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KINETICS OF MALONAMIDE INDUCED HEMOLYSIS OF HUMAN ERYTHROCYTES, AN AUTOCATALYTIC EFFECT

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INTRODUCTION

Studies by Coldman and Good [1, 2] have advocated use of the Arrhenius and Eyring equations for interpretation of hemolysis reactions and their thermodynamic activation parameter. The authors do not, however, suggest any form for the general kinetics of the hemolysis reaction, which causes us to question the validity of their choice of a reaction rate constant. In the present study, hypotonic solutions of malonamide were used to induce hemolysis of several human whole blood samples. The observed hemolysis appears to exhibit an autocatalytic behavior in relation to the concentration of hemoglobin released. Our data can indeed be shown to fit autocatalytic kinetics equations. The resultant rate constant allows a kinetically valid usage of the Arrhenius and Eyring equations with activation parameters expressed per mole of hemoglobin released.

A recent study by DeTraglia et al. [3] has indicated that changes occur in the erythrocyte fragility distribution as a function of age of the blood donor. The present study does not indicate an aging correlation in the kinetics of hemolysis with malonamide. Variance in calculated activation energies, enthalpies and entropies suggests great variety in the behavior of individual human samples in hemolysis. A correlation between the activation energy and the Arrhenius frequency factor indicates that the variety of human hemolysis responses encountered is related through a common reaction mechanism.

EXPERIMENTAL

Hemolysis experiments were conducted by modification of the method of Good [4]. Human blood was classified by erythrocyte count, hemoglobin concentration, hematocrit and corpuscular constants. All experiments were run no later than 4 days after the fresh blood was drawn. Samples were oxygenated for 10 min immediately prior to hemolysis.

Malonamide, NaCl and NaOH were "Analyzed Reagent" grade from J. T. Baker. Solutions of malonamide were prepared as 2.2477, 2.1846, and 2.125 g/100 ml to provide the same tonicity (approx. 2/3 isotonic) at 20, 12 and 4 °C, respectively. NaCl (6.3 g/100 ml) and NaOH (0.4%) solutions were prepared in advance. Experi-

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ments, conducted at 4, 12 and 20 °C in baths thermostated to ±0.1 °C, were initiated by adding 2 ml of oxygenated blood to 40 ml of malonamide lysing solution. At given time intervals, 1.5-ml aliquots were withdrawn from the hemolyzing mixture which was gently stirred to maintain homogeneity and were quenched with 0.25 ml of NaCl solution which introduced sufficient tonicity to prevent subsequent hemolysis for several hours. Adequate sampling intervals were found to be 3, 2 and 1 min with a duration of 100, 48 and 30 min at 4, 12 and 20 °C, respectively. The aliquots were then centrifuged at $1207 \times g$ for 3 min (International Centrifuge, size 2, Model 2, 12 inch head diameter, International Equipment Co., Boston, Mass.). 1 ml of the supernatant from the centrifuged aliquots was added to 2 ml of NaOH solution to convert the hemoglobin to alkali hematin. After 15 min, spectrophotometric absorbance was read on each aliquot at 540 nm (Bausch and Lomb Spectronic-88, Rochester, N.Y.). A totally hemolyzed standard from each hemolysis was withdrawn after several hours and was treated likewise. The hemolyzed fraction for each aliquot was then calculated as:

$$H = \frac{\text{Absorbance of aliquot}}{\text{Absorbance of standard}} \tag{1}$$

KINETIC REPRESENTATION OF HEMOLYSIS

The appearance of curves representing the malonamide-induced hemolysis of human erythrocytes at 4, 12 and 20 °C suggested a time dependence similar to that of a general autocatalytic reaction. In this type of reaction, the rate is dependent upon the concentration of the reacting species, which decreases with time, and upon the concentration of a product which catalyzes the reaction, and whose concentration increases in proportion to the decrease in the reacting species. In hemolysis experiments with human erythrocytes, intracellular hemoglobin can be treated as a reacting species. After hemolysis, extracellular hemoglobin appears as the observed product of the reaction, and is measured spectrophotometrically. The limiting maximum concentration of extracellular hemoglobin is reached at the time of complete hemolysis. The concentration of extracellular hemoglobin at complete hemolysis is the quantity measured as a standard for construction of hemolysis curves. The molar concentration of hemoglobin at complete hemolysis may be calculated from the clinically measured hemoglobin concentration (in g/100 ml), the molecular weight of hemoglobin (68 000 g/mole), and the known blood dilution used in the hemolysis experiments. Thus:

$$\mathrm{Hb}_\infty = \mathrm{Hb}\left(\frac{\mathrm{g}}{\mathrm{dl}}\right) \cdot \frac{1}{68\,000}\left(\frac{\mathrm{moles}}{\mathrm{g}}\right) \cdot \frac{10}{1}\left(\frac{\mathrm{dl}}{\mathrm{l}}\right) \cdot D \tag{2}$$

where Hb is the clinically determined hemoglobin concentration, D is the dilution factor (2 ml blood/42 ml solution, for our studies), and Hb_∞ is the limiting molar extracellular hemoglobin concentration in the reaction vessel.

Consistent with this form, the fractional hemolysis (H) at any time is:

$$H = \frac{\mathrm{Hb}_t}{\mathrm{Hb}_\infty} \tag{3}$$

where Hb_t is the extracellular hemoglobin concentration at time/2.

We may now write the differential form for the equation of autocatalysis as:

$$\frac{dHb_t}{dt} = k(Hb_\infty - Hb_t)(Hb_t) \tag{4}$$

in which we have expressed the rate of disappearance of intracellular hemoglobin in terms of the rate of appearance of extracellular hemoglobin. The remaining intracellular hemoglobin at any time is given by $Hb_\infty - Hb_t$, and the extracellular hemoglobin concentration hemoglobin seems to participate as a catalyst. The kinetic rate constant is k. Eqn 4 may be integrated by partial fractions and the integration constant evaluated at the half-life (t_{50}) of the reaction ($Hb_{t_{50}} = 1/2\ Hb_\infty$). The resulting equation is

$$H = \frac{Hb_t}{Hb_\infty} = \frac{1}{1+e^{-kHb_\infty(t-t_{50})}} \tag{5}$$

and expresses the time dependence of hemolysis as a function of the autocatalytic rate constant (k), the half-life (t_{50}), and the known limiting hemoglobin concentration (Hb_∞). Eqn 5 may be fit to experimental hemolysis data through its linear form:

$$\ln\left(\frac{1-H}{H}\right) = kHb_\infty(t-t_{50}) \tag{6}$$

to determine the rate constant and half-life explicitly (DeTraglia, M. C., Cook, F. B., Stasiw, D. M. and Cerny, L. C., unpublished). A typical fit is shown in Fig. 1.

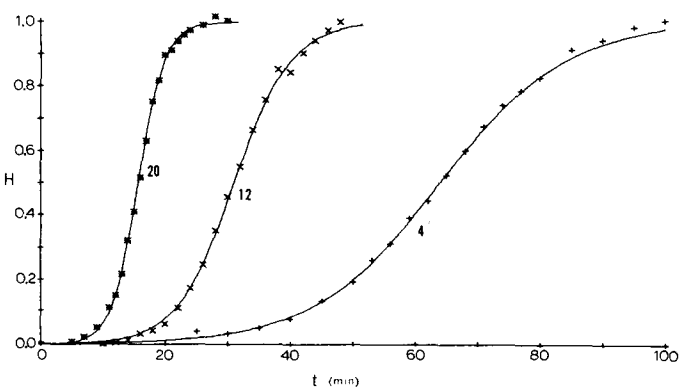

Fig. 1. Typical fit of hypotonic (2/3 isotonic) malonamide-induced hemolysis data to Eqn 5. The donor is a 29-year-old male with clinical blood hemoglobin concentration of 15.7 g/100 ml ($Hb_\infty = 1.099 \cdot 10^{-3}$ moles/l).

THERMODYNAMIC ACTIVATION PARAMETERS

Previous work by Coldman and Good [1, 2] suggested analysis of hemolysis kinetics data via the Arrhenius and Eyring equations. A serious shortcoming of their work was an inability to express the hemolysis rate constant in a form consistent with accepted kinetics evaluations. The approach presented here, however, yields k

TABLE I

Blood morphology					Temperature-dependent parameters				Temperature-independent parameters			
Age (years)	Sex	RBC* (10_6/mm^3)	Hb** (g/100 ml)	HCT*** (%)	T (°C)	k (l · mole^{-1} · min^{-1})	t_{50} (min)	$\Delta G^{\ddagger\dagger}$ (kcal/mole)††	E_a† (kcal/mole)	ln A	$\Delta H^{\ddagger\dagger}$ (kcal/mole)††	$\Delta S^{\ddagger\dagger}$ (cal/mole per °K)
Young												
17	F	4.9	14.0	41.0	4	541	51.8	14.95	17.87	34.66	17.3	8.43
					12	1386	24.6	14.9				
					20	3175	12.6	14.85				
20	F	4.7	14.7	44.0	4	768	51.6	14.8	14.91	29.62	14.35	−1.57
					12	1670	23.9	14.8				
					20	3363	12.0	14.8				
29	M	5.7	15.7	47.5	4	903	63.9	14.7	15.61	31.05	15.05	1.27
					12	2003	31.0	14.7				
					20	4241	15.7	14.65				
33	M	4.4	14.5	41.5	4	546	120.9	14.9	17.44	33.92	16.85	6.97
					12	1529	52.7	14.9				
					20	3065	25.0	14.85				
Old												
77	M	5.5	15.0	58.0	4	553	56.0	14.95	14.76	29.03	14.2	−2.75
					12	1227	23.8	14.95				
					20	3175	13.9	15.0				

81	M	4.9	15.3	45.5	4	935	47.5	14.6	14.94	29.92	14.35	−0.98
					12	2328	20.8	14.65				
					20	4099	12.0	14.65				
82	M	4.4	13.5	44.0	4	864	68.3	14.7	15.48	31.83	14.5	2.81
					12	2074	31.3	14.7				
					20	4233	17.1	14.65				
82	M	4.7	13.5	43.0	4	738	74.8	14.8	16.78	32.98	16.2	5.11
					12	1727	36.1	14.75				
					20	3896	17.0	14.7				
89	M	4.2	13.2	39.5	4	793	77.8	14.8	15.82	31.27	15.25	1.70
					12	1588	35.5	14.75				
					20	3809	18.3	14.75				
90	F	4.4	13.8	41.0	4	1090	52.4	14.6	16.76	33.25	16.2	5.64
					12	2044	26.5	14.6				
					20	5763	12.1	14.55				
ST†††												
12	F	4.7	13.3	41.0	4	582	69.5	14.95	15.62	30.62	15.05	0.40
					12	1234	34.0	14.95				
					20	2737	16.5	14.95				

* RBC, red blood cell count.
** Hb, hemoglobin (clinical determination).
*** HCT, hematocrit.
† All thermodynamic values are per mole of hemoglobin released.
†† Rounded to the nearest 50 cal/mole.
††† ST, sickle trait blood.

in $[Hb]^{-1} \cdot min^{-1}$, which are the expected units of a second-order rate constant.

The Arrhenius equation expresses the temperature dependence of reaction rate as

$$k = Ae^{-E_a/RT} \tag{7}$$

where k is the rate constant, A is the Arrhenius frequency factor, E_a is the reaction activation energy, R is the gas constant (1.9872 cal/mole per °K), and T is the absolute reaction temperature. In linear form,

$$\ln k = \frac{-E_a}{R}\left(\frac{1}{T}\right) + \ln A, \tag{8}$$

the Arrhenius equation was analyzed by least squares ($\ln k$ vs $1/T$) at 4, 12 and 20 °C to determine E_a and $\ln A$ for each blood sample. The activation enthalpy, ΔH^* and activation entropy, ΔS^*, were calculated for each sample by the Eyring absolute rate equation:

$$k = \frac{\boldsymbol{k}T}{h} e^{-\Delta H^*/RT} e^{\Delta S^*/R} \tag{9}$$

where $\boldsymbol{k}$ and h are Boltzman's and Planck's constant, respectively. Eqn 9 facilitates calculation of ΔH^* and ΔS^* by least squares analysis of the linear form:

$$\ln\left(\frac{k}{T}\right) = \frac{-\Delta H^*}{R}\left(\frac{1}{T}\right) + \left(\ln\frac{\boldsymbol{k}}{h} + \frac{\Delta S^*}{R}\right) \tag{10}$$

with (X, Y) pairs corresponding to $1/T$ and $\ln(k/T)$, respectively. After having determined ΔH^* and ΔS^*, the free energy of activation, ΔG^* was determined at each temperature from the relation:

$$\Delta G^* = \Delta H^* - T\Delta S^* \tag{11}$$

A data summary of kinetics constants from Eqn 6 and thermodynamics activation parameters from Eqns 8, 10 and 11 for malonamide-induced hemolysis of 11 human whole blood samples is presented in Table I.

DISCUSSION

The concept of autocatalysis in hemolysis may seem quite unrealistic to some investigators. The fact that the observed process does adhere to autocatalytic kinetics leads us to the following explanation of the rate controlling factor.

When a suspension of erythrocytes is rapidly mixed with a hypotonic concentration of malonamide, water enters the cells to relieve the initial osmotic imbalance. This process is relatively fast and induces prehemolytic swelling. The slow (observed) swelling process ensues as both malonamide and water enter the cells due to the activity gradient of the extracellular permeant solute (malonamide). The extracellular malonamide concentration ($[M]$) approaches a minimum as does its activity gradient just prior to the first cell's lysis. Thereafter, $[M]$ increases as cells hemolyze, releasing their intracellular hemoglobin and the malonamide which has permeated them. Thus $[M]$ will approach its initial value upon completion of all cells' lysis. We can then visualize a prehemolytic phase which is characterized by no hemolysis and $[M]$

approaching a minimum; this phase would not, presumably, adhere to autocatalytic kinetics. The observed hemolytic phase, however, is characterized by $[M]$ approaching a maximum and extracellular hemoglobin concentration approaching Hb_∞; thus we see the permeant solute increasing as more cells hemolyze and driving all subsequent hemolysis with an increased activity gradient (see Fig.2). We may rewrite the differential Eqn 4 as

$$\frac{dHb_t}{dt} = k(Hb_\infty - Hb_t)(Hb_t) = k'(Hb_\infty - Hb_t)[M] \tag{13}$$

wherein participation by $[M]$ is expressed as a function of Hb_t, that function being incorporated into the rate constant (k) of the observable "autocatalytic" form.

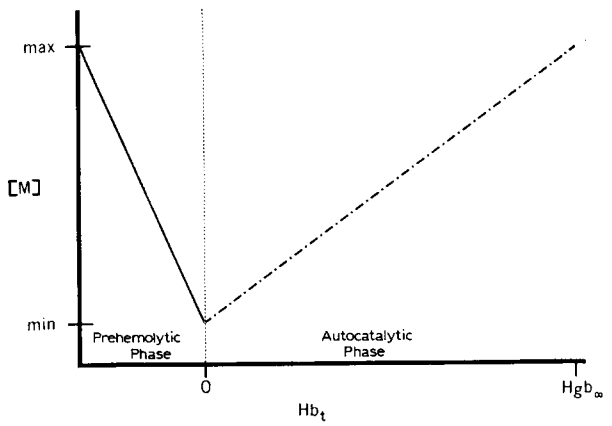

Fig. 2. Schematic representation of the change in extracellular malonamide concentration $[M]$ as a function of extracellular hemoglobin concentration Hb_t. The autocatalytic effect is encountered after $[M]$ has reached a minimum, at the onset of visible hemolysis.

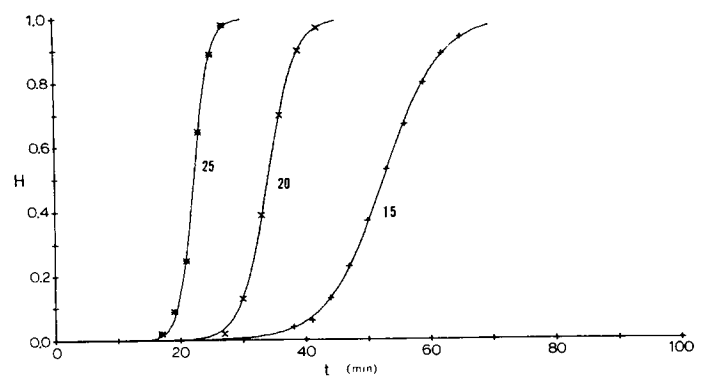

Fig. 3. Hemolysis curves for guinea pig erythrocytes constructed from published data by Good [4]. Explicit determination of rat constants was not possible, lacking a value for Hb_∞ in the reference. Values for kHb_∞ (min^{-1}) and t_{50}(min) are 0.2191, 52.57; 0.4530, 34.12; and 0.7973, 22.33 at 15, 20 and 25 °C, respectively.

Accepted methods for analysis of hemolysis data involve treating the median portion of hemolysis vs time curves as a straight line, the slope of which is used as a rate constant for the hemolysis reaction [4]. The present interpretation utilizing a computed exponential fit (Eqns 5 and 6) provides a more comprehensive analysis of the rate process witnessed in hypotonic lysis. Published values by Good [4] for hemolysis of guinea pig erythrocytes at 15, 20 and 25 °C analyzed by Eqn 6 (see Fig. 3) yielded an acceptable fit, suggesting that the present technique may lend itself well to studies involving species other than humans, and at temperatures not investigated here.

Results indicate that a logarithmic linear correlation exists between the activation energy, E_a, and the frequency factor A for the human samples studied. Plotting E_a versus ln A, in the form

$$\ln A = \frac{E_a}{R\beta_0} + \ln A_0 \tag{14}$$

derived from the Arrhenius equation, allows the determination of β_0, the isokinetic temperature of the reaction family, and A_0 the intrinsic rate of the reaction. The isokinetic temperature for the samples in Table I was determined to be 297 °K (24 °C), and the intrinsic rate was found to be 5453 $l \cdot mole^{-1} \cdot s^{-1}$. Theoretically, at this temperature, malonamide-induced hemolysis of all erythrocytes in this group occurs at the same rate. The fact that a linear relation between E_a and ln A was established (Fig. 4) leads us to question the validity of reporting species averaged E_a values and ln A values independent of individual donors' contribution [5] (at least in considering the variety of human samples presented here).

Investigation by Coldman and Good [2] has suggested that the hemolysis of erythrocytes is controlled by the hydration structure of the membrane. The process of hemolysis then requires the free passage of unbounded water molecules through the membrane. The thermodynamic activation process would then involve freeing a

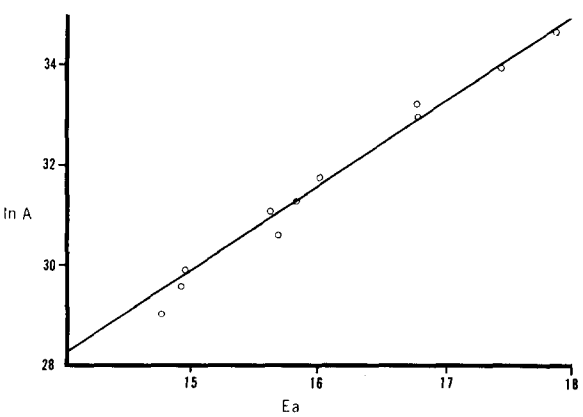

Fig. 4. Linear correlation between E_a (activation energy in kcal/mole of hemoglobin released) and ln A (frequency factor) from the Arrhenius equation (Eqn 7). The correlation indicates that activation of hemolysis for human erythrocytes may vary appreciably with the individual, all reactions belonging to the same characteristic family.

water molecule from its neighboring hydration lattice before it may permeate the membrane. This process predicts a positive $\Delta S^{\ddagger}$ for water molecules which break from their hydrated structures in the activated state. It follows then that a more positive $\Delta S^{\ddagger}$ may be expected for more extensively hydrated membrane structures. Results of this investigation indicate that $\Delta S^{\ddagger}$ varies both in sign and in magnitude for the human samples studied. The magnitude of $\Delta S^{\ddagger}$ in all cases is quite small as compared to either $\Delta H^{\ddagger}$ or $\Delta G^{\ddagger}$, the free energy of activation remaining nearly constant at all temperatures. If Coldman and Good's hypothesis is valid in the present examination of hemolysis thermodynamics, our results are suggestive of great variety in the membrane hydration structure of humans.

Recent work has shown that whole blood from old people exhibits higher fragility and a broader distribution of cell types than blood from young people [3]. The possibility of an age related change in the kinetic or thermodynamic activity of whole blood in malonamide-induced hemolysis was evaluated in this study. With respect to blood morphology and donor age, it does not appear at this time that an aging correlation can be detected.

ACKNOWLEDGEMENT

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