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THE HAEMOLYSIS OF HUMAN ERYTHROCYTES IN RELATION TO THE LATTICE STRUCTURE OF WATER*

I. DELAYED HAEMOLYSIS IN HYPOTONIC MALONAMIDE SOLUTIONS

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SUMMARY

The haemolysis of human erythrocytes in hypotonic aqueous malonamide has been examined in relation to the quasi-crystalline properties of liquid water. It is suggested that the controlling factor in the mechanism of this process is the order promoting effect of malonamide on the water lattice; this suggestion is supported by a study of the kinetics of malonamide induced haemolysis.

INTRODUCTION

During an investigation of the permeability of human erythrocytes to organic nitrogenous substances, in which the haemolysis technique was employed, a delayed action effect was observed with hypotonic solutions of malonamide.

A similar effect occurs with hypotonic solutions of glucose and this has been described by HENDRY[1] as delayed haemolysis.

The results of an investigation of delayed haemolysis in hypotonic malonamide solutions are described.

MATERIALS AND METHODS

Preparation of the blood

The experiments reported here were carried out on fresh, normal human blood. Immediately after its withdrawal by venepuncture, a 20-ml sample of blood was

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discharged from the syringe into a clean, dry test tube containing 2 mg heparin powder; the tube was stoppered and the contents thoroughly mixed by repeated gentle inversion. After mixing, the blood was transferred to a tonometer and agitated gently for at least 20 min, at room temperature and pressure, in an atmosphere of moist oxygen. The blood received no treatment other than oxygenation and remained in the tonometer until required.

Preparation of malonamide solutions

The malonamide was purified by boiling with active charcoal and recrystallising twice from acetone. The purified material was dried *in vacuo* and stored over conc. sulphuric acid. Fresh solutions were prepared daily.

Since the erythrocyte is highly osmosensitive the potential osmotic pressure of the suspending medium is a major factor in this work. Accordingly osmotic pressures were derived from freezing point depression data by the equation[2]

$$\pi = -\frac{LT}{V_1 T_0^2} \Delta T$$

L denotes the molar latent heat of fusion of the solvent, $\overline{V}_1$ is the partial molar volume, and is, for dilute solutions, equivalent to V′, the molar volume of the solvent; T is the absolute temperature of the solution, T_0 the freezing point of the pure solvent and ΔT the freezing point depression due to added solute.

The equation simplifies to,

$$\pi_t = (-12.02)\frac{273 + t}{273}(-\Delta t)$$

where π_t is the osmotic pressure, in atmospheres at t^0, of a dilute aqueous solution having the freezing point depression Δt.

TABLE I

OSMOTIC PRESSURES OF MALONAMIDE SOLUTIONS

Osmotic pressure (Atm)	*Temperature °C*	*Malonamide (g/100 ml)*
3.5	10	1.539
	20	1.489
	30	1.439
5.0	10	2.196
	20	2.125
	30	2.056
6.5	10	2.855
	20	2.761
	30	2.670

The osmotic pressure–solute concentration–temperature relationships for some of the malonamide solutions used are illustrated in Table I.

The use of this equation is advocated because it provides a common denominator for expressing an important physical property of solutions, irrespective of the solute species.

The haemolysing system

A standard dilution of 1 part by volume whole blood to 20 parts by volume solu-

tion was used throughout this work. With normal blood therefore, of packed cell volume 45 %, the plasma is diluted 1:38 and the osmotic contribution of the plasma solutes is about 0.2 atm.; the effect of temperature change on this value is small enough to be neglected. It should be remembered, however, that although the osmotic pressure of the solution is quoted in the following experiments, the osmotic pressure of the haemolysing system is greater by 0.2 atm.

In aqueous solution malonamide has a weakly acid reaction and this, together with dissolved carbon dioxide, gives a pH range of 4.0–5.5 at the concentrations used. The buffering power of the plasma is sufficient to bring the pH of the system up to the alkaline side of neutrality. In the experiments reported here the pH was checked at intervals and found to be within the range 7.2–7.7 units.

Experimental techniques

At the beginning of each experiment the malonamide solution, contained in a large test tube with ground glass stopper, was placed in a thermostatic water bath to attain working temperature. Fully oxygenated whole blood in the required proportion, was then added by pipette; the system was thoroughly mixed and the tube returned to the water bath.

At suitable intervals of time, 3-ml sampleswere with drawn and the intact cells centrifuged down at 4000 rev./min for 30 sec in an MSE Minor centrifuge. After centrifugation a 2.0-ml sample of the supernatant was withdrawn by pipette and its haemoglobin content measured by the alkaline haematin method of CLEGG AND KING[4]; colour intensity was measured in the Spekker photoelectric absorptiometer, using a 1.00-cm cell and No. 604 spectrum green filters. At the end of the experiment an uncentrifuged 2.0-ml sample was treated in the same way, giving a value for complete haemolysis; the degree of haemolysis in each of the partially haemolysed samples was expressed as a percentage of this figure. The rate curves were obtained by plotting percent haemolysis to a base of time.

The experimental rate was taken as the average slope of the haemolysis curve between 25 % and 75 % haemolysis. The time factor is very important, so a stop clock was employed and a standard procedure for the withdrawal and centrifugation of samples was established and adhered to throughout the work.

For the determination of rates in rapidly haemolysing systems the following procedure was adopted. Using a 1.00-ml graduated pipette. 0.15 ml oxygenated whole blood was added to 3.0 ml distilled water in a small test tube carried in a rack in the thermostatic water bath; the instant the addition of blood was complete, the tube was removed from the bath, shaken twice and returned. The end point, complete haemolysis, was obtained by periodically removing the tube from the water bath and holding it against a printed page in strong light; at 100 % haemolysis, when the system becomes quite clear, the watch was stopped. The rate was expressed as percent haemolysis per second. This method, of course, can only be employed when haemolysis is rapid and complete.

RESULTS

Haemolysis in hypotonic malonamide solutions at constant temperature

When fully oxygenated whole blood is diluted in the ratio 1:21 with slightly hypotonic malonamide solution (6.5 atm. at 20°) 55–60 min elapse before the ery-

throcytes are completely haemolysed. Of this period there is a zero haemolysis lag phase lasting 15 min, after which haemolysis begins and accelerates into the main lytic phase of about 20 min duration; the rate then decreases as the system approaches complete haemolysis. The graph of the process (Fig. 1) is a sigmoid curve.

In a solution of 5.0 atm. at 20° the lag phase is reduced to 10 min; the main lytic phase occupies about 10 min and haemolysis is complete in 35 min. At 3.5 atm the lag phase is reduced to around 5 min and the process is complete in 21 min. When the osmotic pressure of the solution is 2.0 atm there is no lag phase, about 50 % haemolysis occurs within 1 min of adding the blood and the remaining cells are completely haemolysed within 5 min.

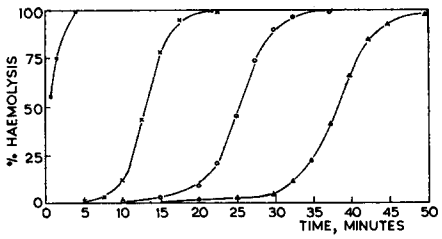

Fig. 1. The haemolysis of human erythrocytes in hypotonic malonamide solutions at 20°. ●—●, 2.0 atm.; ×—×, 3.5 atm.; ○—○, 5.0 atm.; ▲—▲, 6.5 atm.

Immediately after mixing blood and solution, in the range 3.5–6.5 atm, the system is opaque to the naked eye and has the characteristic sheen of a discoid cell suspension. On standing the cells swell, the system becomes translucent and soon afterwards a trace of haemolysis is detectable.

All existing evidence shows that osmotic swelling, which is governed by the relative intra- and extracellular osmotic pressures, is complete within a matter of seconds; it is the result of the movement of water, to which the membrane offers no apparent resistance. This accounts for the initial 50 % haemolysis observed in the system of lowest osmotic pressure; it accounts also for the decrease in the duration of the lag phase with decreasing osmotic pressure, since the lower the osmotic pressure of the solution, the greater the initial rapid swelling.

The occurrence of delayed haemolysis in aqueous malonamide, irrespective of the degree of hypotonicity, shows that swelling continues beyond the point necessary for the restoration of simple osmotic equilibrium. This continued swelling is due to displacement of the rapidly established osmotic equilibrium by the penetration of malonamide, which diffuses into the cell in the direction of its concentration gradient. The increase in intracellular malonamide necessitates a corresponding increase in intracellular water to maintain osmotic equilibrium, the excess water causes further swelling and the process continues until haemolysis occurs.

In the main lytic phase the rate of haemolysis increases as the osmotic pressure decreases. The reason for this is not immediately obvious since the cells become prolytic spheres, irrespective of the tonicity of the medium, before this stage of haemolysis is reached. Haemolysis is the result of stresses imposed by the accumulation of excess water within the cell and this accumulation of excess water is induced by the penetration of malonamide; the rate of haemolysis therefore will depend on the conditions which control one or other, or both of these factors. The experiments

indicate that the volume of water required to haemolyse the cell is less in more dilute solutions and supports the observations of PONDER AND ROBINSON[5], who have shown that the haemolytic or critical volume of erythrocytes depends on the composition of the suspending medium.

The sigmoid shape of the haemolysis curve is interpreted as being due to the statistical nature of blood samples. Any sample of normal whole blood contains a proportion of effete cells, which are, for various reasons, more fragile than the remainder of the cell population; it also contains a proportion of more robust cells, those for example which have newly become erythrocytes. The majority of the cells in the sample have characteristics which are intermediate between the two extremes. That part of the curve between 25 % and 75 % may therefore be regarded as the interquartile range of the cumulative frequency distribution of red cell fragility.

The effect of adding solid solute during the main lytic phase

The effect is shown in Fig. 2 of allowing haemolysis to proceed for 10 min at 3.5 atm and 20° and then adding sufficient solid malonamide to raise the osmotic pressure of the solution to 6.5 atm. Immediately following the addition of solid malonamide haemolysis ceases abruptly, the system becomes opaque to the naked eye and there is a considerable lag before haemolysis begins again.

The same pattern of events occurs when that quantity of solid malonamide required to raise the osmotic pressure of the solution to 5.0 atm is added.

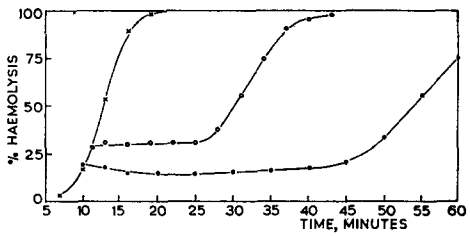

Fig. 2. The effect of adding solid malonamide during the main lytic phase. ×—×, 3.5 atm; ●—●, solid malonamide added 10 min after addition of blood. Final osmotic pressure 6.5 atm.; ○—○, solid malonamide added 11 min after addition of blood. Final osmotic pressure 5.0 atm.

The addition of solid malonamide stops haemolysis abruptly and causes immediate rapid shrinking of the swollen erythrocytes. The prolytic sphere therefore is relatively impermeable to malonamide, for, if this were not so, the addition of solid material—all of which does not dissolve instantaneously—should reduce the rate of haemolysis rather than cause reversal of the process. In terms of classical osmosis the mechanism may be described as follows.

As the added solute goes into solution the extracellular osmotic pressure increases; the resulting osmotic imbalance is corrected by an increase in intercellular solute concentration which is brought about by a reduction in cell water. As the intracellular water leaves, the cells shrink and continue to do so very rapidly until osmotic equilibrium is restored. From this point the slow penetration of malonamide induces swelling once more and the events are repeated until haemolysis recurs.

From these experiments it is evident that the human erythrocyte is permeable to malonamide, but the rate of penetration of this solute is so low relative to water, that it has the property of inducing osmosis.

The effect of temperature change on malonamide induced haemolysis

The effect of temperature change at constant osmotic pressure has been examined and representative experiments are shown graphically in Figs. 3, 4 and 5.

From these results it may be concluded that, at constant osmotic pressure, the rate of haemolysis increases with increasing temperature and, that the effect of temperature change becomes greater as the osmotic pressure of the malonamide solution is increased.

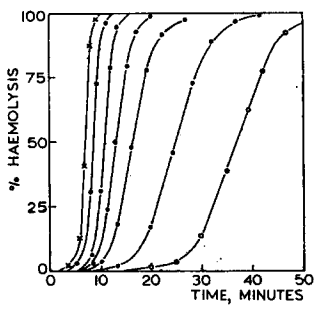

Fig. 3.

Fig. 3. The effect of temperature change at constant osmotic pressure, 3.5 atm. Temperature decreasing from left to right in steps of 5°. ×—×, 3.5 atm. at 35°; O—O, 3.5 atm. at 5°.

Fig. 4. The effect of temperature change at constant osmotic pressure, 5.0 atm. Temperature decreasing from left to right in steps of 5°. ×—×, 5.0 atm. at 35°; O—O, 5.0 atm. at 5°.

Fig. 5. The effect of temperature change at constant osmotic pressure, 6.5 atm. Temperature decreasing from left to right in steps of 5°. ×—×, 6.5 atm. at 35°; O—O, 6.5 atm. at 5°.

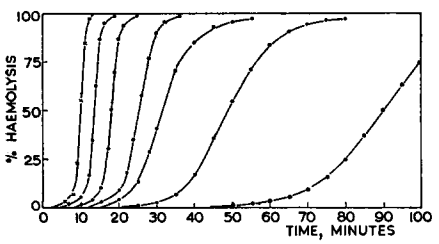

Fig. 4.

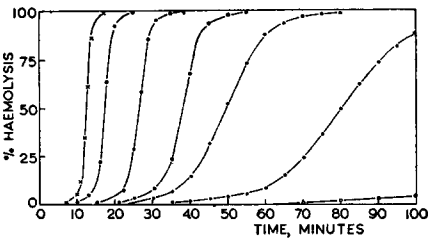

Fig. 5.

The effect of temperature change on the rate of haemolysis at constant osmotic pressure suggested that the data might fit the Arrhenius equation. Although this equation has its origin in the field of chemical kinetics, the exponential law which it expresses describes the effect of temperature change on many rate processes in both physical and chemical systems. In logarithmic form the equation is

$$\log V = \log A - \frac{E}{2.303\,RT}$$

where V is the rate of the process, in appropriate units, at the absolute temperature T and R is the universal gas constant; A is a constant and E is the energy of activation of the process.

For the interpretation of these experiments the rate of haemolysis, V, is defined as the average slope of the haemolysis curve, between 25 % and 75 % haemolysis.

The Arrhenius plot of the data obtained from two series of experiments carried out at different times with blood from the same donor is shown in Fig. 6. From this graph, of slope — 2855, E was found to be 12,970 cal and $\log A$ had the value 10.80.

In order to reduce fortuitous error and to allow also for inherent biological

variation, the data from a number of similar experiments were subjected to statistical analysis. The parameters of the regression equation

$$\log V = -m\,(1/T) + C$$

were found by the method of least squares; the standard error of estimate and the correlation coefficient were calculated, and the level of significance was assessed by Student's "t" Test, as shown in Table II.

TABLE II

STATISTICAL ANALYSIS OF EXPERIMENTAL DATA

Osmotic pressure (Atm)	*Number of observations*	*Parameters of regression equation*		*Correlation coefficient (—)*	*Standard error of estimate*	*Student's t test*
		−m	*c*			
3.5	41	2815	10.7361	0.978	0.0515	29.21
5.0	22	3482	12.8896	0.981	0.0560	22.66
6.5	14	4236	15.3702	0.965	0.0937	12.70

In each of the three sets of results there is a high degree of negative correlation which is also very highly significant since, at the 0.1 % probability level, the values of "t" for 40, 22 and 14 degrees of freedom are 3.55, 3.79 and 4.14 respectively.

Values for E, the energy of activation, were calculated from the slopes of the corresponding regression equations and $\log A$ was evaluated from the respective intercepts.

Because of partial haemolysis it was not possible to measure the rate of the process at osmotic pressures much below 3.5 atm. However, by the simple technique described, rate data at different temperatures were obtained for the haemolysis of erythrocytes at a 1:21 dilution of whole blood with distilled water. The energy of activation and $\log A$ were evaluated as before and found to be 5,100 cal and 4.60 respectively. The information is summarized in Table III.

TABLE III

EXPERIMENTAL VALUES OF E AND LOG A

Osmotic pressure (Atm)	*Energy of activation (cal)*	*Log A*
0	5,100	4.60
3.5	12,830	10.74
5.0	15,880	12.90
6.5	19,300	15.37

Seeking a correlation between the energy of activation of the process and the osmotic pressure of the haemolysing solution, a graph was drawn, as shown in Fig. 7.

The linear relationship derived from this graph may be expressed

$$E = 2{,}180\,\pi + 5{,}100$$

The value of 5,100 cal for the intercept is of considerable interest since the activation energy for the self-diffusion of water is near this value, being quoted by MOELWYN-HUGHES[6] as 4,400 cal and by ORR AND BUTLER[7] as 5,300 cal.

There is also a linear relationship between $\log A$ and the osmotic pressure of the haemolysing system,

$$\log A = 1.7\pi + 4.60$$

Thus, if the osmotic pressure in atmospheres, between 0° and 35°, of a hypotonic aqueous solution of malonamide is known, the activation energy and $\log A$ may be derived from the two equations given above; by substituting the derived values in the Arrhenius equation, the rate of haemolysis may be calculated for any temperature within the range quoted. A comparison between observed and calculated rates of haemolysis is illustrated in Table IV.

Considering the number of variables involved, the agreement is fair. By applying the Arrhenius equation therefore, a considerable body of experimental data has been summarised in convenient form.

Since E and $\log A$ are each linearly related to the osmotic pressure of the solution, it might be expected that they should have a linear relationship with each other; that this is so is shown in Fig. 8.

TABLE IV

COMPARISON OF OBSERVED AND CALCULATED VALUES FOR THE RATE OF HAEMOLYSIS

Osmotic pressure (Atm)	*Temperature °C*	*Rate of haemolysis*	
		(obs.)	*(calc.)*
0	0	2.7 %/sec	3.3 %/sec
0	35	9.1 %/sec	10.3 %/sec
0	20	6.3 %/sec	6.3 %/sec
2.3	20	9.1 %/min	7.9 %/min
2.4	30	16.7 %/min	14.1 %/min
3.5	10	5.6 %/min	5.6 %/min
3.5	30	31.2 %/min	24.9 %/min
3.5	35	40.7 %/min	43.5 %/min
5.0	30	28.2 %/min	37.3 %/min
6.5	30	20.0 %/min	28.8 %/min

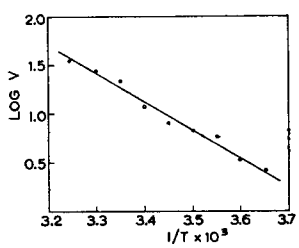

Fig. 6.

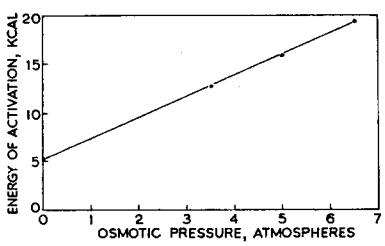

Fig. 7.

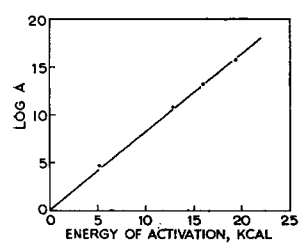

Fig. 8.

Fig. 6. Graph of log V on $1/T$ for blood from a single donor. –m, 2855; E, 12,970 cal.; log A, 10.80. ●—●, Expt. 1; ○—○, Expt. 2.

Fig. 7. The relationship between the energy of activation and the osmotic pressure of the solution.

Fig. 8. The relationship between log A and the energy of activation.

The interesting feature of this graph is that the equation of the line passes through the origin. The interpretation of this relationship involves the consideration of other factors which are better discussed in a later section.

DISCUSSION

The structure of water

The experiments have been described in terms of classical osmosis and although some conclusions are possible, the mechanism remains obscure. It would therefore seem pertinent to reconsider the facts in the light of modern theories of the structure of water.

It has been appreciated for many years that the unique properties of water depend largely on the association of its molecules. BERNAL AND FOWLER[8], however, were first to postulate that an ice-like, orderly arrangement of the molecules persisted in the liquid state; although later work has shown the need for detail modifications, their general picture of extensive tetrahedral co-ordination remains valid.

It is not within the scope of this paper to review the work of the many individuals who have contributed to the understanding of water structure, but mention must be made of FORSLIND's water model[9], and his discussion of the problem.

From a study of the wave mechanical aspects of the water molecule FORSLIND has developed a structural model of water which has proved most useful in interpreting the experiments reported here. This author has shown that the electronic distribution of the oxygen valence state gives rise to hybrid bond maxima which are approximately tetrahedrally directed and further, that the formation of a water molecule causes only slight modification of the oxygen hybridisation. Thus, while the water molecule is usually depicted as an oxygen atom with two hydrogens at an angle of 104.5°, there are also, in the plane perpendicular to the first, two unfilled bonds at an angle of 131.8° (see ref. 9). Since the true tetrahedral angle is 109.5°, the formation is possible of an extensive, though not ideal, tetrahedrally co-ordinated structure, which depends on the linking of oxygen atoms by hydrogen bonds; in these bonds the proton oscillates between two equilibrium positions on a line joining the oxygen nuclei, but each oxygen atom is associated only with two hydrogen atoms.

The consequences of this structure are discussed by FORSLIND and it suffices here to say that the stability of the water lattice is enhanced by attenuation of the anharmonic intermolecular vibrations of the hydrogen bond, which may be achieved by cooling, or by the formation of hydrogen bonds between the water lattice and a specific substrate surface. A specific substrate surface is defined as one in which the molecular surface groups are of such a nature and so placed that hydrogen bonding is possible without undue deformation of the water lattice; that is, the better the fit between the substrate surface groups and the basal molecules of the water lattice, the greater the stability conferred on the lattice. The stability conferred in this way may extend 300 Å and more from the surface concerned.

FORSLIND has employed this water model to account for the hydration of certain clay sols[10] and JACOBSON[11] has used the concept to explain the properties of deoxyribonucleic acid solutions. Since both authors provide reasonable explanations of these phenomena, it seems not unreasonable to suggest that, given a specific molecular structure, small molecules also will stabilise the water lattice.

Water stabilished in this way, in the vicinity of a small molecule, is not true water of hydration; it is, rather, a zone in which there are more intact hydrogen bonds than in the body of the liquid. Because of the more extensive hydrogen bonding within the stabilized zone the water molecules remain longer in their positions in the lattice and so have less freedom of movement than the molecules outside the zone.

It will be shown in the following sections that the concept of water structure, and its consequences provide a reasonable explanation of the experiments reported here.

The movement of water in blood

Before considering possible mechanisms in detail it is worth recalling that osmotic equilibrium is a dynamic state in which there is a continuous exchange of water across a semipermeable membrane. This dynamic exchange, or turnover has been demonstrated in normal blood by GOVAERTS AND LAMBRECHTS[12], who found that HDO added to whole blood is equally distributed between the cells and plasma within 1 min. There is no reason for supposing that the osmotic equilibrium attained in aqueous malonamide solutions is in any way different from this.

When whole blood is diluted with hypotonic aqueous malonamide there occurs the immediate redistribution of water which restores osmotic equilibrium and induces cell swelling. In the same way there is an immediate redistribution of water—which induces cell shrinkage—when haemolysis is arrested by adding solid solute to the haemolysing system. There is, in these two statements, the vaguely implied notion that water is drawn across the semipermeable membrane into the more concentrated solution. This is not necessarily true, however, because the effect—swelling or shrinking—would be the same if the volume of water required to maintain osmotic equilibrium was merely retained on the more concentrated side of the membrane. The experiments are re-examined below with this point in mind.

With regard to the addition of solid malonamide, the instant before the solid material is added the cells are swollen and in osmotic equilibrium with the solution; water is entering and leaving the cells at the same rate. The addition of solid malonamide to the extracellular side of the membrane could, by increasing the stability of the extracellular water lattice, reduce or even stop the flow of water into the cell. In this circumstance water would continue to leave the cell until a new dynamic osmotic equilibrium is attained. Thus the addition of solid solute to the extracellular phase causes the cells to shrink, not by withdrawing water, but by retaining in the extracellular phase that volume of water necessary to maintain the original osmotic equilibrium.

The same mechanism could account for the haemolysis of erythrocytes when blood is diluted with water. With sudden dilution of the plasma the outgoing cell water virtually ceases to flow, but the ingoing water continues and may do so at an increased rate, since the stability of the extracellular water lattice is reduced. If the dilution of the plasma is sufficiently great the combined effects of water retention and continued water uptake cause the swelling which culminates in haemolysis. The idea that the swelling or shrinking of erythrocytes depends on the structural integrity of the extracellular phase suggests that the rate of haemolysis may be correlated with the viscosity of that phase.

The energy of activation

It has been shown that a linear relationship exists between the energy of activation of the process being measured and the osmotic pressure of the haemolysing solution and that this relationship holds good to zero osmotic pressure. The explanation offered for the behaviour of erythrocytes when whole blood is diluted with water points to a connection between the observed effect and viscous flow. Viscosity is a measure of resistance to flow and decreases with increasing temperature, whereas the rate of haemolysis increases with increasing temperature; fluidity, which measures tendency to flow and increases with increasing temperature, is more closely related to the experimental observations.

It would appear then, that the energy of activation derived from observations on the effect of temperature change on water induced haemolysis refers to the fluidity of water. This possibility has been examined by determining E and $\log A$ for the fluidity of water in the temperature range 5°–35°. The graph of $\log \phi$ on $1/T$ is slightly convex to the x-axis but, for the purpose of this work, was taken as linear. The energy of activation found in this way was 4,200 cal and $\log A$ has the value 4.14; these values compare not too unfavourably with 5,100 cal and 4.60 obtained by experiment for water induced haemolysis. By extrapolating the E/π and $\log A/\pi$ graphs a further 0.2 atm, thereby allowing for the contribution of the plasma solutes to the osmotic pressure of the haemolysing system, the agreement is quite striking, for E then becomes 4,300 cal and $\log A$ has the value 4.25.

The existence of a linear relationship between the energy of activation and the osmotic pressure of the solution suggests that the process upon which E depends is essentially the same throughout the range of the experiments, namely the effect of temperature change on the fluidity of the extracellular phase.

On re-examining malonamide induced haemolysis the following picture emerges. When whole blood is added to a hypotonic malonamide solution there is considerable dilution of the plasma; the extracellular water lattice stability is reduced and so the fluidity of the extracellular phase is increased. Up to the point of addition the cells were in dynamic osmotic equilibrium with the plasma. The decrease in lattice stability of the extracellular water—but not the intracellular water—results in the whole or partial retention of water within the cell, together with an increased flow of water from the solution into the cell. Dynamic osmotic equilibrium is re-established when water is once again entering and leaving the cell at the same rate. As shown experimentally the establishment of this new equilibrium, which accounts for the initial swelling, is extremely rapid and takes place in a few seconds. Water continues to exchange between the cell and the solution until the new equilibrium is disturbed by the penetration of a molecule of malonamide. The loss of a molecule of malonamide has a negligible effect on the stability of the extracellular water lattice, but the effect of that molecule on the relatively small volume of the intracellular phase is considerable in comparison. The cell therefore, retains a proportion of its outgoing water, which is incorporated in the stablised intracellular water lattice, and osmotic equilibrium is restored by a compensating increase in the ingoing water from the solution. Each time a molecule of malonamide enters the cell a proportion of the outgoing equilibrium water is retained and osmotic balance is restored by additional water moving in from the extracellular phase. The ultimate stage in the process is haemolysis and it is evident that the rate of swelling and therefore of haemolysis is governed

mainly by the fluidity of the extracellular phase. If the extracellular phase is highly fluid, as it becomes when whole blood is diluted 1:21 with water, haemolysis is very rapid indeed. When the extracellular phase is less fluid, through stabilisation of the water lattice by solute, the rate of haemolysis is less. This accounts for the relationship between the energy of activation and the solution osmotic pressure, since the lower the energy of activation the more rapid the process.

In terms of the collision theory of reaction rates the Arrhenius activation energy refers to kinetic energy only. Later work, however, associates E with potential energy in that it is the measure of a potential energy barrier to the fulfilment of a rate process. The activation energy derived in these experiments refers to the fluidity of the extracellular phase, so E could be considered a measure of the potential energy of the water lattice, since it represents the energy which must be expended before a molecule of water becomes completely detached from its neighbours and attains the active state for flow. The fact that the activation energy increases with increasing osmotic pressure shows that, within the range of these experiments, the potential energy or stability of the water lattice increases with increasing solute concentration.

The entropy of activation

For the further evaluation of the results it is necessary to interpret the non-exponential factor of the Arrhenius equation, because E depends only on the effect of heat on the process, and the factor A covers all the remaining variables.

In a consideration of the temperature dependence of viscosity ANDRADE[13] interprets A as a measure of the rate of the process at infinite temperature. While infinite temperature as such has no meaning for real systems, the artifice does suggest that A represents the rate of the process in the absence of intermolecular forces.

The theory of absolute reaction rates has been applied to viscous flow[14], and the equation derived has, for fluidity, the form

$$\phi = \frac{h\mathrm{N}}{V} e^{\Delta S^{\ddagger}/R} e^{-\Delta H^{\ddagger}/RT}$$

where N is the Avogadro number, h is Planck's constant and V is the molar volume of the liquid; $\Delta S^{\ddagger}$ is the entropy of activation and $\Delta H^{\ddagger}$ is the heat of activation.

Since $\Delta H^{\ddagger}$ is very nearly the same as E, the experimental activation energy, when the Arrhenius equation and this one are compared, it is seen that

$$\log A = \log \frac{h\mathrm{N}}{V} + \frac{\Delta S^{\ddagger}}{2.303\,R}$$

Log A is therefore, a function of the entropy of activation of the process.

The process of activation therefore, involves an increase in the randomness of the system; this is in agreement with the supposition that liquid water has an extensive quasi-crystalline structure, for, if the unit of flow is the individual water molecule, activation for flow necessitates the breaking of hydrogen bonds and disruption of the structure. The linear relationship between log A and the osmotic pressure of the solution shows that the stability of the water lattice increases with increasing solute concentration, since the more ordered the initial structure the greater the number of bonds to be broken in order to attain the active state.

The linear relationship between $\log A$ and E confirms the interpretation already suggested. It is to be expected that lowering of the potential energy of the water lattice will loosen the structure and increase the entropy of the system; the entropy of activation would therefore decrease. At zero activation energy, which occurs at infinite temperature, the system is completely random in the initial state and so the entropy of activation is also zero.

The free energy of activation

Numerical values of $\Delta S^{\ddagger}$ were calculated from the logarithmic form of the above equation. Using the thermodynamic equation,

$$\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$$

and substituting the experimental values of E for $\Delta H^{\ddagger}$, the free energy of activation, $\Delta F^{\ddagger}$, was calculated for the different osmotic pressures employed. This information is tabulated below.

TABLE V

THE ENTROPY AND FREE ENERGY OF ACTIVATION

Osmotic pressure (Atm)	*E (cal)*	*$\Delta S^{\ddagger}$ (Cal deg^{-1}mole^{-1})*	*$\Delta F^{\ddagger}$ (cal)*
0	5,100	35.5	—5,300
3.5	12,830	63.6	—5,800
5.0	15,880	73.4	—5,630
6.5	19,300	84.8	—5,540

This table is particularly interesting for it shows that the free energy of activation is negative, relatively small and, within the limits of these experiments, virtually constant irrespective of the concentration of the solution. The small negative value of the free energy of activation accounts for the fact that, even with the comparitively high activation energies found at higher osmotic pressures, haemolysis in the main lytic phase occurs spontaneously and rapidly at room temperature. That the free energy of activation is small and negative is the result of the compensating effect of the relatively large positive entropy of activation; as already pointed out this implies the existence of an extensive orderly arrangement when the system is in the initial state, even at zero osmotic pressure. The constant value of the free energy of activation shows that the driving force in the co-ordinate of reaction, in this case fluid flow, is the same whatever the concentration of the solution; it is likely therefore that the mechanism will be the same also and that the unit of flow is the single water molecule.

As suggested at the beginning of the discussion, the concept of water as an ordered lattice structure opens up a new approach to the mechanism of haemolysis. In these experiments at least, the study of the kinetics of the process strongly supports the view that the stabilizing effect of the solute on the water lattice is the principal factor in the mechanics of malonamide induced haemolysis.

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THE CONSEQUENCES OF SYSTEMATIC ERROR IN ENZYME KINETICS

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SUMMARY

The consequences of systematic error in enzyme kinetics was considered by introducing a first order error term into the MICHAELIS–MENTEN–BRIGGS–HALDANE equation. The situations investigated were those involving over- or undercorrection of an enzyme or substrate blank reaction in the absence and presence of product inhibition and in the presence of a totally competitive, non-competitive or uncompetitive inhibitor. In addition, consideration was given to errors arising from a departure from the BEER-LAMBERT relationship and those proportional to the velocity or substrate concentration. Finally, attention has been called to the questionable validity of using weighting procedures to correct for random error in the presence of systematic error.

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