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THE KINETICS OF MALONAMIDE-INDUCED HAEMOLYSIS OF MAMMALIAN ERYTHROCYTES

I. THE ARRHENIUS ACTIVATION PARAMETERS

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SUMMARY

1. The haemolysis of erythrocytes of rabbit, guinea pig, rat, cattle, sheep, dog, cat and pig has been examined in hypotonic solutions of malonamide at several temperatures between 15° and 25°.

2. The temperature dependence of the haemolytic process has been evaluated in terms of the activation parameters E and $\log A$ of the Arrhenius equation.

3. Both Arrhenius parameters vary in a like and linear manner with the osmotic concentration of the haemolysing solution for all eight animal species, and the graph of $\log A$ *vs.* E is collinear for the erythrocytes of rabbit, guinea pig, rat, sheep, cat and pig, with a separate collinear relation—from a common intercept—for cattle and dog.

4. The implications of these findings are discussed and it is concluded that linearity between the Arrhenius activation parameters depends more on the hydration structure of the red-cell membrane than on any other single factor.

INTRODUCTION

In previously reported work[1] it was shown that the kinetics of haemolysis of human erythrocytes in hypotonic solutions of malonamide obey the Arrhenius law and that the parameters E and A of the Arrhenius equation can be informative about the mechanism of the process; these studies have now been extended to the erythrocytes of 8 other mammals and the results are presented here, where the intention is to study the kinetics of malonamide-induced haemolysis of mammalian red cells in the most direct way, and to evaluate the results in terms of the activation parameters of the Arrhenius equation without recourse to preconceptions about the mechanism of the process. As an alternative to this the assumption can be made that haemolysis kinetics depend only on the rate of penetration of malonamide and the process can be studied on this basis[2]. In kinetics, however, all postulated mechanisms are essentially theories devised to explain the results of rate measurements and there are often

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several conceivable mechanisms consistent with the factual data; one mechanism becomes more acceptable than another only in so far as it can keep pace with the advance of knowledge in general, and in related fields in particular.

A brief preliminary note on the significance of the linear relationship between the Arrhenius activation parameters has already been published[3].

MATERIALS AND METHODS

Collection and preparation of the blood

In all experiments fresh, normal, fully oxygenated whole blood was used, collected from each animal species by the most convenient method. Samples of cattle, sheep and pig blood were obtained from the abattoir and were collected immediately after death; guinea pigs and rats were bled after decapitation and rabbit blood was obtained from an ear-vein incision. Dog blood was withdrawn by leg venipuncture and blood from the cat was taken under nembutal anaesthesia from a cannula in the femoral vein. All blood samples were collected in clean, dry vessels with approx. 100 units of low sodium heparin per 100 ml blood as the anticoagulant. At soon as possible after collection the blood was exposed to an atmosphere of moist O_2 to reduce the fragility variable[4] and was maintained thus for at least 20 min before use; apart from oxygenation the blood received no other treatment prior to experiment.

Preparation of malonamide solutions

Laboratory reagent grade malonamide (British Drug Houses) was found to have a melting point of 178°, so no further purification was considered necessary, but the material was dried *in vacuo* over conc. H_2SO_4 before use.

The volume of the mammalian erythrocyte is highly sensitive to osmotic pressure change and in any solution of given concentration osmotic pressure is proportional to the absolute temperature; in kinetic studies at different temperatures therefore, it is necessary to maintain constant osmotic pressure rather than constant concentration. The concentrations of the haemolysing solutions employed here are such as to provide constant osmotic pressure at the different temperatures of experiment; these were derived in the way previously described[1].

Experimental methods

In most respects the experimental methods are the same as those employed in the earlier work[1], including the standard 1:21 dilution of whole blood. For those cells which haemolysed more rapidly, however, another technique was adopted. The malonamide solution was distributed in 3.0-ml volumes in a number of test-tubes in the water bath; to one of these tubes 0.15 ml whole blood was added, and the system mixed. Haemolysis was allowed to proceed for the appropriate time period and was then arrested by adding 1.0 ml 3.6% NaCl solution. This procedure was repeated for different time intervals and percentage haemolysis was determined as before.

The rate of haemolysis for all species, the slope of the percent haemolysis *vs.* time curve, was measured at 15, 17.5, 20, 22.5 and 25°, temperature control being maintained at $\pm$ 0.2° in a Weyco thermostatic water bath.

RESULTS

Species variation

At constant temperature and constant osmotic concentration the haemolysis curves of erythrocytes of all eight animal species have the characteristic sigmoid shape (Fig. 1) of the cumulative frequency distribution of red-cell fragility[5].

The figure shows that sheep and cat cells haemolyse rapidly in hypotonic malonamide and that pig and rat erythrocytes are only slightly less rapidly destroyed. With the erythrocytes of the rabbit the rate of haemolysis is appreciably less and the lag phase is greater; with the guinea pig, although the rate of haemolysis is similar to that of rabbit cells, the lag phase is much longer. Cattle and dog erythrocytes differ markedly from the others, for although they have a much lower rate of haemolysis, the lag phase is relatively short.

The effect of concentration

The effect on the rate of haemolysis of increasing the osmotic concentration of malonamide is illustrated in Fig. 2 by cattle and sheep haemolysis curves. For both species increasing the osmotic concentration increases the lag phase and reduces the rate of haemolysis, but the effect is much greater with cattle cells than with those of the sheep.

The effect of temperature

The sensitivity of the rate of haemolysis to temperature change at constant osmotic pressure is depicted in Fig. 3 for cat and guinea-pig erythrocytes. The figure

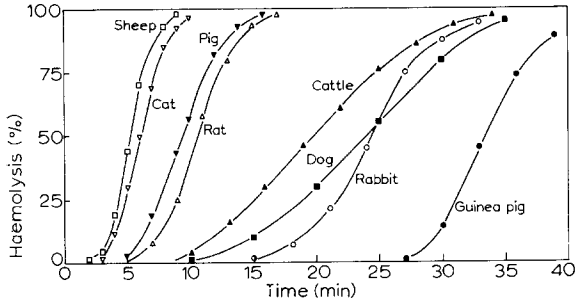

Fig. 1. A selection of typical haemolysis curves at constant temperature (20°) and constant osmotic concentration (5 atm) of malonamide for the various animals.

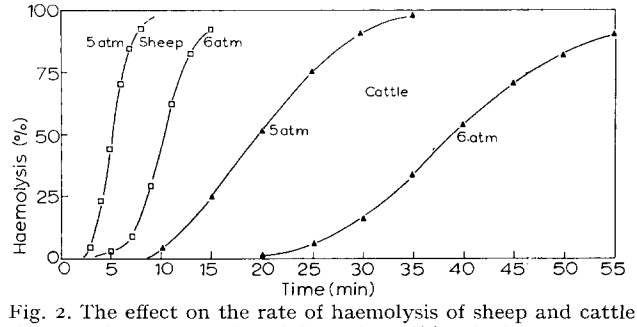

Fig. 2. The effect on the rate of haemolysis of sheep and cattle erythrocytes caused by changing the osmotic concentration of the malonamide solution at a constant temperature of 20°.

shows that guinea-pig cells haemolyse much more slowly, but are more responsive to temperature change than those of the cat. Pig and sheep erythrocytes respond to temperature change in a similar way to the cat, while rabbit and guinea-pig cells resemble each other in this respect.

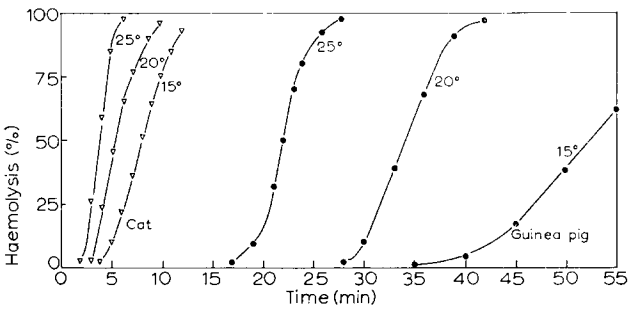

Fig. 3. The effect on the rate of haemolysis of cat and guinea-pig erythrocytes caused by changing the temperature of the malonamide solution at a constant osmotic concentration equivalent to 5.0 atm.

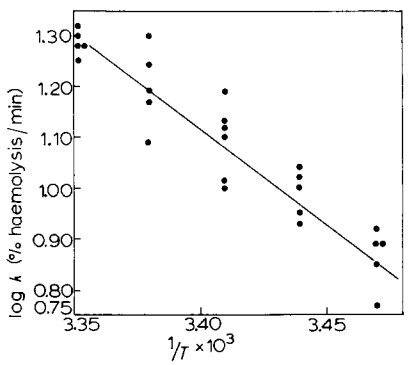

Fig. 4. The Arrhenius plot, log percent haemolysis per min *vs.* $1/T$, for guinea-pig erythrocytes at 3.5 atm constant osmotic concentration of malonamide.

These experiments show clearly that the rate of haemolysis of mammalian erythrocytes in hypotonic malonamide solutions varies with the animal species, the osmotic concentration of the haemolysing solution and the temperature of the haemolysing system.

The kinetic evaluation

Since the method employed to determine the rate of haemolysis is simple, and because of normal biological variation between individuals within species, a number of rate measurements were made on several blood samples from each species at five temperatures in the range 15–25°; the log of the rate of haemolysis was then plotted against $1/T$, as shown in Fig. 4, to derive the slope ($-m$) and the intercept (C). These parameters were calculated by regression analysis for each species at several osmotic concentrations and the statistical data are presented in Table I.

The table shows that for a given species at each osmotic concentration the slope of log k on $1/T$ is negative and the intercept is positive, and that both slope and inter-

TABLE I

REGRESSION ANALYSIS OF THE RATE EQUATION $\log k = -m/T + C$

Animal species	*Osmotic concn. (atm)*	*Slope (—m)*	*Intercept (C)*	*Correlation coefficient (—r)*	*Standard error of estimate*	*Total variance*	*Variance due to causes other than the regression*	*Student's t-test*		
								Degrees of freedom (N—2)	*t (Calc.)*	*t (tables) (P = 0.01)*
Rabbit	3.5	3.8968	14.4070	0.8831	0.0878	0.0350	0.0077	16	7.530	2.921
	4.25	4.3144	15.7743	0.8547	0.1323	0.0649	0.0175	8	4.655	3.355
	5.0	4.5956	16.6783	0.9527	0.0598	0.0400	0.0036	14	11.923	2.977
	5.75	5.0604	18.2525	0.9844	0.0405	0.0530	0.0016	8	15.809	3.355
	6.5	5.3114	19.0464	0.8990	0.0996	0.0517	0.0099	19	8.949	2.861
Guinea pig	3.5	3.7560	13.9206	0.8484	0.0828	0.0245	0.0068	24	7.851	2.797
	4.25	4.0322	14.8107	*						
	5.0	4.4132	16.0638	0.9571	0.0523	0.0326	0.0027	24	16.180	2.797
	5.75	4.8580	17.5564	*						
	6.5	5.1938	18.5844	0.9857	0.0271	0.0401	0.0011	22	26.161	2.845
Rat	4.5	3.1203	11.9426	1.0000	0	0.0265	0	8		3.355
	5.0	3.3768	12.7664	0.9695	0.0438	0.0254	0.0019	9	9.258	3.499
	6.0	3.7970	14.1193	*						
	7.0	4.2442	15.6362	*						
Cattle	4.0	2.5970	9.8040	*						
	4.5	2.9171	10.7274	0.9933	0.0012	0.0104	0.0001	14	32.098	2.977
	5.0	3.0655	11.1925	0.9722	0.0351	0.0225	0.0012	8	11.744	3.355
	5.5	3.4346	12.3721	0.9600	0.0362	0.0167	0.0013	18	14.547	2.878

	6.0	3.5741	12.8020	0.8825	6.0641	0.0186	0.0041	10	5.933	3.109
	6.5	3.8135	13.5229	0.7590	0.1401	0.0463	0.0196	32	6.595	2.750
	7.0	4.0548	14.2735	0.9219	0.0619	0.0255	0.0038	9	7.139	3.250
Sheep	5.0	2.8165	10.9051	0.9851	0.0252	0.0214	0.0006	8	16.197	3.355
	5.5	3.1619	11.9809	0.9996	0.0035	0.0150	0.0002	12	122.440	3.055
	6.0	3.4108	12.8696	0.9846	0.0275	0.0248	0.0008	7	14.896	3.499
	6.5	3.6443	13.4904	0.9500	0.539	0.0298	0.0029	11	10.093	3.106
	7.0	4.1176	14.9895	0.9594	0.506	0.0321	0.0026	7	8.999	3.499
Dog	3.5	2.0863	8.1109	0.8235	0.0614	0.0117	0.0038	15	5.622	2.947
	4.5	2.6059	9.7655	0.9556	0.0345	0.0137	0.0012	12	11.236	3.055
	5.0	2.7923	10.3341	0.8400	0.0701	0.0167	0.0049	13	5.583	3.012
	5.5	3.0952	11.2347	0.8194	0.0879	0.0235	0.0077	8	4.043	3.355
	6.5	3.4923	12.5275	0.8548	0.0826	0.0253	0.0068	16	6.588	2.921
Cat	4.5	2.2545	9.1424	*						
	5.0	2.5934	10.2252	*						
	5.5	2.8910	11.1846	*						
	6.0	3.1353	11.9155	*						
	6.5	3.2921	12.3752	*						
	7.5	3.6090	13.3514	*						
Pig	5.0	1.8922	7.6890	0.9000	0.0396	0.0083	0.0016	14	7.726	2.977
	6.0	2.3082	9.1307	0.9588	0.0321	0.0128	0.0010	10	8.071	3.169
	6.5	2.7432	10.3888	0.9556	0.0403	0.0187	0.0016	11	10.759	3.106
	7.0	3.0202	11.2365	0.9688	0.0365	0.0217	0.0013	11	12.968	3.106

* Insufficient data to permit full statistical analysis.

cept increase with increasing osmotic concentration. The correlation coefficient confirms the high degree of negative correlation between the rate of haemolysis and temperature and the uncertainty in the rate, given by the standard error of estimate, is not unreasonable in view of the extreme simplicity of the experimental methods.

TABLE II

THE NUMERICAL VALUES OF THE ARRHENIUS PARAMETERS E AND $\log A$ CALCULATED AT 20°

Animal species	*Osmotic concn. (atm)*	E *(kcal/mole)*	$\log A$ *(k = % haemolysis/sec)*
Rabbit	3.5	17.83	12.6288
	4.25	19.75	13.9961
	5.0	21.03	14.9001
	5.75	23.16	16.4743
	6.5	24.31	17.2682
Guinea pig	3.5	17.19	12.1424
	4.25	18.45	13.0325
	5.0	20.20	14.2856
	5.75	22.23	15.7782
	6.5	23.77	16.8062
Rat	4.5	14.28	10.1644
	5.0	15.45	10.9882
	6.0	17.38	12.3411
	7.0	19.42	13.8580
Cattle	4.0	11.89	8.0258
	4.5	13.35	8.9492
	5.0	14.03	9.4143
	5.5	15.72	10.5939
	6.0	16.36	11.0238
	6.5	17.45	11.7447
	7.0	18.55	12.4953
Sheep	4.5	12.69	9.1043
	5.0	12.89	9.1269
	5.5	14.47	10.2027
	6.0	15.61	11.0914
	6.5	16.68	11.7122
	7.5	18.85	13.2113
Dog	3.5	9.55	6.3327
	4.5	11.93	7.9873
	5.0	12.78	8.5559
	5.5	14.17	9.4565
	6.5	15.98	10.7493
Cat	4.5	10.32	7.3632
	5.0	11.87	8.4470
	5.5	13.23	9.4064
	6.0	14.35	10.1373
	6.5	15.07	10.5970
	7.5	16.52	11.5732
Pig	5.0	8.66	5.9108
	6.0	10.56	7.3525
	6.5	11.54	7.8892
	7.5	13.82	9.4583

Between 70 and 80 % of the total variance is due to the regression itself, that is to the effect of temperature on the rate of haemolysis, and the *t*-test confirms statistical significance at the 1 % level of probability. It is therefore concluded that this approach and the methods employed are quite adequate for the evaluation of haemolysis kinetics.

The numerical values of the Arrhenius parameters were found from $-m$ and C, for $E = 2.303\ Rm$ and $\log A = C$, where the rate k is expressed in units of percent haemolysis per min. Log A in terms of percent haemolysis per sec is found by subtracting log 60 from C. The values of these parameters, calculated at the mean temperature of 20° for the different animals and osmotic concentrations are listed in Table II.

This table shows that the Arrhenius parameters vary with animal species and that within a species, both E and log A vary in the same sense with the osmotic concentration of the haemolysing solution.

The relationship between the Arrhenius activation energy E and the osmotic concentration in atmospheres is illustrated in Fig. 5, where the values for man[1] are included for the purpose of comparison. The figure shows that E decreases in magnitude in the order rabbit > guinea pig ⋙ man > rat > cattle > sheep > dog > cat ⋙ pig and there is the unexpected feature that the individual curves are very nearly parallel; individual slopes differ from the mean slope of 2.15—calculated by least squares—by only ± 0.14, that is by 6.5 %. In the graph of log A *vs.* osmotic concentration, which is not illustrated, log A follows the same order as E and the curves are again virtually parallel, with a mean slope of 1.49 ± 0.14, a deviation of ± 9.4 %. This finding, that the numerical values of the Arrhenius parameters have a common linear relationship with osmotic concentration suggests that there may be some

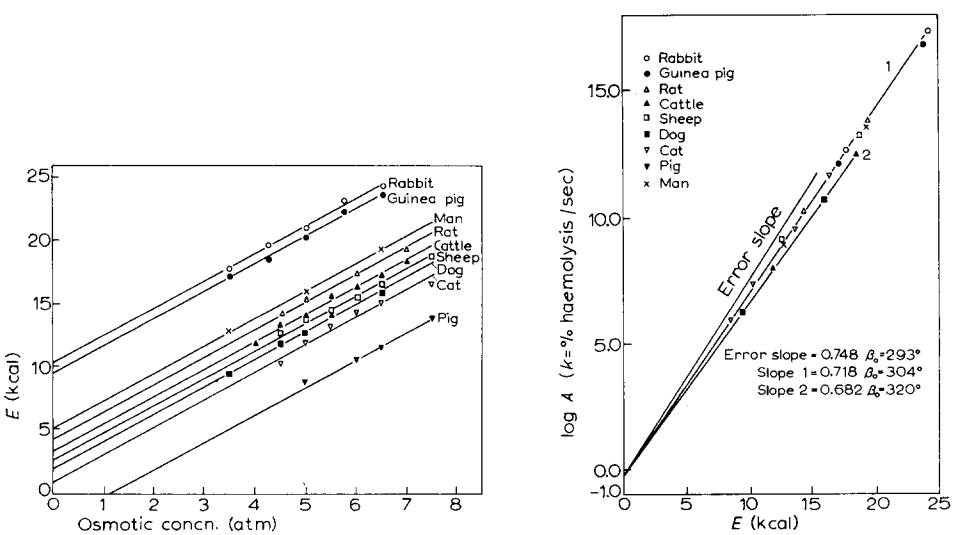

Fig. 5. The relationship between the Arrhenius activation energy (E) and osmotic concentration of malonamide for the various animals.

Fig. 6. The relationship between the Arrhenius activation parameters, log A and E, for the various animals. In this figure only the highest and lowest pairs of values are recorded for each species.

common basic mechanism underlying the haemolysis of all these cells and that this is related to the osmotic properties of the system. There is, too, the further expectation that within species $\log A$ and E should be linearly related to each other; that this is in fact the case, is shown in Fig. 6.

For the sake of clarity in presentation only two points are given for each animal, which suffices to show that these appear to be not merely linear within species, but are probably collinear for the majority of species, with a separate collinear relationship for cattle and dog. The existence of two separate correlations is supported by the statistical data of Table III, which shows that although there is a high degree of correlation when all species at every concentration are taken together, the precision—from the standard error, the variance due to causes other than the regression and the t-test—is much higher when the data of Curves 1 and 2 are treated separately.

Correlations like this are not uncommon in chemical kinetics[6] and are generally taken to indicate common process mechanisms. The derivation of these relationships, however, involves a transformation of coordinates and there can arise, purely from the mathematical treatment, a high degree of correlation which is directly related to the mean temperature of experiment and which has no physical significance[7,8]. Fortunately, the validity of $\log A$ *vs.* E relationships can be readily checked against the mean temperature or error slope[9,10], which are here 293°K or 0.7478, respectively. The experimental correlations derived in this work differ significantly from each other and from the error slope (Table III), so it is concluded that these are valid examples of linear relationships between $\log A$ and E.

DISCUSSION

The most striking and unexpected results of this work are the collinear relationships between $\log A$ and E for the two groups of animals; these are all the more surprising when it is recalled that mammalian erythrocytes differ considerably from one species to another. Cell size is an obvious difference, but cell contents differ too, electrolytes and glucose for example[11], and most probably many other cell solutes as well. Perhaps more significantly—in the light of lipid solubility theories of cell permeability—the lipid consituents of red-cell membranes vary appreciably between species[12,13]. When these differences are considered in the context of accepted views on cell permeability there is little guidance for interpreting statistically significant linear relationships between the activation parameters of haemolysis of different cell species; fortunately, however, a lead can be obtained from chemical kinetics.

The general equation of linearity between the Arrhenius parameters is,

$$\log A = \frac{1}{2.303\, R\beta_0} E + \log A_0$$

where $\log A$ and E have the same meaning as before, R is the gas constant, $\log A_0$ is an intrinsic rate of haemolysis for all cells examined here and β_0 is a group or family constant that is sometimes referred to as the isokinetic temperature[14]. The meaning of β_0 has been interpreted in several ways[6], but perhaps the most useful approach for this work is the view that the Arrhenius activation energy, E, is an overall energy parameter which is composed of two terms, an intrinsic activation energy and a

TABLE III

REGRESSION ANALYSIS OF THE RELATIONSHIP $\log A = mE + C$

Data for all species at each osmotic pressure, including man[1].

Curve examined	*Species included*	*Slope (m)*	*Intercept (C)*	*Correlation coefficient (r)*	*Standard error of estimate*	*Total variance*	*Variance due to causes other than the regression*	*Student's t-test*		
								Degrees of freedom (N−2)	*t (Calc.)*	*t (P=0.001)*
1 + 2	Man Guinea pig Rabbit Sheep, cat Rat, pig Dog, cattle	0.7243	—1.5774	0.9970	0.2253	8.4624	0.0507	44	44.689	3.551
1	Man Guinea pig Rabbit Sheep, cat Rat, pig	0.7183	—1.7912	0.9994	0.0982	9.6643	0.0097	32	178.912	3.646
2	Dog, cattle	0.6818	—1.8519	0.9998	0.0340	2.8866	0.0012	10	158.068	4.587
3 (Error slope)		0.7478	—1.7912	1.0000	—	—	—	—	—	—

structural activation energy, where the structural component is determined by β_0 and the entropy. The constant β_0 becomes then a measure of the sensitivity of E to log A and shows that the structural contribution is linearly dependent on entropy. Linear relationships of this kind are examples of the compensation law and are very commonly observed in kinetic studies, but particularly in related reactions that differ from each other mainly in the extent to which solvational changes are involved[15]; this concept, that changes in solvation—or here hydration—are largely responsible for compensation between log A and E is an attractive one for interpreting the relationships discovered in this work.

Although the erythrocytes studied here are highly variable in many respects, they have in common free permeability to water and swell or shrink according to the osmotic concentration of the suspending medium, so that when they are placed in hypotonic malonamide they attain osmotic equilibrium within a few seconds. That they should continue to swell—and sooner or later haemolyze—in hypotonic malonamide shows that the cells are permeable to this solute. It might therefore be supposed that because the movement of water is extremely rapid, the rate-determining step is the penetration of malonamide and the kinetics described here refer to that process. This, however, presupposes that these erythrocytes behave as perfect osmometers; that cell swelling is invariably in uniform stoichiometric proportion to the intracellular concentration of the haemolytic solute. Evidence will be presented in a later paper to show that this is an untenable assumption. The possibility has not been considered that menbrane-bound carrier transport or permease activity may be concerned in the mechanism of malonamide-induced haemolysis because there is no evidence of saturation kinetics. It is difficult, moreover, to formulate a common mechanism to account for the transfer of the same polar organic solute across membranes that differ considerably in lipid composition; the kinetics indicate the existence of such a mechanism, but the concept cannot be reconciled with a significant lipid solubility factor in membrane permeability. If, on the other hand, it is assumed that the hydration of the membrane plays a more important part in permeability processes, the hydration changes do provide a reasonable basis for a common mechanism with linearity between the activation parameters.

It has recently been postulated that the cell membrane contains an interconnected hydrogen-bonded framework—a hydrate continuum—that permeates the ordered lipoprotein structure[16] and it has also been proposed that changes in the configuration of the lipids may determine the water content of the membrane structure[17]. More recently a model has been put forward[18] which assigns to water an important role as an integrated structural component of the membrane protein, and which provides also for extensive cell surface hydration. There are, therefore, grounds for supposing that ordered water of hydration is just as important a constituent of the cell membrane as it is of the intracellular phase, and it may be that cell swelling in a hypotonic medium is not due merely to an increase in volume of the intracellular phase, but also to an increase in volume of the membrane.

In this circumstance the more extensive and highly ordered the membrane hydration structure, the more sensitive it will be to temperature change and consequently the higher will be the structural component of the overall activation energy; the more extensive and ordered the hydration structure the greater the disorder created by passage of solute, and the greater the entropy gain. The converse is true

where hydration structure is less extensive and less ordered. In a process mechanism which is based on hydration structure change, both E and log A (a measure of the entropy term) vary together in the same sense; that is to say they compensate and behave in the same way as do the parameters evaluated in this work.

According to this interpretation the dependence on osmotic concentration of E and log A within a given species follows from changes in membrane hydration. Decreasing the tonicity reduces the stability of the hydration structure so that passage of solute is allowed at a lower structural activation energy and with a smaller increment in disorder. The variation at constant osmotic concentration of E and log A between species suggests that the hydration structure of erythrocyte membranes is yet another cell variable that has not hitherto been recognised. It is inferred from these results that ordered water of hydration is most stable—and extensive—in the membrane of the rabbit erythrocytes and least stable and extensive in the red-cell membrane of the pig, with the other species in this correlation ranged between these two extremes. The separate curve for cattle and dog cells is important, for the common intercept and similar behaviour of E and log A within this correlation suggest that the mechanism of the process is intrinsically the same as the others; log A is less sensitive to E, which suggests that the entropy term may be less significant. This could mean that their hydration structures are less stable, but it could also indicate the intervention of an extraneous effect that is not directly involved in the process mechanism.

It is concluded from these results that linearity between the Arrhenius activation parameters depends more on cell membrane hydration than on any other single factor, but, for the further elucidation of these data an evaluation of the kinetics in terms of the Eyring equation is presented in the next section.

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