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THE KINETICS OF HAEMOLYSIS OF HUMAN ERYTHROCYTES IN HYPOTONIC SOLUTIONS OF GLUCOSE

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SUMMARY

1. A study has been made of the kinetics of haemolysis of human erythrocytes in hypotonic solutions of glucose and values are presented for the Arrhenius and Eyring parameters.

2. Linear correlations between the activation parameters indicate that glucose-induced haemolysis kinetics conform with the compensation law and that hydration plays a major role in the mechanism of the process.

3. Collinearity between the activation parameters of glucose- and malonamide-induced haemolysis suggests that both solutes penetrate by a common mechanism that depends on their hydration characteristics.

INTRODUCTION

A re-examination of earlier data[1], made in the light of newer knowledge of the compensation law[2], suggests that the basic mechanism of haemolysis of human erythrocytes in hypotonic solutions of glucose is at least similar to, but is more probably identical with that already proposed[3] for haemolysis in hypotonic solutions of malonamide. In order to obtain further information about this point, and to facilitate direct comparison between the two systems, the kinetics of haemolysis of human erythrocytes in glucose solutions have been examined by the methods previously employed for malonamide. The results which are presented here support the view that the two substances do indeed effect haemolysis by a common mechanism.

MATERIALS AND METHODS

The blood for these experiments was obtained from normal, healthy maternity patients with heparin as the anticoagulant and was stored overnight at 4°. Before use the blood was allowed to come to room temperature and was equilibrated with moist O_2 at atmospheric pressure until required for experiment.

The haemolysing solutions were made up from BDH Analar grade D-glucose, previously dried *in vacuo* over conc. H_2SO_4; osmotic concentrations were derived from freezing-point depressions[3] and selected values are tabulated below.

In the haemolysis experiments 1 vol. whole blood was added to 20 vol. haemolysing solution, contained in a suitable vessel in a Grant waterbath at the desired

TABLE I

OSMOTIC CONCENTRATIONS OF GLUCOSE SOLUTIONS

Osmotic concn. (atm.)	*Molality*		
	25°	*30°*	*35°*
2.9	0.120	0.118	0.116
4.0	0.164	0.161	0.158
4.9	0.202	0.198	0.195
5.5	0.227	0.222	0.219
6.3	0.259	0.254	0.250

temperature, and haemolysis was allowed to proceed under thermostatic control (± 0.2°). At appropriate time intervals a 3.0-ml sample was withdrawn from the haemolysing system and transferred to a centrifuge tube containing 0.5 ml 3.15 % NaCl; this gives a final concentration of 0.45 % NaCl which, in itself, is sufficient to prevent further haemolysis. After centrifugation for 2 min at 2000 rev./min (MSE Minor), 2.0 ml supernatant were transferred to a test tube and 8.0 ml 0.1 M NaOH added. At the end of the experiment an uncentrifuged sample of the haemolysing system was treated in the same way, giving a measure of complete haemolysis. The test tubes were heated in a boiling waterbath for 10 min and, after cooling, the colour development was read in the Unicam SP 1300 colourimeter, using No. 4 filter (yellow-green, 510–590 nm). The degree of haemolysis in each tube was expressed as a percentage of total haemolysis.

Rate curves were constructed by plotting per cent haemolysis against time and the rate of haemolysis was taken as the average slope of the curve between 25 and 75 % lysis. Between 2 and 8 rate determinations were made for each concentration and at each temperature within the chosen range.

RESULTS

Typical examples of haemolysis curves are shown in Fig. 1, which illustrates how the rate of haemolysis in hypotonic glucose solutions varies with the osmotic concentration of the system. The rates are appreciably lower than those in corresponding concentrations of malonamide[3]. The second figure shows the enormous effect

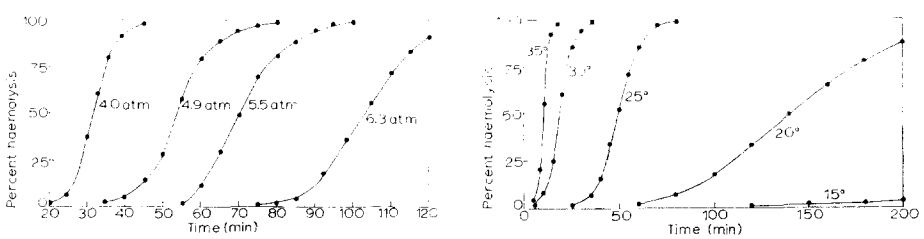

Fig. 1. The effect of osmotic concentration on the rate of haemolysis of human erythrocytes in hypotonic solutions of glucose at 35°.

Fig. 2. The effect of temperature on the rate of haemolysis of human erythrocytes at a constant osmotic concentration of glucose, equivalent to 2.9 atm.

of temperature on glucose-induced haemolysis; this, of course, indicates that the heat of activation of the process is extremely high. The temperature dependence of haemolysis in glucose solutions is therefore very much greater than in malonamide systems[3].

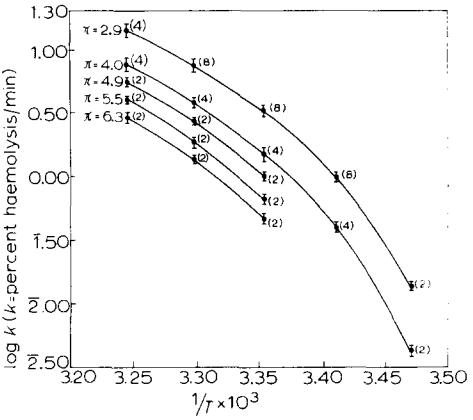

Fig. 3. Arrhenius plots for the haemolysis of human erythrocytes in hypotonic solutions of glucose. The numbers in parenthesis refer to the number of rate determinations made and the range of values is denoted by the vertical lines.

The kinetic evaluation

The tremendous temperature dependence of glucose-induced haemolysis is perhaps more strikingly obvious in the Arrhenius plots of the rate coefficients, which are depicted in Fig. 3. The pronounced curvature in the graphs of log k *vs.* $1/T$ is not evident in the less temperature dependent malonamide system within the same temperature range[3]. Because of this curvature the Arrhenius activation parameters cannot be calculated by linear regression analysis and instead the following equations[4] were employed:

$$E = \frac{2.303\ RT_1T_2}{T_1 - T_2}(\log k_1 - \log k_2) \qquad (1)$$

$$\log A = \frac{T_1}{T_1 - T_2}\left(\log k_1 - \frac{T_2}{T_1}\log k_2\right) \qquad (2)$$

In these equations E is the Arrhenius activation energy at the mean absolute temperature $(T_1 + T_2)/2$; T_1 and T_2 are respectively the upper and lower absolute temperatures of experiment and k_1 and k_2 are respectively the upper and lower average rates of haemolysis, expressed as per cent haemolysis per min. A is the non-exponential Arrhenius parameter and log A in terms of per cent haemolysis per second is found by subtracting log 60 from the value given by Eqn. 2. The numerical values of the essentially empirical activation parameters thus derived are recorded in Table II.

The primary criterion for establishing identity of mechanism in related rate processes is a linear correlation between the rate constants k_1 and k_2 (refs. 4, 5) and the hitherto more usual definition of isokinetic relationships—linearity between the Arrhenius activation parameters—is in fact a secondary criterion. More seriously, this secondary relationship may be subject to inherent error, because a linear relationship that does not correspond with the true isokinetic relation can arise purely from

TABLE II

THE ARRHENIUS ACTIVATION PARAMETERS FOR GLUCOSE-INDUCED HAEMOLYSIS AT 20, 25 AND 30° AND FOR MALONAMIDE AT 20°

Mean experimental temp.	*Osmotic concn. (atm.)*	*E (kcal/mole)*	*log A (k = % haemolysis/sec)*
20°	2.9	54.2	38.6197
	4.0	60.3	42.7712
25°	2.9	35.4	24.7334
	4.0	40.1	27.7817
30°	2.9	26.5	18.2238
	4.0	29.4	20.0090
	4.9	31.1	21.0626
	5.5	32.5	21.8987
	6.3	33.5	22.5136
20°	3.5	12.9	8.9579
	5.0	15.9	11.1114
	6.5	19.4	13.5920
	3.5 + 1.5	22.2	15.7418

the method of computation[4,5]. This situation, however, is easily recognised for, in the circumstance, the value of T in the expression $10^3/2.303RT$—the gradient of the correlation log A *vs.* E—turns out to be the mean temperature of the experiment and this gradient is termed the error slope. Significant deviation from the error slope therefore implies validity in correlations between E and log A, and an example of this is presented in Fig. 4.

The figure shows quite clearly that the slope of the calculated regression line

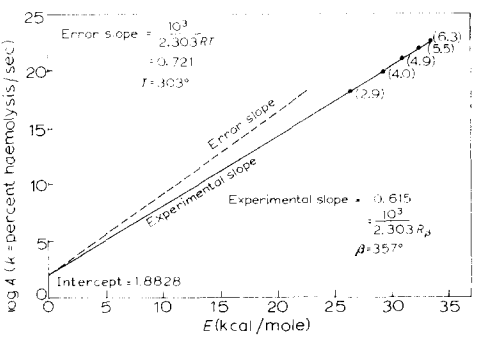

Fig. 4. Graph of log A *vs.* E for haemolysis in hypotonic solutions of glucose at 30°. The numbers in parenthesis refer to the osmotic concentrations at 30°.

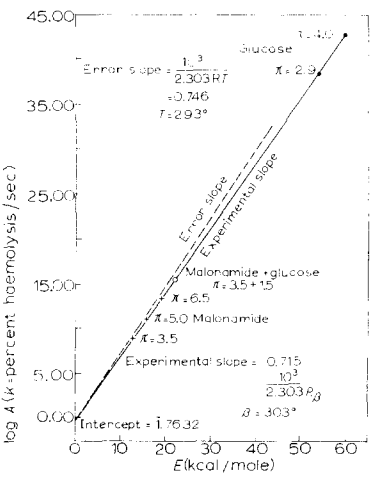

Fig. 5. Graph of log A *vs.* E for haemolysis in hypotonic solutions of glucose and hypotonic solutions of malonamide at 20°.

differs substantially from the error slope and that the isokinetic temperature (β) is, at 357°, much higher than the mean temperature of 303°. This is, therefore, a valid isokinetic relationship from which it may be inferred that the basic mechanism of haemolysis in hypotonic solutions of glucose at 30° does not vary with the osmotic concentration of the medium. Data are not available to compare with malonamide haemolysis at 30°, but the comparison has been made at 20°, as shown in Fig. 5.

In this graph the experimental slope is much closer to the error slope, but is still obviously different from it; the isokinetic temperature is no less than 13° higher than the mean temperature, so that once more the experimental relationship can be considered valid. The striking feature here is that the points for malonamide alone at three different osmotic concentrations, the point for glucose + malonamide and the two points for glucose alone are collinear, which means that haemolysis in all six systems occurs by an identical mechanism.

For the further evaluation of the haemolysis kinetics the EYRING activation parameters[6,37] have been derived from the rate data in the following way:

$$\Delta H^{\ddagger} = E - RT \tag{3}$$

$$\Delta S^{\ddagger} = 2.303R\left(\log A - \log \frac{V}{hN}\right) \tag{4}$$

In Eqn. 3 $\Delta H^{\ddagger}$ is the heat or enthalpy of activation, E is the Arrhenius activation energy and R and T are the gas constant and the absolute temperature respectively. In Eqn. 4 $\Delta S^{\ddagger}$ is the entropy of activation, R is again the gas constant, A is the non-exponential Arrhenius parameter, V is the molar volume of water, h is Planck's constant and N the Avogadro number. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, calculated from Eqns. 3 and 4 are recorded in Table III below. There are, of course, linear relationships

TABLE III

THE EYRING ACTIVATION PARAMETERS FOR GLUCOSE-INDUCED HAEMOLYSIS AT 20, 25 AND 30° AND FOR MALONAMIDE AT 20°

Mean experimental temp.	*Osmotic concn. (atm.)*	$\Delta H^{\ddagger}$ *(kcal/mole)*	$\Delta S^{\ddagger}$ *(cal/degree·mole)*
20°	2.9	53.6	160.0
	4.0	59.7	179.0
25°	2.9	34.8	96.4
	4.0	39.5	110.4
30°	2.9	25.9	66.7
	4.0	28.8	74.8
	4.9	30.5	79.6
	5.5	31.8	83.5
	6.3	32.9	86.3
20°	3.5	12.2	24.2
	5.0	15.5	35.0
	6.5	18.8	45.6
	3.5 + 1.5	21.6	55.3

between $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ that correspond with those between log A and E, but a plot of $\Delta H^{\ddagger}$ as the ordinate and $\Delta S^{\ddagger}$ as the abscissa (Fig. 6) is more informative with

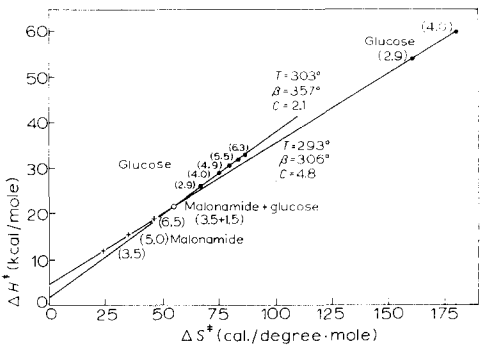

Fig. 6. Graph of $\Delta H^{\ddagger}$ *vs.* $\Delta S^{\ddagger}$ for glucose and malonamide and glucose + malonamide at 20°, and for glucose at 30°. Numbers in parenthesis refer to the respective osmotic concentrations.

regard to interpretation. As the figure shows the numerical values of the slopes of the graphs are the isokinetic temperatures and the general equation is,

$$\Delta H^{\ddagger} = \beta \Delta S^{\ddagger} + C \tag{5}$$

which very obviously resembles the thermodynamic relationship

$$\Delta H = T\Delta S + \Delta G \tag{6}$$

It is therefore inferred that the intercept C corresponds with the experimental free energy of activation $\Delta G^{\ddagger}$, so that this parameter is properly calculated from the equation

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - \beta \Delta S^{\ddagger} \tag{7}$$

and not as hitherto from

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \tag{8}$$

TABLE IV

THE EYRING FREE ENERGY OF ACTIVATION FOR GLUCOSE-INDUCED HAEMOLYSIS AT 20, 25 AND 30° AND FOR MALONAMIDE AT 20°

Mean temp. (°K)	*Isokinetic temp.* (°K)	$\Delta G^{\ddagger}$ (*from regn.*) (*kcal/mole*)	*Osmotic concn.* (*atm.*)	$\Delta G^{\ddagger}$ (*calc.*) (*kcal/mole*)
293	306	4.8	2.9	4.7
			4.0	4.9
298	336	2.4	2.9	2.5
			4.0	2.4
303	357	2.1	2.9	2.2
			4.0	2.2
			4.9	2.2
			5.5	2.1
			6.3	2.2
293	306	4.8	3.5	4.8
			5.0	4.8
			5.5	4.8
			3.5 + 1.5	4.7

which is, in effect, an error slope derivation. The free energies of activation, calculated from Eqn. 7, are recorded in Table IV. With both glucose and malonamide alone and glucose and malonamide together, $\Delta G^{\ddagger}$ is virtually constant at 4.8 kcal/mole and in the glucose systems $\Delta G^{\ddagger}$ decreases with increasing temperature. Although these results are a direct consequence of linearity between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, they add weight to the view that the mechanism of haemolysis in both glucose and malonamide solutions is identical, for it is the free energy of activation that governs reaction rates under specified conditions. That $\Delta G^{\ddagger}$ should decrease with increasing temperature suggests that the mechanism of haemolysis changes as the temperature rises.

DISCUSSION

The linear correlation

Linear enthalpy–entropy relationships are not uncommon in chemical kinetics and usually indicate conformity with the compensation law; linearity—and constant values of $\Delta G^{\ddagger}$—derive from mutual compensation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, since any change in the one parameter is exactly balanced by a corresponding change in the other. Compensation of this kind appears most frequently in sets of basically similar reactions which are accompanied by differing degrees of solvational change, and it is now believed that the solvation or hydration changes are the principal source of the effect[7]. According to this interpretation $\Delta H^{\ddagger}$ represents an overall heat of activation that is made up of two contributions, ΔH^{r}, the heat of activation of the basic reaction and ΔH^{h}, the head of activation of the associated hydration reaction, with $\Delta S^{\ddagger}$ similarly divided. The relative contributions of the two components can be appreciated by considering a simple example, the hydrolysis of a family of substituted aromatic esters. In the basic reaction the ester is converted to an acid and an alcohol and two molecules are produced from one, irrespective of the kind of substituent groups present; ΔS^{r} is essentially the same for the whole family. The presence of substituents, however, alters the strength of the ester bond, so that ΔH^{r} may be greater or less according to the effect of a particular substituent group. In this situation there is little correlation between ΔS^{r} and ΔH^{r}, because the former is largely independent of the latter. With ΔS^{h} and ΔH^{h}, on the other hand, there is a direct correlation, for both parameters refer to the same hydration structure; ΔS^{h} is a measure of the number of hydrogen bonds broken in the course of activation and ΔH^{h} is the heat content of these bonds. Despite the gross oversimplification, this picture suffices to show that the basic or intrinsic components of activation seldom contribute significantly to linearity within a group, whereas the hydrational contributions almost invariably dominate the overall correlation.

This generalization is probably no less valid in respect of the results presented here, for membrane penetration can be considered as equivalent to the intrinsic reaction and hydrational changes are undoubtedly involved; the kinetic approach therefore offers the possibility of separating hydration changes from other effects. It is, however, as well to remember that these hydrational changes are the net result of a variety of complex interactions that include water with water, water with solute and water with membrane, none of which are as yet fully understood.

The physical interactions of water

Although it is now fairly well accepted that water is a quasi-crystalline liquid[8], the fine details of its structure remain obscure, for it is uncertain whether the structure takes the form of discrete molecular clusters[9], or exists as a framework of hydrogen bonds extending throughout the liquid[10,11]. It has recently been suggested[12], however, that both types of structure occur in water, with the clusters predominating above 35° and the network persisting at lower temperatures. Because the half-life of these structures is several hundred times the period of a molecular vibration, the concept of order in water is valid at molecular level, but the structure that the order represents is dynamic rather than static. This flickering, statistical order depends on the intrinsically co-operative nature of the hydrogen bond[13] and is, on that account, extremely sensitive to temperature change; it is therefore not unlikely that many of the high temperature coefficients encountered in biological processes are due mainly to the effect of temperature on the structure of water within the system.

The interaction of solute with water is even more complex than water with water but, in general, it appears that solutes destroy the intrinsic co-operative order of water. This is particularly true of simple electrolytes[14], where the water dipole is radially orientated by the ion field, although there is evidence[15,16] that Li^+ and Mg^{2+} may promote structure in water at concentrations below 0.05 M. Other solutes, notably some inert gases, impose their own structure on water, which is essentially a network of clathrate cages[17]. The situation becomes still more complicated when the solute is capable of interacting directly with water by accepting or donating hydrogen atoms to form hydrogen bonds. In the simplest liquid–liquid systems of this kind (monohydric alcohols in dilute aqueous solution) the profound thermodynamic and other physical changes which occur can be related to the structural interactions of the two components[18]. With increasing numbers of hydroxyl substituent groups the physical anomalies become less prominent, so that polyhydric alcohols exhibit fewer abnormalities with increasing molecular size. This does not mean that the water interactions of the hydroxyl groups become less intense, but rather that the combined effect of multiple interactions confers increasing uniformity on the physical behaviour of their solutions. The total hydration of H-bonding solutes is, in principle, analogous with that of electrolytes[19] and may with advantage be similarly divided, for water hydrogen-bonded directly to the solute corresponds with primary hydration and that hydrogen-bonded to the primary hydration represents secondary hydration. In this context physical uniformity in solution depends mainly on secondary hydration, which of course tends to mask the primary hydration structure. The behaviour of polyhydric alcohols and sugars in the aqueous environment of biological systems could therefore be determined largely by their hydration characteristics.

Membrane hydration is perhaps the most difficult type of water interaction to elucidate, for there is as yet no entirely satisfactory way of measuring this property; however, developments in electron microscopy may soon contribute to the solution of this problem by providing direct visualization of biological systems in their natural hydrated state[20]. There is nevertheless a substantial body of opinion which supports the hypothesis that ordered water of hydration is a structural component of biological systems, including all membranes. KAVANAU[21] has postulated that the structural integrity of the cell membrane depends on an equilibrium between hydration and

lipids and FERNANDEZ-MORAN[22] suggests that membrane lipoproteins contain an interconnected hydrogen-bonded framework of water which permeates the entire structure. HECHTER[23] has proposed that the membrane surface protein consists of plane hexagonal subunits held together in two layers and attached to the polar heads of membrane lipids by double layers of wholly ordered water; moreover, since the external surfaces of cells incorporate sialomucins[24] which are intensely hydrophilic, the cell surface must also be heavily hydrated. Although it is at present hypothetical, the concept of ordered water as a structural component of cell membranes offers a new and hitherto unexplored approach to problems involving the permeability and integrity of cells.

The relevance of water interactions in glucose- and malonamide-induced haemolysis

In these experiments there is an initial prolytic lag phase in which the cells swell from biconcave discs to prolytic spheres. The rates as measured here refer therefore to the haemolysis of prolytic spheres in the relatively uniform cell population that constitutes the interquartile range of erythrocyte fragility[25]; for that reason it is assumed that the cell membrane is in a state of maximum hydration which is presumed to be constant. The concentrations of glucose range from 0.1 to 0.25 M, so that in these solutions there are from 550 to 220 molecules of water for each molecule of glucose; it is therefore assumed that glucose (and malonamide) are in their maximum hydration states also. Glucose is, of course, more heavily hydrated than malonamide, because its heat of solution, a measure of total solute–water interaction, approaches —3 kcal at 20–25° (ref. 26), whereas that of malonamide is more likely to be around —1 kcal[27]. Total water interaction depends, of course, on molecular structure and conformation. Glucose has five groups, all hydroxyls, which are capable of hydrogen bonding with water; malonamide has four, two amino and two carbonyl groups. In solution glucose adopts the pyranose ring structure and the C1 chair conformation[28], which is compatible with the hexagonal arrangement of nearest neighbours in the water structure[29]. This does not mean that glucose can substitute for a water hexagon in an undisturbed water lattice, because the planes in which the oxygen atoms of primary hydration are located, one above and one below the plane of the pyranose ring, are only about half as far apart as the corresponding planes in the native water structure[29]; it does mean, however, that heavy secondary hydration, in the form of stabilized water structure, will extend from each surface of the pyranose ring. The conformation of malonamide is not known, but it might reasonably be supposed that rotation about C–C bonds will permit the adoption of that arrangement which leads to the formation of the maximum number of hydrogen bonds, and hence to the minimum potential energy with respect to solute and solvent; the configuration of the primary hydration could well be helical, so that the secondary hydration could again be stabilized water structure[30]. The physical properties of these solutes are therefore such that their presence at the cell surface and in the cell membrane can hardly but increase the degree of order at these sites; the high temperature dependence of haemolysis in these systems and their conformity with the compensation law are the direct consequences of this. The increasing deviation (at 30° compared with 20°) of the isokinetic temperature from the mean experimental temperature (Fig. 6) is another consequence of the predominance of hydration structure effects in the mechanism of haemolysis. Although $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are both much lower at 30° than at 20°,

the effect on $\Delta S^{\ddagger}$ is proportionally greater, so the slope is greater and this implies that order–disorder transitions assume lower significance at 30°. That is to say the process becomes less hydrationally controlled at the higher temperature, which is to be expected from the thermal instability of hydration structures.

The hydrational components, which provide the major contributions to $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, are largely self-cancelling in $\Delta G^{\ddagger}$, so this parameter is more independent of solvent participation than the other two and reflects more nearly the effect of factors other than hydration on the basic mechanism of the process[7]. A decrease in $\Delta G^{\ddagger}$ with increasing temperature implies, therefore, a change in basic mechanism as the temperature rises and it may be that this change is connected with membrane stability. The molecular architecture of the membrane of the prolytic sphere is considerably distorted and probably exists in a state of metastable equilibrium even at the lower temperature; in this circumstance increasing the temperature will increase instability and contribute to reducing $\Delta G^{\ddagger}$. The constant values of $\Delta G^{\ddagger}$ for glucose and malonamide at 20° also support this view, for they indicate that the membrane is unable to discriminate between the two solutes as primary hydrated entities.

The proposition that both glucose and malonamide enter the human erythrocyte by an identical mechanism is less at odds with current thought[31–33] than may be apparent at first sight, for it is well known that even among the sugars transfer specificity depends more on molecular conformation than on molecular configuration[34,35]. LEFEVRE[34] has in fact suggested that attention to the physical properties of molecular assemblies may be more rewarding than preoccupation with specific chemical groupings. In this context, however, it is usually overlooked that the predominant form of intermolecular association in biological systems is water and that the simplest and most common type of molecular assembly occurs between solute and water hydration. The suggestion made here is that molecular species which appear to be radically different in configuration may adopt conformations in solution that differ less, and thus have secondary hydration structures that actually resemble each other.

The principal conclusion of this work is that hydration is an extremely important factor in haemolysis kinetics and there is nothing to suggest that it should be any less significant in erythrocyte transport processes generally. It may be that the discrepancies discovered by a recent computerized approach to monosaccharide transfer in human erythrocytes[36] are due mainly to the absence of terms for the hydration variables.

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