes a critical target is somewhat obscure. In this kind of model, a higher-order process such as lysis is controlled by the inactivation of, or damage to, a molecular target, without precisely defining whether the target is an individual molecule or a supramolecular structure.

The temperature dependence for lysis obeys the Arrhenius relation (Fig. 2). A single target is suggested by the straight line on the Arrhenius plot. The activation energy (E_A) for lysis and, hence, for inactivation of the critical target is 304 ± 22 kJ/mol. This is a rather high value indicative of the rapid increase in lysis with increasing temperature and suggests that a transition, such as a protein conformational change, rather than increased metabolism, constitutes the rate-limiting step.

The leakage of hemoglobin from artificial liposomes made from erythrocyte lipids was measured to determine if membrane protein is necessary for the measured leakage rates and the high activation energy.

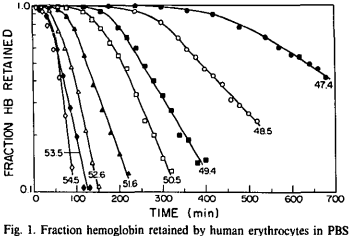

Fig. 1. Fraction hemoglobin retained by human erythrocytes in PBS vs. time of incubation at 47.4°C (●), 48.5°C (○), 49.4°C (■), 50.5°C (□), 51.6°C (▲), 52.6°C (△), 53.5°C (◆), and 54.5°C (◇).

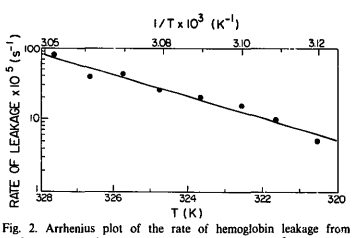

Fig. 2. Arrhenius plot of the rate of hemoglobin leakage from erythrocytes over the temperature range of 47.4 to 54.5°C. (Rates of leakage derived from curves in Fig. 1.)

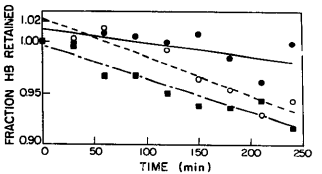

Fig. 3. Fraction hemoglobin retained by liposomes made from erythrocyte lipids vs. time of incubation in PBS (semi-log plot) at 45 °C (●), 50 °C (○), and 60 °C (■).

Hemoglobin was encapsulated as described by Hunt et al. [18]. The liposomes were incubated at 45, 50, or 60 °C and the hemoglobin retained as a function of time (Fig. 3) measured as for the erythrocytes. The rate of hemoglobin leakage, which is two orders of magnitude smaller than for erythrocytes, was determined by linear regression analysis of these curves, and the values are given in Table II. There is little change in k with temperature; an activation energy of 50 ± 49 kJ/mol was obtained from an Arrhenius plot. The large error occurs because of the large fluctuations in the value of k due to the small amount of leakage occurring, but it is apparent that the temperature dependence of leakage from liposomes is very different than that from erythrocytes. The value of 50 kJ/mol only barely differs significantly from zero and is in the range of values measured for membrane properties controlled by the weak interactions between lipids [23,24]. This value is much smaller than the activation energy expected for protein denaturation.

There is no accumulation of sublethal damage in liposomes. The value of D_q is about the same as the error in this parameter, and n is very close to unity. Thus, leakage commences immediately upon exposure to elevated temperature, and there appears to be only a single target per liposome. From these results, we infer that membrane protein is involved in the high-temperature hemolysis of erythrocytes.

Many compounds affect the stability of proteins as defined by the denaturation temperature T_m. Short chain alcohols decrease and for most proteins glycerol increases stability [25]. Ethanol is a potent sensitizer of hemoglobin leakage at 52.6 °C (Fig. 4 and Table III). There is a greater than 200% increase in k for erythrocytes in 2.2% ethanol. Glycerol has little effect: D_q is lengthened but k is also increased slightly (Fig. 4 and Table III). The effect of these compounds can be expressed as the change in temperature necessary for an equivalent change in k. The value of k in 2.2% ethanol is the same as that at 55.9 °C (from Fig. 2), equivalent to a 3.3 K temperature shift. Exposure in glycerol (10%) is equivalent to a 0.8 °C increase in temperature as determined from the increase in k, but the increase in D_q corresponds to a similar reduction in temperature. Thus, glycerol has little effect overall. These compounds should have a corresponding effect on the transition temperature of the critical target.

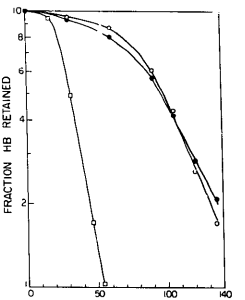

Fig. 4. Fraction hemoglobin retained by human erythrocytes vs. time of incubation in PBS at 52.6 °C: control (●), ethanol 2.2% (w/v) (□), and glycerol 10% (w/v) (○).

Differential scanning calorimetry

Thermal transitions in erythrocytes were determined by DSC in order to ascertain if there is a relation between thermally-induced protein conformational changes and hemolysis. Fig. 5 illustrates a DSC scan of

TABLE II

The rate of hemoglobin leakage at 45 to 60 °C from liposomes made from erythrocyte lipids. The definitions of k, D_o, D_q, and n are given in the legend of Table I.

T(°C)	$k(s^{-1}) \times 10^5$	D_o(min)	D_q(min)	n
45	2.08 ± 1.23	8000 ± 4700	100 ± 90	1.01 ± 0.01
50	6.23 ± 1.02	2700 ± 400	60 ± 30	1.92 ± 0.01
60	5.68 ± 0.72	2900 ± 400	10 ± 30	1.00 ± 0.01
			$n_{avg} = 1.01 \pm 0.01$	

TABLE III

Rates of hemoglobin leakage (k), percent change in leakage, predicted shift in the transition temperature of the critical target (ΔT), and D_q of erythrocytes at 52.6 °C in the presence of ethanol and glycerol.

Additive	$k(s^{-1}) \times 10^4$	Δk(%)	ΔT(K)	D_q(min)
Control	3.81 ± 0.38	–	–	66.3
Ethanol 2.2 %	11.6 ± 0.1	204 ± 29	−3.3	21.1
Glycerol 10%	4.97 ± 0.40	30.6 ± 14.4	0.8	75.6

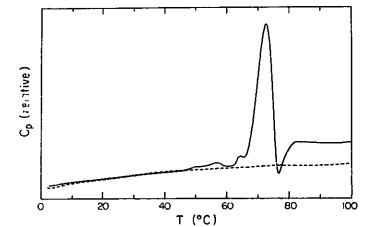

Fig. 5. DSC scans (C_p^{excess} vs. temperature) of human erythrocytes ($3.3 \cdot 10^8$ cells/ml) in PBS (solid line) and a rescan of the same cells (broken line).

erythrocytes in PBS, the same solution in which erythrocyte lysis was measured. There are three small peaks between 50 and 65 °C and a major peak at about 72 °C. The last is due to hemoglobin denaturation and is followed by a dip or exotherm at about 75 °C. The baseline shifts to a higher value of specific heat (C_p) following hemoglobin denaturation, which is commonly observed following the denaturation of nearly all proteins [26].

A rescan was obtained from the same sample and is also shown in Fig. 5. It matches in the low temperature region, below the onset of any transitions, very well and also matches in the region above 80 °C except for the offset in specific heat (ΔC_p). The rescan was used as an instrumental baseline and subtracted from the original scan. The increase in specific heat (ΔC_p) between the native and denatured states was corrected by the procedure suggested by Hemminger and Höhne [26].

The scan corrected for the baseline is shown in Fig. 6. The major peak, transition Hb, with a transition temperature of 72.5 °C (defined as the temperature of maximum C_p), was identified as hemoglobin denaturation from the large enthalpy associated with this transition and the fact that hemoglobin is the principle protein species of the erythrocyte. The identification was verified by obtaining a DSC scan of the supernatant remaining after the lysis of erythrocytes in 5 mM potassium phosphate (pH 7.4) and centrifugation to pellet the ghosts. The denaturation temperature (T_m) of the free hemoglobin was 70.1 °C and transitions A–C were not present (results not shown). The T_m of hemoglobin in 5 mM phosphate is about two degrees lower than that found in whole cells, probably because of the low osmotic strength of the lysis buffer.

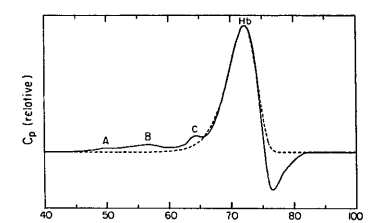

Fig. 6. DSC scan of human erythrocytes corrected for the baseline (solid line) and the best fit curve of the hemoglobin transition (broken line). The original scan is shown in Fig. 5. Transitions A, B and C, and the transition due to hemoglobin denaturation (Hb) are labelled.

The exotherm at 76 °C is most likely due to the aggregation of hemoglobin following denaturation. Aggregation is abetted by the high concentration of hemoglobin in the erythrocyte and is probably one of the factors contributing to the irreversibility of denaturation.

The hemoglobin transition was fitted using the model for denaturation described in the Materials and Methods. The region from 66 to 74 °C was used in the fitting to eliminate contributions from the low temperature transitions and the high temperature exotherm. Fitting parameters of $E_A = 455$ kJ/mol and $A = 154$ were obtained and used to generate the best fit shown in Fig. 6.

The best-fit curve was subtracted from the corrected scan of the erythrocytes to obtain a profile of transitions A, B, and C with the contribution from hemoglobin removed (Fig. 7A). Three well-defined transitions (A, B, and C) with transition temperatures of 50, 57, and 64 °C, respectively, are found (Table IV).A DSC scan of isolated ghosts in the same solution is also shown in Fig. 7A. Erythrocyte ghosts have three corresponding transitions in this temperature region, al-

TABLE IV

Transition temperatures (T_m) of human erythrocyte ghosts and whole erythrocytes determined at a scan rate of 1 K/min. in PBS and PBS plus ethanol or glycerol. The T_m values for transitions A, B, C, and Hb were determined from scans of C_p vs. temperature (e.g., Figs. 7a and 8), and the T_m values for transitions B1, B2 and B3 were determined from 4th-derivative curves (e.g., Fig. 7B). The reproducibility of the T_m values of erythrocytes is approx. ±0.2 K.

Transition	T_m(°C)			
	Ghosts	Erythrocytes		
		Control	Ethanol 2.2%	Glycerol 10%
A	49.5	50.0	49.7	51.5
B	53–58	56.8	55.8	57.9
B1	–	52.3	–	–
B2	–	57.2	56.3 (B3)	58.2
B3	–	60.8	–	61.1
C	65.5	63.8	59.8	64.4
Hb	–	72.4	69.9	72.2

though the transition temperatures vary somewhat from those found for intact erythrocytes. Thus, the erythrocyte transitions A, B, and C are primarily membrane associated transitions. This conclusion is supported by the lack of these transitions in the non-membrane, soluble fraction of lysed cells (not shown). The erythrocyte ghost transitions have been referred to as transitions A, B, and C by Brandts and colleagues [10].

In addition to the slight differences in transition temperatures, the relative enthalpies of the three transitions vary dramatically between the ghosts and whole cells. Specifically, transition B has a greater relative enthalpy in whole cells and transition A is stronger in the ghosts. This indicates a weakening of transition B in ghost membranes, possibly due to a loss of some proteins that contribute to transition B or other undefined membrane damage occurring during membrane isolation.

The profile of the transition of the critical target for hemolysis was predicted as described in the Materials and Methods and is shown in Fig. 7A (dotted curve). A transition temperature of 60°C is predicted from E_A and A for hemoloysis (from Fig. 2). There is a suggestion of an inflection representing a transition at about 60°C, partially hidden under the B transition, in the scan from erythrocytes in Fig. 7A. This inflection is weak and suggests but does not unambiguously demonstrate the existence of a transition of this temperature correlating with the predicted transition.

Derivatization can be used to remove the broad background from peaks consisting of several components to reveal the individual components. Second derivatives of DSC scans of mixtures of dimyristoyl- and dipalmitoylphosphatidylcholine have been used to better resolve the multiple components of DSC scans [27]. The fourth-derivative curve, which has the advantage that the original transitions are present as positive peaks, of the erythrocyte scan (Fig. 5) is shown in Fig. 7B. Derivatization does not introduce additional information that is not already present, but it can make weak transitions that are present as barely discernible inflections more apparent. Transitions B appears to consist of three components, transitions B1, B2, and B3. The B transition in membrane ghosts is split into two transitions, previously referred to as transitions B_1 and B_2, in solutions near isotonic ionic strength and after treatment with some inhibitors of anion transport [28]. (Subscripts will be used to differentiate the membrane transitions from intact erythrocyte transitions). Thus, there is evidence that the B transition region contains more than one transition; however, the B1 and B2 transitions in intact erythrocytes are not necessarily the same as the B_1 and B_2 transitions in isolated ghosts.

The peak labelled B2 ($T = 57.2$°C) in the fourth-derivative curve matches very well the B transition ($T = 56.8$°C) in the original scan. In addition, transition A and C are represented by peaks at the proper temperatures in the fourth-derivative curve. Two new peaks present in the fourth-derivative curve are peaks B1 and B3 at transition temperatures of 52.3°C and 60.8°C.

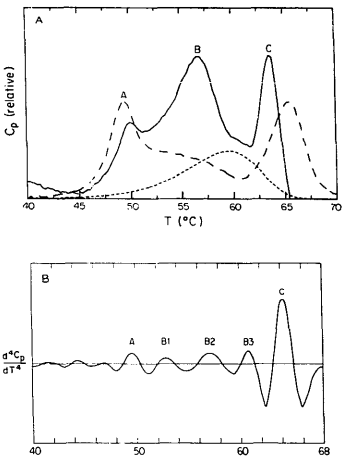

Fig. 7. (A) DSC scans (40–70°C) of human erythrocytes with the hemoglobin transitions subtracted (solid line), erythrocyte ghosts (broken line), and the predicted transition of the critical target of the rate-limiting step for hemoglobin leakage (dotted line). Erythrocytes and ghosts were suspended in PBS. (B) The fourth-derivative curve of the DSC scan of control erythrocytes from (A).

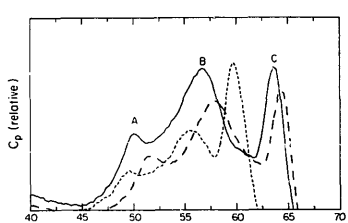

Fig. 8. DSC scans of human erythrocytes in PBS with the hemoglobin transition subtracted: control (———), ethanol 2.2% (- - - - -), and glycerol 10% (— — —).

respectively. Peak B1 does not correspond to any obvious inflection in the original scan, but peak B3 is due to the weak inflection at 60–62 °C, further supporting the presence of an unresolved transition at this temperature that may represent the denaturation of the predicted critical target.

If the rate-limiting step of hemolysis is a transition at 60 °C and if the inactivation of the critical target is represented by transition B3, then any shift in the transition temperature of B3 should correspond to a change in the rate of hemolysis. Conversely, any agent that alters the rate of hemolysis should shift the transition temperature of B3. As shown in Fig. 4, ethanol causes a dramatic increase in the rate of lysis. The effects of glycerol are more complex, with both slight protection (increase in D_q) and slight sensitization (increase in k) occurring. The effects of these compounds on the thermal transitions of the erythrocyte are shown in Fig. 8 and Table IV. Ethanol lowers the T values of transitions A and B by 0.3 and 1.0 K, respectively. The T_m of hemoglobin is decreased by a greater amount, 2.5 K, and transition C is lowered the most, 4.0 K. Transition B3 is not present in the fourth-derivative scan of erythrocytes in ethanol, presumably because it is shifted sufficiently to overlap with the B2 peak. Thus, its T_m would be approximately 56.3 °C, giving a shift of 4.5 K, comparable to the shift in transition C.

The expected decrease in the transition temperature of the initial target can be calculated from the value of k for erythrocytes in ethanol assuming that the activation energy (E_A) is unchanged. Up to 10% ethanol has little effect on the E_A of inactivation of the Ca^{2+}-ATPase [20]. Moderate changes in E_A will not significantly affect the shift in T_m since there must be a compensating change in A. An increase in k from $3.81 \cdot 10^{-4}$ to $11.6 \cdot 10^{-4}$ s^{-1} (Table III) corresponds to a temperature increase of 52.6 to 55.9 °C, that is a difference of 3.3 K (from Fig. 2). This is approximately equal to the shift in transition C and the probable shift in transition B3 but significantly greater than the shift in transitions A and B.

Glycerol is a general protein stabilizer and is known to increase the denaturation temperature of most proteins. Except for the denaturation temperature of hemoglobin, which is unaffected or slightly lowered (0.2 K), all the erythrocyte transition temperatures are increased by glycerol. The magnitude of the increase is greatest for the transitions of lowest temperature ($\Delta T_m = 1.5$ K for transition A) and decreases as the transition temperature increases. A similar observation was made for the effect of glycerol on the thermal transitions of cultured fibroblasts [21].

The T_m of transition B3 is increased by only 0.3 K. Glycerol also has very little effect on hemolysis; the increase in k is compensated by an increase in D_q (Table III). Only the value of k affects the T_m of the critical target, and the small increase in k leads to a predicted decrease of 0.8 K in the T_m of the critical target. This shift is small but opposite to that observed. Thus, the observed and predicted shifts are opposite but both are sufficiently small that they are consistent with the lack of a significant effect of glycerol on the T_m of transitions near 60 °C. If the T_m of the critical target was at a lower temperature, one would expect protection from glycerol, as is observed for the increase in the T_m of spectrin and as is true for the hyperthermic killing of fibroblasts which has a predicted T_m of the critical target at 46 °C [21].

Discussion

Lysis of erythrocytes with release of hemoglobin occurs at elevated temperature. For a period of time, designated by D_q, there is little leakage, indicative of accumulation of damage insufficient to cause lysis. This is often referred to as sublethal damage. Eventually, leakage increases and obeys pseudo-first-order kinetics, suggesting that there is a step in the sequence of molecular damage occurring at the elevated temperature that is rate-limiting and has similar kinetics. Erythrocyte survival, plotted as fraction unlysed cells vs. time of exposure at elevated temperature, has a similar curve shape [4]. A likely scheme, on which the following analysis is based, is a series of steps leading to lysis, one of which, the rate-limiting step, primarily determines the rate of lysis. This is analogous to a series of chemical reactions, each with a different rate constant, leading to a final product. In the case of chemical reactions, there is seldom one rate-limiting step, but usually several steps influence the overall rate of product formation depending on the specific conditions present [29]. A similar scheme may occur for temperature-induced hemolysis, but for a start, a simple rate-limiting step which describes damage to a critical target will be assumed. The sequence of morphological changes occurring during exposure before lysis supports the existence of a sequence of steps at the molecular level [1–6].

The relatively high activation energy of lysis (304 kJ/mol) suggests that the rate-limiting step is not metabolic but is due to a transition in the erythrocyte membrane. The most apparent transitions are those representing protein denaturation. An obvious candidate for the rate-limiting step is the denaturation of spectrin ($T_m \approx 50$ °C), but this will be shown to occur at too low a temperature to be the rate-limiting step. Since sublethal damage can accumulate, more than one copy of the critical target must be inactivated or inactivation must result from multiple hits before lysis commences. Multiple copies of the critical target seem more likely if protein denaturation is the rate limiting step. A value of

n (from strict target theory the number of initial targets per cell) of approx. 5 was obtained. This value does not change with temperature, implying that neither the nature of the critical target nor the number of targets that must be inactivated for lysis changes with temperature. Although a specific critical target may exist, the meaning of the value of n, other than as an indication that more than one critical target exists per cell, is unclear. It certainly is not the total number of molecules per cell that must be inactivated for lysis to occur, but probably is representative of a minimal, supramolecular structure that must be inactivated or damaged.

If damage to membrane protein is necessary for hemolysis, then hemoglobin leakage from reconstituted liposomes should be much slower and have a different temperature-dependence, i.e., activation energy, than that of erythrocytes. This was investigated by reconstituting liposomes and measuring leakage as for erythrocytes. It has been shown that liposomes reconstituted by the procedure described by Hunt et al. [18] are unilamellar, range in size from 0.1 to 1.5 μm with an average diameter of about 0.7 μm, are relatively stable when injected into rats, and are capable of oxygen transport. The rate of hemoglobin leakage from liposomes over the temperature range of 45–60°C is two orders of magnitude less than for erythrocytes, the activation energy of lysis (50 ± 49 kJ/mol), while difficult to determine accurately, is much less than for erythrocytes, and there is no accumulation of sublethal damage ($n = 1.01 \pm 0.01$). These observations are consistent with leakage from liposomes being due to rupture of the lipid bilayer controlled by the weak interactions between lipids. Membrane protein is necessary for the much greater rate of leakage from erythrocytes.

The liposomes results suggest a general model for hemolysis. A membrane transition, possibly the denaturation of a membrane protein or proteins forming part of the membrane skeleton [7], results in a loss of stabilization. Since liposomes of 1–2 μm maximum diameter are formed during reconstitution, this is probably the maximum stable size in the absence of a supporting membrane protein skeleton. This conclusion rests on similar shear forces occurring during liposome formation and hemolysis. Thus, destabilization of the membrane skeleton leads to rupture of the erythrocyte membrane. This will occur spontaneously if the destabilization of the membrane skeleton is such that the large diameter of the erythrocyte can no longer be maintained. Alternatively, the possibility that denatured protein may stimulate lysis should be considered. For example, denatured protein is amphipathic, due to exposure of hydrophobic amino acids, and thus should have detergent-like properties which might actively destabilize the membrane bilayer. This general model of rupture due to destabilization of the membrane skeleton is supported by the observation that a much higher temperature is needed for lysis of erythrocytes in microcapillaries, which presumably reduce the shear flow [6] necessary for rupture of the destabilized erythrocyte.

The question remains as to the identity of the critical target. Differential scanning calorimetry (DSC) scans were obtained of the erythrocytes in the same solution used for hemolysis. The major transition is due to the denaturation of hemoglobin ($T_m = 72.5$°C) with three transitions (A–C) present at lower temperature. The hemoglobin transition was fit from 66 to 74°C and the resulting curve extrapolated to below 66°C and subtracted from the original scan to give a profile of transitions A to C. This profile is similar to that obtained from isolated membrane ghosts, although the relative intensities and transition temperature differ somewhat. Transition B is much stronger in intact erythrocytes, either because denaturation of some cytoplasmic proteins occurs at this temperature or some weakly attached membrane proteins are lost during isolation. Isolation may also disrupt membrane structure, altering the transition temperatures of the protein components. In addition, the transition temperatures are sensitive to salt concentration. After isolation both sides of the membrane ghosts are exposed to isotonic NaCl, which is quite different than the normal, high KCl, intracellular solution.

The DSC profile for inactivation of the critical target was predicted. The transition representing inactivation is assumed to be detectable on a DSC scan; although, it is not possible to predict the calorimetric enthalpy (i.e. intensity) of the transition. A transition temperature of 60°C is predicted for a scan rate of 1 K/min, which is higher than, but includes the overall range of, the B transition. This eliminates the major denaturation of spectrin ($T_m = 50$°C) as the critical target, which is apparent from a casual inspection of Figs. 1 and 7A. Approximately half of the spectrin denatures on scanning to 50°C at 1 K/min and nearly all the remainder denatures in a short period of time (less than 5 to 10 min) at 50°C. At 50°C, D_q is greater than 150 min, and even on the exponential portion of the curve less than 10% hemolysis occurs in 10 min. Thus, the rate of spectin denaturation is much too fast to be rate limiting.

A weak inflection, which appears to be due to an unresolved transition, is present at 60–62°C in the DSC scan of erythrocytes (Fig. 7A). The fourth derivative of this scan has a definite peak at 60.8°C, supporting the presence of an unresolved transition. This transition is not resolvable in membrane ghosts. As shown by thermal gel analysis, several proteins are involved in the B transition [13]. These include bands 2.1, 4.1, 4.2 and 3. In 310 mosM phosphate, transition B is split into transitions B_1 and B_2; band 3 is then involved in the B_2 transition and the other proteins in the B_1 transition [13]. Under these conditions, the cytoplasmic portion of band 3 denatures during the B_2 transition and the

transmembrane domain denatures during the C transition [14]. Since these transitions are dependent on salt content, pH, etc., which vary between the ghost suspension and the intracellular milieu, it is not possible to relate any of them to the B3 transition in intact erythrocytes.

Most, if not all, of the erythrocyte membrane transitions are due to protein denaturation (A-spectrin, B_2-band 3, and C-band 3). In addition, the Hb transition in intact erythrocytes is due to hemoglobin denaturation. The conformational changes in bands 2.1, 4.1 and 4.2 during the B_1 transition have not been elucidated, but the formation of disulfide crosslinks is consistent with unfolding or denaturation [13]. The term denaturation is used to refer to thermally-induced conformational changes to a more disordered state, which is difficult to define exactly since it is not possible to precisely determine the structure of the denatured state. For most denatured proteins, the denatured state is probably a distribution of partially unfolded conformations rather than a unique structure. The denaturation of spectrin at 50 °C involves a substantial loss of helical structure, as shown by circular dichroism (CD); however, considerable helical structure remains above 80 °C [12].

Thus from the characteristics of the other membrane transitions, transition B3 most likely represents protein denaturation, but this interpretation is not required to link it to the critical target. Transition B3 may be some other endothermic, structural rearrangement or conformational change involving membrane protein, e.g., the interaction of several proteins possibly induced by the unfolding on partial unfolding of one or more proteins. In addition, transition B3 could be the further unfolding of a protein partially unfolding in transition A or B. The Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum denatures through two steps, each representing the unfolding of a discrete domain [20]. CD measurements of spectrin extracted from the erythrocyte membrane appears to show a very weak transition at about 60 °C representing a further unfolding; however, this potential transition was not detected by DSC [12]. Thus, transition B3 could be due to an additional transition in spectrin, which would explain the relationship between defective spectrin and hereditary pyropoikilocytosis [10].

The decrease in the transition temperatures in the presence of ethanol is consistent with the increased rate of hemolysis in the presence of ethanol. The shifts in the transition temperatures of transitions B3 and C (4.5 and 4.0 K, respectively) correlate with the predicted shift in the transition temperature of the critical target (3.3 K). The shifts in transitions B3 and C are much greater than for any other transitions, indicating a possible coupling or similarity between these transitions. Photohemolysis of erythrocytes with eosin-isothiocyanate labelled band 3 implies that oxidative damage to this protein or another protein in close proximity greatly increases hemolysis [30]. In addition, a number of hydroxychloroaromatic compounds greatly stimulate hemolysis and decrease the transition temperature of band 3 (transition C) in ghosts [31]. These observations support the coupling of band 3 to the critical target.

Thus, upon exposure to elevated temperature a number of transitions occur in the erythrocyte membrane. Spectrin is the least thermostable major membrane protein and undergoes a major denaturation at 50 °C. This leads to destabilization of the membrane skeleton and results in sphering of the erythrocytes [12]. However, this destabilization is not sufficient for lysis. With longer exposure or higher temperatures, several other proteins (bands 2.1, 4.1 and 4.2) of the membrane skeleton are involved in an undefined transition; although, it seems likely that the denaturation of at least one of these proteins triggers this transition. Most of this transition also occurs at too low a temperature to be be rate-limiting for hemolysis. The denaturation of the cytoplasmic protein of band 3 is part of the B transition under some conditions (low salt), but it is shifted to a higher temperature of 62–63 °C (transition B_2) in isotonic phosphate [16]. Thus, it is possible that transition B_2 in membrane ghosts is the same as transition B3 in intact erythrocytes; however, this seems unlikely since transition B_2 is not present in isolated ghosts in isotonic saline (Fig. 7A). Hence, the T_m of the cytoplasmic portion of band 3 will have to be measured in intact erythrocytes to ascertain if it is identical to transition B3. An additional possibility is that transition B3 is due to a further unfolding of spectrin.

Another important point that emerges from this study is that not only is it possible to detect membrane transitions in intact erythrocytes, but the denaturation of hemoglobin can be detected and quantitated. Fitting of Hb transition (Fig. 6) gives an activation energy (E_A) and frequency factor (A) of 455 kJ/mol and 154, respectively, which are different than the values for isolated hemoglobin. Assuming that the Arrhenius relating holds from 72 to 37 °C, it is possible to predict the rate of thermal denaturation of hemoglobin in the body. A rate constant of $4.15 \cdot 10^{-11}\ s^{-1}$ is obtained, which gives approximately 0.04% denaturation of hemoglobin during the normal 120 day lifespan of the human erythrocyte. Denatured hemoglobin in aged erythrocytes, in the form of Heinz bodies, has been linked to the clustering of band 3 protein which provides the recognition site for antibodies directed against senescent cells [32,33]. Thus, denatured hemoglobin may act as the signal for removal of aged erythrocytes. For studies of this sort, hemoglobin is usually denatured by oxidation [32,34], and oxidation has been proposed as the mechanism for the formation of denatured hemoglobin in vivo [34]. However, at least a partial contribution from the thermal denaturation of hemoglobin at 37 °C cannot be dismissed.

Acknowledgements

This investigation was supported by PHS grant number CA40251 awarded by the National Cancer Institute, DHHS to J.R.L. The assistance of Miriam Heynen is gratefully acknowledged.

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