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THE KINETICS OF MALONAMIDE-INDUCED HAEMOLYSIS OF MAMMALIAN ERYTHROCYTES

II. THE EYRING ACTIVATION PARAMETERS

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SUMMARY

1. The temperature dependence of the malonamide-induced haemolysis of erythrocytes of rabbit, guinea pig, rat, cattle, sheep, dog, cat and pig has been further evaluated by an empirical application of the EYRING equation.

2. Numerical values are presented for the EYRING parameters, $\Delta H^{\ddagger}$, enthalpy of activation, $\Delta S^{\ddagger}$, entropy of activation, $\Delta G^{\ddagger}$, free energy of activation and $K^{\ddagger}$ the activation equilibrium constant.

3. From a consideration of the dependence of these parameters on the concentration of the haemolysing solution, and on their variation with animal species, it is concluded that these data fully substantiate the view that the hydration structure of the erythrocyte membrane is the predominant factor in the mechanism of haemolysis.

INTRODUCTION

In the Arrhenius equation the activation parameters are interpreted on the basis of simple collision theory, so that E is a kinetic energy term and the non-exponential factor refers to collision frequency and steric or probability states[1]. In many rate processes, however, simple collision theory is inadequate for the purpose of interpretation and a more sophisticated statistical-mechanical approach has been developed. The EYRING equation[2] is an example of one such development and in this the activation energy is a potential energy term; activation is seen as the formation of a transition state, or active complex at the top of a potential energy barrier between the initial (unreacted) and final (reacted) states. The rate of reaction then depends on the velocity at which the active complex crosses the potential energy barrier. The EYRING equation is employed in this work because it has enjoyed considerable success not only in the field of chemical reactions, but also in the study of many physical processes[3]; it would therefore seem to be particularly appropriate for an investigation of haemolysis kinetics.

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The EYRING equation is usually expressed in the form,

$$k = \frac{\boldsymbol{k}T}{\boldsymbol{h}} \exp\left(\frac{-\Delta G^{+}}{RT}\right) = \frac{\boldsymbol{k}T}{\boldsymbol{h}} \exp\left(\frac{\Delta S^{+}}{R}\right) \exp\left(\frac{-\Delta H^{+}}{RT}\right)$$

where k is the specific reaction rate, $\boldsymbol{k}$ is the Boltzmann constant, T is the absolute temperature, $\boldsymbol{h}$ is Planck's constant and R is the gas constant; ΔG^{+}, ΔS^{+} and ΔH^{+} are the free energy of activation, the entropy of activation and the enthalpy of activation, respectively. The Arrhenius parameters of activation are, of course, related to those of EYRING and for reactions in solution at constant temperature and pressure, $E = \Delta H^{+} + RT$ and $A = \boldsymbol{k}T/\boldsymbol{h}$ $(\exp \Delta S^{+}/R)$; the latter relationship brings out the point that A is a function of entropy.

The EYRING equation in this original form has already been employed—with extremely interesting results[4]—in the study of haemolysis kinetics. This usage, however, confers on the prolytic sphere, which is the terminal stage of prehaemolytic swelling, the status of an 'active complex'. In principle at least this status seems undesirable, for it is a central postulate of transition-state theory that the active complex is in equilibrium with the initial state[5] and the most careful studies of cell swelling[6] suggest that, at the stage of the prolytic sphere, swelling becomes irreversible.

In an alternative approach to the evaluation of activation parameters, the EYRING equation for fluid flow[7] has been used[8].

$$\phi = \frac{V}{\boldsymbol{h}N} \exp\left(\frac{-\Delta G^{+}}{RT}\right) = \frac{V}{\boldsymbol{h}N} \exp\left(\frac{\Delta S^{+}}{R}\right) \exp\left(\frac{-\Delta H^{+}}{RT}\right)$$

Here ϕ is fluidity, V is the molar volume of the liquid, $\boldsymbol{h}$ is Planck's constant, N is the Avogadro number and R and T are the gas constant and the absolute temperature, respectively; ΔG^{+}, ΔS^{+} and ΔH^{+} are the activation parameters as before. There are several reasons for adopting this form of the equation. With human erythrocytes the linear relations between E or log A and osmotic concentration extrapolate to the values for water-induced haemolysis at zero osmotic concentration. This suggests that the process of haemolysis is basically the same from zero osmotic concentration; that the mechanism is altered in degree—but not in principle—by increasing the proportion of solute. This view is substantiated by the observations presented in the previous section. If the process does depend more directly on the movement and net accumulation of water it is not unreasonable to look on the haemolysing cell as a microfluidometer and percent haemolysis per sec as a measurement which depends directly on volume flow per sec.

It is worth noting, in this context, that a mechanism based on the fluid flow of water within the system leads to a less hypothetical concept of the transition state. In a system where extensive hydrogen bonding prevails, such as the water of hydration of the cell and its membrane, it might be questioned whether the active complex or transition state has any real meaning. If, however, the mechanism depends on the fluid motion of water then, because the unit of flow is the individual water molecule, the active state is unbonded water. In the initial state an individual water molecule may be hydrogen bonded to 1, 2, 3 or 4 neighbours, whereas, in the transition state this molecule is unbonded and in a condition of maximum potential energy; translation occurs at this stage and hydrogen bonds are subsequently reformed in the final state, with these states in equilibrium throughout the process.

The dimensional discrepancy, which has distressed some critics of the earlier work is probably more apparent than real, and although it must be borne in mind that the EYRING parameters derived here are strictly empirical, they are quite adequate for the purpose of comparing malonamide solutions of different concentrations and one species with another. It is from empirical studies like these that the true basis of the mechanism of malonamide-induced haemolysis will be ascertained, and only this will permit the proper evaluation of the activation parameters.

METHODS AND RESULTS

The EYRING enthalpy of activation $\Delta H^{\ddagger}$ and entropy of activation $\Delta S^{\ddagger}$ were calculated from the Arrhenius parameters E and $\log A$, respectively; not, however, directly from the experimental values of E and $\log A$, which are subject to scatter, but from mean values determined by least square analysis of E *vs.* osmotic concentration; these mean slopes and intercepts are recorded in Table I.

TABLE I

THE MEAN SLOPES (m) AND INTERCEPT (C) OF THE RELATIONSHIPS E *vs.* π AND $\log A$ *vs.* π

Animal species	$E = m_1\pi + C_1$		$\log A = m_2\pi + C_2$	
	m_1	C_1	m_2	C_2
Rabbit	2.18	10.31	1.57	7.2155
Guinea pig	2.26	9.08	1.61	6.3601
Man	2.19	5.14	1.56	3.4968
Rat	2.03	5.19	1.46	3.6310
Cattle	2.18	3.33	1.47	2.2246
Sheep	2.15	2.55	1.46	2.2118
Dog	2.17	2.11	1.47	1.2570
Cat	2.04	1.65	1.38	1.5367
Pig	2.14	−1.97	1.46	−2.8672

The mean values of E and $\log A$ were derived by inserting appropriate values of π, the osmotic concentration in atmospheres, in these equations and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were evaluated as follows.

$$\Delta H^{\ddagger} = E - RT \tag{1}$$

$$\Delta S^{\ddagger} = 2.303\, R \left(\log A - \log \frac{V}{hN}\right) \tag{2}$$

The free energy of activation $\Delta G^{\ddagger}$ was then found from,

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

and the activation equilibrium constant $K^{\ddagger}$ from

$$\log \frac{1}{K^{\ddagger}} = \frac{\Delta G^{\ddagger}}{2.303\, RT} \tag{3}$$

The EYRING activation parameters thus derived for the haemolysis of erythrocytes of different species at the several osmotic concentrations are presented in Table II.

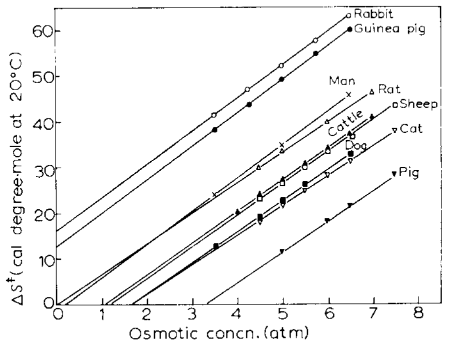
TABLE II

THE EYRING ACTIVATION PARAMETERS FOR THE HAEMOLYSIS OF MAMMALIAN ERYTHROCYTES AT DIFFERENT OSMOTIC CONCENTRATIONS

Animal species	*Osmotic conc. (atm)*	$\Delta H^{\ddagger}$ (*kcal/mole*)	$\Delta S^{\ddagger}$ (*cal/°K · mole*)	$\Delta G^{\ddagger}$ (*kcal/mole*)	$K^{\ddagger}$
Rabbit	3.5	17.36	41.41	5.23	0.9328
	4.25	18.99	46.80	5.28	0.9319
	5.0	20.63	52.18	5.34	0.9310
	5.75	22.26	57.57	5.39	0.9302
	6.5	23.90	62.96	5.45	0.9293
Guinea pig	3.5	16.41	38.14	5.24	0.9319
	4.25	18.10	43.66	5.31	0.9310
	5.0	19.80	49.19	5.39	0.9302
	5.75	21.49	54.71	5.46	0.9293
	6.5	23.19	60.23	5.54	0.9285
Sheep	4.5	11.64	23.44	4.77	0.9380
	5.0	12.72	26.78	4.87	0.9363
	5.5	13.79	30.12	4.97	0.9354
	6.0	14.87	33.46	5.07	0.9337
	6.5	15.94	36.80	5.16	0.9328
	7.5	18.09	43.48	5.35	0.9302
Dog	3.5	9.12	12.56	5.44	0.9293
	4.5	11.29	19.28	5.64	0.9267
	5.0	12.38	22.64	5.75	0.9259
	5.5	13.46	26.01	5.84	0.9250
	6.5	15.63	32.73	6.04	0.9216
Cat	4.5	10.25	18.71	4.77	0.9380
	5.0	11.27	21.87	4.86	0.9363
	5.5	12.29	25.02	4.96	0.9354
	6.0	13.31	28.18	5.05	0.9345
	6.5	14.33	31.33	5.15	0.9328
	7.5	16.37	37.65	5.34	0.9302
Pig	5.0	8.15	11.49	4.78	0.9372
	6.0	10.29	18.16	4.97	0.9354
	6.5	11.36	21.50	5.06	0.9345
	7.5	13.50	28.18	5.24	0.9310
Man	3.5	12.22	24.24	5.12	0.9328
	5.0	15.51	34.95	5.27	0.9310
	6.5	18.79	45.65	5.42	0.9293
Rat	4.5	13.74	29.94	4.97	0.9354
	5.0	14.76	33.27	5.01	0.9345
	6.0	16.79	39.95	5.09	0.9337
	7.0	18.82	46.63	5.16	0.9328
Cattle	4.0	11.47	20.35	5.51	0.9285
	4.5	12.56	23.71	5.61	0.9276
	5.0	13.65	27.07	5.72	0.9259
	5.5	14.74	30.43	5.83	0.9250
	6.0	15.83	33.79	5.93	0.9233
	6.5	16.92	37.16	6.03	0.9216
	7.0	18.01	40.52	6.14	0.9208

From the table it is evident that $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ vary with animal species and osmotic concentration in much the same way as do E and log A, from which they were derived. $\Delta H^{\ddagger}$ differs from E by only 0.58 kcal so that E may conveniently be used instead in empirical comparative studies of this kind. With $\Delta G^{\ddagger}$, however,—and $K^{\ddagger}$, which is derived from $\Delta G^{\ddagger}$—both the variation between species and the variation with osmotic concentration within species is very much less.

The graph of $\Delta S^{\ddagger}$ *vs.* osmotic concentration, depicted in Fig. 1 shows the virtually parallel relationships that were noted before, except with rat and cat, where the deviation appears to be more marked; it is not known whether this difference has any real significance because the scatter in log A *vs.* π was greater with these two species. The graph of $\Delta H^{\ddagger}$ *vs.* π, which is not shown, is similar to that of $\Delta S^{\ddagger}$ and the order in which the animals are arranged is the same.



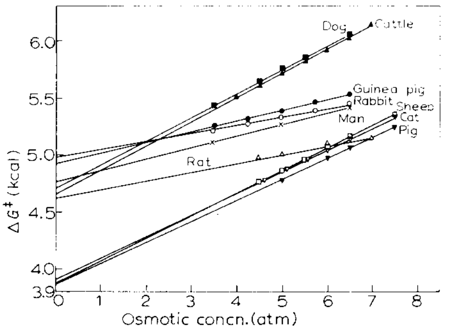


Fig. 1. The dependence of the entropy of activation ($\Delta S^{\ddagger}$) on the osmotic concentration of the malonamide solution.

Fig. 2. The dependence of the free energy of activation ($\Delta G^{\ddagger}$) on the osmotic concentration of the malonamide solution.

The graph of $\Delta G^{\ddagger}$ *vs.* π (Fig. 2) shows that, like $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, $\Delta G^{\ddagger}$ increases with increasing osmotic concentration, but that the magnitude of the increase is very much smaller. The order in which the animals are arranged is quite different and the species fall into three distinct groups that appear to be inter-related. Dog and cattle cells form one group, with sheep, cat and pig as another; these two groups are virtually parallel, with $\Delta G^{\ddagger}$ increasing in the same proportion with increasing osmotic concentration although the group values of $\Delta G^{\ddagger}$ differ by about 1 kcal. Between these two groups is the third, comprising guinea pig, rabbit, man and probably rat, in which $\Delta G^{\ddagger}$ varies much less with osmotic concentration, but which apparently intersects with the first group at approximately zero osmotic concentration and with the second around 7.5 atm osmotic concentration. This same pattern of variation and interrelationships is also exhibited by the activation equilibrium constant and is shown in Fig. 3.

There are, of course, linear relationships between $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ for the same two groups of animals (Fig. 4). The constant β_0 has the same values, 304° for the major correlation and 320° for the minor, and these values differ appreciably from the mean temperature of 293°.

The respective intercepts, however, are perhaps more meaningful in Fig. 4. The intercepts on $\Delta S^{\ddagger}$ are practically identical at —15.39 and —15.94 cal/°K·mole and would seem to represent an intrinsic entropy of activation $\Delta S^{\ddagger}$ common to all cells examined here. TRINCHER[4] has pointed out—in a related context—that such negative values are numerically close to the standard entropy of water (16.66 cal/°K·mole at 298°) and infers from this that intracellular water in erythrocytes is in the wholly ordered state. The intercepts on $\Delta H^{\ddagger}$ are also amenable to interpretation for the equation of the graphs can be written,

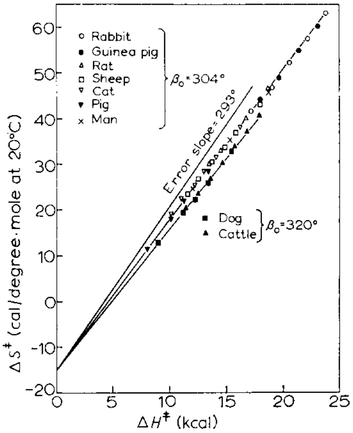


Fig. 3. The relationship between the EYRING parameters, $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$, for the various animals.

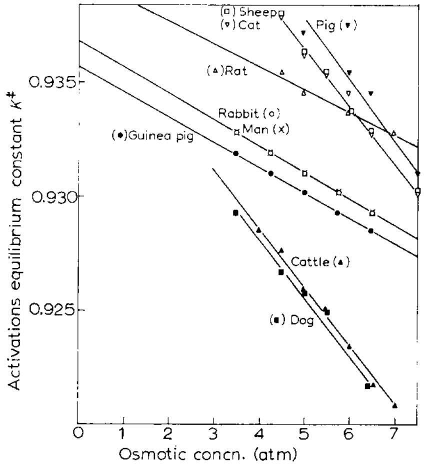


Fig. 4. The dependence of the activation equilibrium constant $K^{\ddagger}$ on the osmotic concentration of the malonamide solution.

$$\Delta H^{\ddagger} = \Delta H_0^{\ddagger} + \beta_0 \Delta S^{\ddagger}$$

where $\Delta H_0^{\ddagger}$ is an intrinsic enthalpy of activation and $\beta_0 \Delta S^{\ddagger}$ represents the structural contribution to the overall heat of activation. It is noteworthy that the structural component of $\Delta H^{\ddagger}$ is greater in the main correlation than it is for the cattle and dog group. The similarity of the above equation to the thermodynamic relation suggests the possibility that $\Delta H_0^{\ddagger}$ may be a free energy parameter; if this is indeed so then the higher value of $\Delta H_0^{\ddagger}$ for cattle and dog cells would account for the appreciably lower rate of haemolysis observed with these species, because it is the free energy of activation that governs the velocity of a rate process.

DISCUSSION

In the preceding section the Arrhenius kinetics were interpreted on the basis of hydration structure change and the EYRING evaluation presented here adds substance to that view.

The entropy of activation, $\Delta S^{\ddagger}$, is a linear function of the osmotic concentration and varies considerably between animal species; the mechanism of activation is related to this. If activation consists in freeing individual water molecules from hydrogen bond attachment to their neighbours, then the higher the positive value of

ΔS^{+}, the greater the initial extent of hydrogen bonding in the system. That is to say, the more extensive the hydration structure the greater the amount of disorder created by activation and conversely, the less extensive the hydration, the more disordered the initial state and the lower the value of ΔS^{+}. Furthermore, since hydration structure depends on a multiplicity of weak hydrogen bonds that are very sensitive to temperature change, extensive hydration is associated with higher values of ΔH^{+} and *vice versa*. For those reasons it is inferred here too that the numerical values of ΔS^{+} and ΔH^{+} reflect the extent of hydration in the system. Both decrease with decreasing osmotic concentration—for this tends to disrupt hydration structure—and the decreasing numerical order of both parameters with animal species is taken as evidence of diminishing hydration structure in these cells.

With regard to linear relationships between ΔS^{+} and ΔH^{+}, these are often encountered in chemical kinetics and are currently the object of intensive study in that field[9,10]. It appears that linearity most frequently occurs in sets of reactions with a common basic mechanism which involves solvent participation. There are grounds for believing that in those processes where the mechanism is solvent controlled, the constant β_0 is numerically smaller than the mean temperature of experiment, and where the process is solute controlled β_0 is numerically greater than the mean temperature[10]. For a large number of reactions, however, the deviation of β_0 from the mean temperature does not exceed $\pm 30\,\%$; this suggests that in these processes there is not an absolute shift in one direction or the other, but there is, rather, a tendency or bias towards solvent or solute control, so the movement of β_0 towards the mean temperature is a measure of the extent to which solvent participation contributes to the compensation implied by linearity[10].

In this context the correlations presented here are interpreted as meaning that membrane-regulated hydration is the principal controlling factor in the mechanism of haemolysis of all these cells, but with those of cattle and dog this mechanism is subject to extraneous interference that acts so as to reduce the role of hydration in the process. The common intercept at ΔS_0^{+}, near to the negative value of the standard entropy of water, suggests that the function on which linearity depends occurs in a wholly ordered water system; there may also be significance in the observation that the values of ΔH_0^{+}—which are obtained at that point where the system requires no increment in disorder for activation, $\Delta S^{+} = 0$—are not far removed from the heat of activation for the fluid flow of water at 20°.

It is not surprising that the free energy of activation should result in the grouping of these animals in an entirely different manner and order from ΔH^{+} or ΔS^{+}, for although the rate of haemolysis depends directly on the magnitude of ΔG^{+}, this parameter does not vary nearly as much as ΔH^{+} or ΔS^{+} with minor changes in reaction conditions. Numerically ΔG^{+} is the difference between ΔH^{+} and $T\Delta S^{+}$ and this difference is greatest with cattle and dog erythrocytes, which have the highest values of ΔG^{+} and the lowest rate of haemolysis; this happens because ΔG^{+} is high in relation to $T\Delta S^{+}$ and the process is less hydration dependent. It may be that the non-structural component of ΔH^{+} is excessively high due to the intervention of some extraneous factor—the outward diffusion of sodium ion, for example—that is not directly involved in the mechanism of haemolysis. With sheep, cat and pig ΔG^{+} is much lower so these cells haemolyse very rapidly in hypotonic malonamide; both ΔH^{+} and ΔS^{+} are low and the difference between them is also low because there is a smaller hydration structure contribution to $\Delta H^{\ddagger}$. With the remainder, guinea pig, rabbit, man and possibly rat, both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are high and since there is a large hydration structure contribution to $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$ is high and changes rather less with concentration because extensive membrane hydration persists in spite of dilution. There may be reservations about including the cells of the rat in this group, for although $\Delta G^{\ddagger}$ for this species and rabbit are virtually parallel with concentration change, the numerical values for rat are appreciably lower than the others. This is almost certainly connected with the unusual paracrystalline state of the rat erythrocyte[11], which is known to be anomalous in its haemolytic response[12].

These free energy relationships are, of course, reflected in the behaviour of the activation equilibrium constant $K^{\ddagger}$, which is markedly favoured by decreasing concentration with pig, sheep and cat cells; in these systems, where hydration is weak and not extensive, activation is much more readily achieved when disorder is increased by dilution. Activation is less favoured with dilution in the systems of rabbit, guinea pig and man because the hydration here is extensive and stable and tends to persist although the concentration is reduced. With the cells of cattle and dog activation is suppressed by what would appear to be a shift in datum rather than a change in mechanism. This kind of change could be induced by the superimposition of a cationic movement—perhaps the extracellular transfer of Na^+—on the existing common mechanism.

It is therefore concluded that although the EYRING parameters derived in this work are empirical, they do permit a more comprehensive evaluation of the temperature dependence of haemolysis than is possible with the Arrhenius equation alone, and further, that the interpretation of these parameters is consistent with the view that membrane hydration is the principal factor in the mechanism of the process.

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