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THE ROLE OF HYDRATION IN THE EFFECT OF BARBITURATES ON THE HAEMOLYSIS OF RABBIT ERYTHROCYTES

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SUMMARY

1. An evaluation has been made of the effect of barbiturates on the temperature dependence of haemolysis of rabbit erythrocytes.

2. It has been found that the kinetics conform with the compensation law, and this shows that hydrational effects predominate in the mechanism of the process.

3. The results indicate that the alkyl groups of barbiturates support apolar hydration, and the suggestion is made that this may be connected with pharmacological activity.

INTRODUCTION

Previous studies have shown that the activation parameters of haemolysis kinetics depend on the molecular structure of the haemolysing solute[1,2], and that the presence of a second solute in small amounts produces measureable changes in the rate of haemolysis which vary according to the solute species added[3-5]; these effects were accounted for in terms of solute–water or hydrational interactions. More recently it has been shown[6] that the malonamide-induced haemolysis of mammalian erythrocytes conforms with the compensation law, and a reappraisal[7] of the earlier work[1,2] indicates that this does so, too. Since the compensation which gives the law its name arises almost exclusively from hydration structure change[8] and because the law appears to hold generally in haemolysis kinetics, this approach offers some prospect of evaluating the role of hydration in biological processes.

In order to investigate this point, a study has been made of the effect of barbituric acid and six of its derivatives on the kinetics of malonamide-induced haemolysis. Rabbit erythrocytes were chosen because they are the most heavily hydrated species of mammalian red cell[6], and the barbiturates were selected because they provide a range of closely related molecular structures that are biologically active at low concentrations.

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MATERIALS AND METHODS

Preparation of the blood

The blood for these experiments was taken by ear-vein incision from normal healthy rabbits, with approx. 1 unit per ml low sodium heparin as anticoagulant. Immediately after its withdrawal, the blood was equilibrated with moist oxygen at atmospheric pressure, to reduce the fragility variable[9], and it was maintained in this condition until required for experiment. Apart from oxygenation, the blood received no other treatment before use.

Preparation of haemolysing solutions

Laboratory reagent grade BDH malonamide (m.p. 178°) was used without further purification for the haemolysing solutions, but the material was dried and stored *in vacuo* over conc. H_2SO_4 before use. Since mammalian erythrocytes are highly osmosensitive and because the osmotic pressure depends on the absolute temperature, it is necessary in kinetic studies to ensure constant osmotic pressure rather than constant concentration; this condition has been met here, and the osmotic concentrations (expressed in atm) were derived in the way already described[2]. The barbiturates employed were commercially available substances of pharmacological purity (British Pharmacopoeia) and included phenobarbitone, barbitone and barbituric acid (British Drug Houses); amylobarbitone and quinalbarbitone (Lilly); butobarbitone and hexobarbitone (May and Baker). Because these substances are relatively insoluble, their effective concentrations make a negligible contribution to the osmotic pressure of the haemolysing system; the concentration employed (0.5 mM) was determined by the least soluble member of the group, and this contributes less than 0.02 atm to the total osmotic pressure. For test purposes the barbiturates were made up in malonamide solutions of appropriate osmotic concentrations, and fresh solutions were prepared daily.

Experimental methods

The methods have been described in detail elsewhere[2] and need only brief mention here. The same dilution of blood was employed, 1 part whole blood to 20 parts haemolysing solution, and the degree of haemolysis was determined by the alkaline haematin method[10]. Haemolysis curves were constructed by plotting per cent haemolysis to a base of time, and the rate of haemolysis was taken as the slope of the graph between 25 and 75% haemolysis; this utilizes the most sensitive section of the rate curve, the interquartile range of the cumulative frequency distribution of erythrocyte fragility[11].

RESULTS

The effect of barbiturates

The action that barbiturates exert on malonamide-induced haemolysis at constant osmotic concentration and constant temperature is illustrated in Fig. 1; at this concentration all seven substances affect both the onset and rate of haemolysis. At lower concentrations of malonamide, the effectiveness of the response decreases, and both the onset and the rate of haemolysis move to the left as the osmotic concentration is reduced.

The effect of temperature on drug-containing systems is illustrated in Fig. 2 with hexobarbitone and phenobarbitone. The former is much less effective than the latter at 15°, so there is a considerable difference in the temperature dependence of the action of these two drugs.

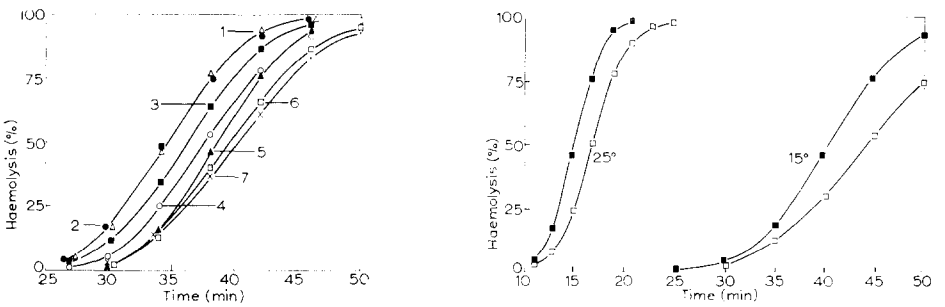


Fig. 1. The effect of 0.5 mM barbiturates on the malonamide-induced haemolysis of rabbit erythrocytes at 6.5 atm osmotic concentration and 20°. 1, amylobarbitone; 2, butobarbitone; 3, hexobarbitone; 4, barbitone; 5, quinalbarbitone; 6, phenobarbitone; 7, barbituric acid.

Fig. 2. The effect of temperature on the haemolysis of rabbit erythrocytes in malonamide solutions of 5.0 atm with 0.5 mM hexobarbitone and phenobarbitone. ■, hexobarbitone; □, phenobarbitone.

The kinetic evaluation

In order to allow for normal biological variation, a number of experiments were conducted with each drug at 15, 20 and 25° and at several concentrations of malonamide. The rate data were then assembled into Arrhenius-type plots in which the ordinate is the common logarithm of the rate of haemolysis (expressed as per cent haemolysis per min), and the abscissa is the reciprocal of the absolute temperature. These graphs represent the logarithmic form of the Arrhenius equation[12], and the Arrhenius activation parameters are found from their slopes and intercepts. For precise evaluation of the kinetics the slopes and intercepts were calculated by regression analysis and are recorded in Table I, together with the relevant statistics.

These correlations (most of which are significant at the 0.1% probability level) show that in the presence of barbiturates, the slope m and the intercept C are lower than the controls, and that with a given drug, decreasing the osmotic concentration decreases both slope and intercept. The Arrhenius activation parameters were calculated at 20° from the relationships $E = 2.303Rm$ and $\log A = C - 1.7782$, where $\log A$ refers to rate measurement in per cent haemolysis per sec. The activation energies thus calculated are illustrated in Fig. 3, which shows that E depends on drug species and varies linearly with the osmotic concentration of the haemolysing solution. $\log A$ varies similarly with concentration, and there are linear correlations between $\log A$ and E (which are not illustrated) that indicate conformity with the compensation law.

In order to provide a more comprehensive evaluation of the haemolysis kinetics, the Eyring activation parameters[13] were derived from their Arrhenius counterparts as follows:

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

$$\Delta S^{\ddagger} = 2.303R\left(\log A - \log \frac{\mathrm{I}'}{\mathrm{h}N}\right) \tag{2}$$

TABLE I

REGRESSION ANALYSIS OF THE EQUATION $\log k = \frac{-m}{T} + C$

Drug (*0.5 mM*)	*Osmotic concn.* (*atm*)	*Slope* (*-m*)	*Intercept* (*C*)	*Correlation coefficient* (*-r*)	*Standard error of estimate* (*Slog k*)	*Degrees of freedom* (*N-2*)	*Significance level* (*P*)
None (control)	3.5	3.8968	14.4070	0.883	0.0878	16	0.001
	5.0	4.5956	16.6783	0.953	0.0598	14	0.001
	6.5	5.3114	19.0464	0.899	0.0996	19	0.001
Pheno-barbitone	3.5	3.6804	13.6530	0.877	0.0905	12	0.001
	5.0	4.3522	15.8051	0.982	0.0420	16	0.001
	6.5	5.0236	17.9745	0.933	0.0868	11	0.001
	7.5	5.4924	19.4590	0.937	0.1098	6	0.001
Barbitone	3.5	3.5572	13.2802	0.854	0.0965	13	0.001
	5.0	4.2601	15.5236	0.839	0.1252	11	0.001
	6.5	5.0135	18.0268	0.783	0.1683	11	0.01
	7.5	5.2543	18.7104	0.944	0.0709	5	0.01
Amylo-barbitone	3.5	3.4962	13.1065	0.916	0.0275	10	0.001
	5.0	4.1466	15.1581	0.937	0.0685	10	0.001
	6.5	4.7260	17.0793	0.858	0.1117	9	0.01
	7.5	5.1256	18.2889	0.933	0.0763	4	0.01
Buto-barbitone	3.5	3.5472	13.2195	0.887	0.0827	11	0.001
	5.0	4.0391	14.8404	0.855	0.1377	9	0.001
	6.5	4.6000	16.6325	0.838	0.1174	9	0.01
	7.5	4.9624	17.7210	0.926	0.1089	6	0.001
Hexo-barbitone	3.5	3.1658	11.9410	0.949	0.0433	9	0.001
	5.0	3.6578	13.5039	0.907	0.0731	10	0.001
	6.5	4.1766	15.1581	0.918	0.0779	10	0.001
	7.5	4.4350	15.8859	0.921	0.0932	4	0.01
Quinal-barbitone	3.5	2.8270	10.8638	0.940	0.0455	10	0.001
	5.0	3.3065	12.1983	0.911	0.0662	7	0.001
	6.5	3.5300	12.9138	0.855	0.0935	10	0.001
	7.5	3.7255	13.4967	0.889	0.1021	6	0.01
Barbituric acid	3.5	3.3308	12.3841	0.912	0.0779	10	0.001
	5.0	3.7738	13.7867	0.986	0.0150	8	0.001
	6.5	4.3006	15.4972	0.915	0.0855	11	0.001
	7.5	4.5413	16.3228	0.891	0.1240	6	0.01

$\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the heat and entropy of activation, respectively, and for reasons that have been discussed elsewhere[14], this derivation employs the Eyring equation in the form developed for fluid flow[15].

These activation parameters vary with concentration in the same way as E and $\log A$, from which they were derived, but the Eyring compensation plot (Fig. 4) is more informative for interpretation than the corresponding Arrhenius correlations, because the equation of the relationship between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ can be written:

$$\Delta H^{\ddagger} = \beta \Delta S^{\ddagger} + \Delta G^{\ddagger} \qquad (3)$$

This permits the calculation of $\Delta G^{\ddagger}$, the free energy of activation, and β, the isokinetic temperature[16]; if the value of β corresponds with the mean temperature of the experiment, the correlation is fortuitous and without physical meaning[17]. Since the figure indicates the probable existence of a number of distinct correlations, each system was examined separately by regression analysis to find the values of β and $\Delta G^{\ddagger}$; the values thus derived together with those of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are recorded in Table II.

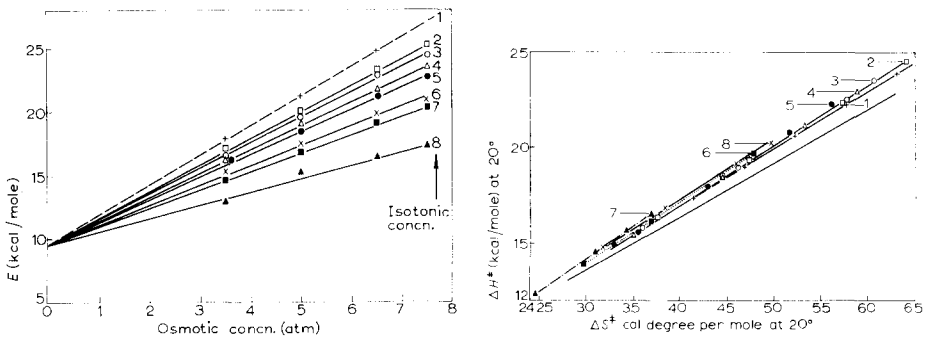


Fig. 3. The relationship between the Arrhenius activation energy, E, and the osmotic concentration of the haemolysing solution for the different barbiturates. 1, control; 2, phenobarbitone 3, barbitone; 4, amylobarbitone; 5, butobarbitone; 6, hexobarbitone; 7, quinalbarbitone; 8 barbituric acid. Arrow: isotonic concn. (7.64 atm at 20°).

Fig. 4. The correlations between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the barbiturates in different osmotic concentrations of malonamide; the arrows indicate malonamide concentrations of 6.5 atm and the error slope is 293° K. 1, control; 2, phenobarbitone; 3, barbitone; 4, amylobarbitone; 5, butobarbitone; 6, hexobarbitone; 7, quinalbarbitone; 8, barbituric acid.

TABLE II

THE EYRING ACTIVATION PARAMETERS FOR 7.5 atm OSMOTIC CONCENTRATION AT 20°

Mean temperature T = 293° K; β = isokinetic temperature.

Drug (*0.5 mM*)	$\Delta H^{\ddagger}$ (*kcal/mole*)	$\Delta S^{\ddagger}$ (*cal/degree·mole*)	β (°K)	$\Delta G^{\ddagger}$ (*kcal/mole*)
None (control)	26.1	70.2	304	4.8
Phenobarbitone	24.6	64.2	312	4.5
Barbitone	23.5	60.7	312	4.5
Amylobarbitone	22.9	58.8	312	4.5
Butobarbitone	22.1	56.2	315	4.4
Barbituric acid	20.2	49.8	312	4.7
Hexobarbitone	19.7	47.8	320	4.3
Quinalbarbitone	16.5	36.9	346	3.7

The variation in the values of the slopes (β) and the intercepts ($\Delta G^{\ddagger}$) confirm that six distinct correlations contribute to the overall trend exhibited in Fig. 4. These are the control, a group comprising phenobarbitone, barbitone and amylobarbitone (identical slopes and intercepts) and separate correlations for the remaining four substances.

Partition coefficients

In view of the possible importance of lipid solubility in this work, the partition coefficients of the substances employed were measured in the chloroform–borate buffer system (pH 7.9) at 20°. The details are not recorded because the order found here is in good agreement with other published work[18,19]. The increasing order of lipid solubility (concentration ratio in chloroform and buffer) is: barbituric acid, 0.015; malonamide, 0.270; barbitone, 0.381; phenobarbitone, 1.13; butobarbitone, 5.62; amylobarbitone, 17.3; quinalbarbitone, 33.6; hexobarbitone, 151. There is no correlation between lipid solubility at 20° and the activation parameters calculated at the same temperature.

DISCUSSION

The linear correlations

The linear relationships presented here differ materially from the error slope and are therefore valid examples of the compensation law. This supports the view that hydrational effects predominate in the basic mechanism of the process, which is the penetration of rabbit erythrocytes by malonamide and barbiturates. At constant values of β, the higher a point on the correlation, the greater the total hydration structure change, and conversely, because $\Delta S^{\ddagger}$ is a measure of the number of hydrogen bonds broken during activation and $\Delta H^{\ddagger}$ refers to their heat content. An increase in β shows that the system has moved further towards solute control[8] and implies a decrease in the role of hydration in the mechanism of the process. Finally, changes in the intercept reflect changes in the intrinsic component of the overall process because the hydrational contribution cancels out in $\Delta G^{\ddagger}$.

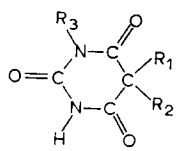
All seven substances used here exhibit these changes, and since the same dilution of rabbit blood and the same haemolysing solute were used throughout, it is inferred that the differences observed are due mainly to the effect of barbiturates on the hydration structure of the rabbit erythrocyte membrane. In this context the decreasing numerical order of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ would follow from increasing hydration-structure breaking by barbiturates, because the more effective the structure breaker the more disordered the preactivation state and the smaller the increment in disorder required for activation. The increasing numerical order of β also corresponds with structure-breaking capacity, for increasing disruption of membrane hydration reduces the sensitivity of $\Delta H^{\ddagger}$ to $\Delta S^{\ddagger}$. The intercept $\Delta G^{\ddagger}$ refers to the hypothetical state in which the hydrational contribution is minimal, so the effect of factors other than hydration, *e.g.*, polarity and Van der Waals forces, becomes more accessible. In this circumstance a relationship between $\Delta G^{\ddagger}$ and the partition coefficient might reasonably have been expected; the absence of any such correlation seems to exclude the possibility that the intrinsic component of barbiturate permeability depends only on lipid solubility.

Although the barbiturates are all membrane hydration-structure breakers, the order in which they effect this is not simply related to molecular weight or structure. Phenobarbitone and hexobarbitone have similar molecular weights but differ greatly in structure-breaking capacity. On the other hand, phenobarbitone and amylobarbitone are structurally distinct from barbitone (Fig. 5), yet the strict collinearity

of their compensation plots indicates an identical mode of action that depends on a common mechanism for the water interactions of these three barbiturates.

The water interactions of barbiturates

The basic structure of barbiturates is a ring condensation product of urea and malonic acid (Fig. 5), the latter being the parent substance of malonamide also. Because urea is a very powerful water-structure breaker[1] and malonamide is a weak structure promoter[2], the derivative is most probably a structure-breaking solute. This would account for the increase in β from 304° (control) to 312° when barbituric acid is present. In the presence of phenobarbitone, barbitone and amylobarbitone β is unchanged at 312°, suggesting that their hydration-structure breaking capacity also depends mainly on the water interaction of the basic ring. Alkylation reduces $\Delta G^{\ddagger}$, however, from 4.7 to 4.5, and this may be connected with the suppression of ionization in the derivatives because, at the pH of these experiments (7 ± 0.2), barbituric acid is fully dissociated, whereas the drugs are only 10–20% ionized[20].



Drug	R_1	R_2	R_3
Phenobarbitone	CH_3CH_2-	C_6H_5-	H–
Barbitone	CH_3CH_2-	CH_3CH_2-	H–
Amylobarbitone	CH_3CH_2-	$(CH_3)_2CHCH_2CH_2-$	H–
Butobarbitone	CH_3CH_2-	$CH_3CH_2CH(CH_3)-$	H–
Barbituric acid	H–	H–	H–
Hexobarbitone	CH_3-	C_6H_9-	CH_3-
Quinalbarbitone	$CH_2=CHCH_2-$	$CH_3CH_2CH_2CH(CH_3)-$	H–

Fig. 5. Barbiturate structures.

The three drugs, which differ only in their R_2 substituents, appear to become less structure breaking in the order amylobarbitone, barbitone, phenobarbitone (the order of increasing $\Delta S^{\ddagger}$) and this anomaly cannot readily be explained without assigning structure-promoting capacity to the alkyl groups. This type of behaviour (apolar hydration) is well known[21], however, and its occurrence depends on the structure of the solute and the absence of a direct interaction between solute and water; the solute behaves essentially as an inert internal support that maintains the first layer of surrounding water molecules in tetrahedral configuration, thus favouring the formation of pentagonal polyhedral hydrogen-bonded structures or clathrate cages of water. The capacity of water to form such cages is almost limitless, and studies with

alkyl-substituted ammonium salts show that entities as large as the tetraisobutyl group[22] and the benzene ring[23] can be enclosed by water in this way. There is good reason for supposing that cages (or partial cages) of this form around the alkyl groups of barbiturates, for with the exception of the *sec.*-butyl, *sec.*-amyl and cyclohexenyl groups (which are pure structure breakers) these alkyl groups are known supporters of apolar hydration[24]. This is the most likely source of increasing hydration structure in the group, amylobarbitone, barbitone, phenobarbitone, and the order of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ depends on the size and type of clathrate cages formed.

The situation is different with the remaining drugs, because each carries a structure-breaking alkyl substituent that disrupts hydration and increases β. The structure-promoting contributions of the apolar hydrate groups become smaller too, for at constant osmotic concentration, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ decrease in the order butobarbitone, hexobarbitone, quinalbarbitone. The extreme position of quinalbarbitone is probably due to interference with the allyl apolar hydrate caused by the presence of the *sec.*-amyl group on the same carbon atom; this effect is less prominent in butobarbitone, where the apolar clathrate is more compact and the structure-breaking group is less effective, and it does not occur at all in hexobarbitone because the three alkyl groups are on separate carbon atoms.

The pharmacological implications

With regard to pharmacological activity, these drugs have been classified in terms of duration of action[25]; phenobarbitone and barbitone are long-acting, amylobarbitone and butobarbitone are intermediate, and hexobarbitone and quinalbarbitone are short- and ultra-short-acting, respectively. The validity of this classification has recently been questioned[26], but it is noteworthy that duration of action corresponds with the decreasing order of the activation parameters; this agreement is hardly fortuitous because the lower these are, the more rapid the passage of the drug through cells and tissues.

It may also be significant that, although the effect of barbituric acid on hydration resembles that of short-acting drugs, this substance is without pharmacological activity; ionization is almost certainly one factor responsible for this, but the absence of alkyl groups could be another because hypnotic activity may depend more directly on apolar hydration, in accord with the PAULING–MILLER hydrate theory of anaesthesia[27,28].

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