The Haemolysis of Human Erythrocytes in Relation to the Lattice Structure of Water

by

William Good, B.Sc.

T h e s i s

presented for the Degree of

Doctor of Philosophy

in

The University of Glasgow.

April, 1954.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgments

My thanks are due to the University of Glasgow for the appointment which enabled me to carry out this work.

I also wish to thank Professor J.N. Davidson, whose continued interest and advice have been to me a constant source of encouragement throughout this period.

I am indebted to Dr. E.B. Hendry for the hospitality of his laboratory and the introduction to the problems of haemolysis: I am grateful also for his advice and criticism during the course of this work.

Contents

Page

Section 4. Discussion

Section 1.

Introduction.

1.1. General

By virtue of their relatively limited metabolism and function, mammalian erythrocytes are suitable subjects for the investigation of permeability processes. Specimens of blood withdrawn from the body contain these cells in their immediate natural environment and are also statistical samples of the total cell population. Physical or chemical alteration of the environment produces certain well defined changes in cell characteristics, e.g., shape, volume, relative translucency or haemolysis. Such changes are amenable to physical measurement and provide methods for the quantitative assessment of the response of the cells to a modified environment. The term, haemolysis, refers to the disorganisation of the erythrocyte - plasma interface and the consequent release of haemoglobin.

The liberation of haemoglobin must be regarded as the extreme case of permeability, since there occurs the passage of solute across an apparently intact membrane. Thus haemolysis produced under controlled conditions gives information about the permeability of the membrane; in some cases the data may suggest membrane structure patterns also. However the loss of haemoglobin may occur, since it is brought about by conditions which are the result of permeability

changes, haemolysis may be regarded as a convenient end point in the permeability process.

1.2. Permeability of Erythrocytes

That the normal human erythrocyte is freely permeable to water has been shown by Wakeman et al. (1927). These authors have shown that defibrinated blood may be diluted up to one part in five with water before haemolysis occurs. More recently Govaerts and Lambrechts (1946) demonstrated that within one minute of adding HDO to whole blood, the intra- and extra-cellular fluid densities are equal. Their observations imply a rapid movement of water, since it is known that HDO penetrates the cell more slowly than water itself (Parpart (1935), Brooks (1935)). The last named authors record that no toxic effects were observed when using deuterium oxide. Hevesy and Jacobsen (1941), using the rabbit, found that within 30 minutes injected HDO is equally distributed between intra- and extra-cellular fluid. From these considerations it appears that a dynamic equilibrium exists, as far as water is concerned, between cells and plasma. Changes in the equilibrium conditions are opposed immediately by a rapid redistribution of water.

1.3. Permeability of Erythrocutes to Ions

(a) Anions

Since the elucidation of the chloride shift about sixty years ago it has been accepted that bicarbonate and chloride ions cross the erythrocyte membrane very rapidly indeed. For this exchange Dirken and Mook (1931) have shown that equilibrium is reached within 0.4 second. It is now held that the red cell is unique in having a high degree of permeability to inorganic monovalent anions, coupled with a very much lower permeability to cations. Experimental evidence points to this although problems of manipulative technique render direct confirmation difficult (Smith et al. (1941); Hahn and Hevesy (1942)).

The inorganic polyvalent anions penetrate more slowly and are rather more complex in their behaviour. Such anions, on penetration, require a corresponding movement of two or more monovalent ions to maintain electrical neutrality, thus inducing cell shrinkage. They have also a dehydrating effect on cell colloids (Höber 1945). Of the polyvalent anions most work has been done with phosphate but the results are extremely difficult to interpret since cellular metabolism seems to be extensively involved. Hahn and Hevesy (1942) found that phosphate penetration has a high temperature coefficient. Raising the temperature from 0°C to 37°C in-

creases the rate of phosphate penetration by a factor of 14. They claim that the concentration gradient seems to have little effect on the direction of transfer. Tulin et al. (1947) confirm that phosphate can move against a concentration gradient and also that the transfer is practically abolished at 4°C. With respect to the effects of metabolic inhibitors the picture is complicated, but there is general agreement that these substances interfere with phosphate transfer (Mueller and Hastings (1951); Gourley (1951)). It has been suggested (Bartlett and Marlow (1951)) that the glycolytic process is so synchronised that there exists an equivalence in glucose uptake, lactic acid production, phosphate intake and phosphate output. It is therefore essential to take into consideration the metabolic state of the cell when interpreting phosphate permeability data.

Sulphate is the only other inorganic polyvalent anion which has been investigated to any extent. A number of authors consider the human erythrocyte to be impermeable to sulphate ion (Bourdillon and Lavietes (1936); Hald et al. (1948)). On the other hand, Mond and Gertz (1929) find the rate of exchange of sulphate for chloride in man exceeds that of the ox. Since the time to reach this equilibrium for the ox is given by Parpart (1940) as 50 seconds, the exchange ought to be faster in man. The evidence either

way is rather poor and no real conclusion can be drawn.

(b) Cations

At one time the erythrocyte membrane was believed to be cation impermeable. This view now has been abandoned. In the case of potassium there is a turnover of about 3 per cent per hour at 37°C (Mullins et al. (1942)), with a slightly lower rate for sodium. With these cations, as with phosphate, metabolic activity seems to be involved (Danowski (1941); Harris (1941)). Sodium and potassium diffuse slowly with their respective concentration gradients at 7°C. At 37°C this process can be reversed to movement against the concentration gradients, provided glucose is present. Further to this view is the fact that metabolic poisons enhance sodium and potassium leakage from the cell (Wilbrandt (1937), Davson (1941)). The problem of potassium transfer has been considered in detail by Maizels and Harris (1952), who conclude that its uptake depends on carriage by chemical groups. They also suggest that potassium movement inwards is possibly related to sodium movement outwards. It therefore appears, in common with phosphate, that potassium permeability is a function of the metabolic state of the cell. This may hold also, though to a lesser degree, with sodium ion. Compared to the monovalent anions, the permeability of sodium and potassium is negligibly low.

Little information exists concerning the permeability of the alkaline earth cations. With magnesium it has been suggested (Wolf, 1939) that increase in the external concentration above that obtaining within the cell causes this ion to penetrate the membrane. This effect may account for the peculiar results of Smith et al. (1939) who could not account quantitatively for parenterally administered magnesium sulphate. Although there is practically no information concerning the permeability of the alkaline earth cations, there does exist a vast amount of literature which discusses the effects of calcium and magnesium on the permeability of other substances. The necessity for these ions, in optimal concentrations, for the normal function of natural membranes, has been appreciated since the introduction of Ringer's solution. Their mode of action however, still remains obscure.

1.4. Permeability of Erythrocytes to Non-electrolytes

Although studies on the permeability of organic non-electrolytes were initiated over fifty years ago (Grijns (1896), Hedin (1897), (1898)), there has been no systematic follow-up of this work. The above authors determined, qualitatively, that substances with similar chemical groupings behave similarly. They found for carbohydrates that the disaccharides do not penetrate, the five and six carbon

sugars penetrate only slowly, if at all, while the three carbon members penetrate more quickly and at rates which vary with their number of substituent hydroxyl groups. The aliphatic aldehydes, ketones and ethers, together with the ureas and amides penetrate very rapidly indeed. Such later work as has been done confirms these findings (Kozawa (1914) and Wilbrandt (1938)) for the sugars and also for the amides and ureas (Höber and Orskov (1933); Jacobs et al. (1935), Klinghoffer (1935)). Orskov (1946) has reported the distribution of some of these substances between cells and plasma.

It is felt that interesting information may be obtained by investigating the haemolytic properties of a series of amides, and substituted ureas, together with certain related substances.

1.5. Theories of Natural Membrane Structure

Certain observations on the permeability of plant and animal cells to non-electrolytes led Overton (1902) to advance the lipid solubility theory of cell penetration. This approach, that cell penetration by a substance is a function of lipid solubility, gained weight from numerous comparisons of solute penetration with solute distribution between ether and water (Ruhland and Hoffman (1925)) or

olive oil and water (Collander and Barlund (1933)). The theory breaks down when the rapid penetration of non lipid soluble substances such as ureas, amides is considered.

Inadequacies of the lipid theory led to the postulation of the pore theory, in which permeability is a function of membrane pore size with respect to solute molecular volume. From this point of view small molecules would be expected to penetrate rapidly. The pore theory is based mainly on work with artificial membranes (Michaelis and Fujita (1926); Höber (1936)) and since pore size is not uniform for such membranes, some form of selection is to be expected. The selective permeability of natural membranes does not vary uniformly with changing molecular volume and the theory breaks down with increasing lipid solubility of the penetrating molecule.

Collander (1926) proposed a combination of the above concepts into the lipid-sieve theory. The cell surface is visualised as a mosaic of lipid and sieve-like areas. Non-lipid soluble material is conceived as penetrating by the sieve-like areas, where molecular volume and pore diameter are the governing factors. Lipid soluble substances will tend to penetrate by these areas for which they have greater affinity.

Danielli (1952) considers that the cell surface may be formed from a double layer of lipid molecules, the hydrocarbon residues of which are arranged inwards to form what amounts to a central film of liquid hydrocarbon within the membrane. The polar residues face towards the inner and outer surfaces of the membrane. These radially orientated lipid molecules carry adsorbed protein in tangential arrangement. Using this model Danielli has made considerable advances in the interpretation of permeability processes from the physico-chemical point of view.

It has been proposed by Parpart and Ballentine (1952) that the plasma membrane is a three dimensional protein stroma possibly two molecules thick. Interspersed in this structure are lipid molecules, bound to the protein and to each other. They suggest that the pores formed by this network are modified by the presence of peripheral "hinged" lipid molecules, able to swing trap door fashion, thus modifying pore size. This model enables the authors to explain the post haemolytic return to semipermeability, the permeability of haemoglobin and several other phenomena.

From the foregoing considerations, it can be seen that no one theory of membrane structure is all-embracing. Each has its defects and each its triumphs. There apparently remains to be discovered a fundamental approach to the

related problems of structure and permeability.

1.6. Erythrocytes and Osmosis

Owing to the semipermeable properties of its membrane, the erythrocyte is a highly sensitive osmometer. It follows, therefore, that experimental conditions must include rigorous control of the osmotic pressure of the red cell environment.

The freezing point depression of normal serum is approximately 0.560°C., and this corresponds to an osmotic pressure of 7.2 atmospheres at 20°C (see Methods Section). When whole blood is diluted the plasma solutes contribute to the osmotic pressure of the resulting suspending fluid. If, for illustration, a packed cell volume of 43 per cent is assumed for whole blood, then at a dilution of 1:21, the plasma contribution will be 0.19 atmosphere. Upon haemolysis the intra-cellular solutes, isotonic with plasma (Mukai (1921); Collins and Scott (1932)), also contribute to the osmotic pressure of a haemolysing system. This factor has been discussed by Hendry (1949) who calculated that the osmotic pressure movement is of the order of 0.015 atmosphere for each 10 per cent haemolysis. Thus, in a haemolysing system of constant dilution, the osmotic effect of added plasma and liberated cell contents is constant and with normal blood it may be neglected.

The physical changes which are the result of osmotic imbalance are relatively clear. Reduction of the extracellular osmotic pressure causes water to pass into the cell at a greater rate than it leaves. Thus the osmotic effect is compensated by the reduction of intra-cellular solute concentration. As the process continues the cell swells, initially without change in surface area, later with increasing surface area and finally undergoes haemolysis. In any hypotonic medium, therefore, an excess of water enters the cell in an endeavour to equalise the internal and external osmotic pressures. Similarly, in any hypertonic medium, excess water will leave the cell, which returns to osmotic equilibrium by concentration of intracellular fluid. Such will be the reaction of erythrocytes in any non-isotonic aqueous medium containing solute to which the membrane is selectively impermeable.

1.7. The Disc-sphere Transformation of Erythrocytes.

The shape changes occurring in the erythrocyte are the result of the capacity of the membrane to undergo mechanical deformation without rupture. This deformation is due to osmotic forces which arise from the semipermeability of the membrane and its concomitant free permeability to water.

Sphering can occur under a variety of different

conditions, not all of which can be attributed directly to osmotic forces. Practically all lysins, whether of biological or other origin, saponins, detergents, etc., induce sphering when present in concentrations of only a few molecules per cell. The phenomenon may be induced by physical means, such as temperature and pH changes.

The transformation appears to take place in the tissues also, as part of the normal mechanism of cell destruction. Knisely (1936) observed the sphering of erythrocytes after they had undergone a period of sequestration in the spleen. Though the spleen is not now regarded as an organ of cell destruction, there is agreement that splenic sequestration selectively increases the mechanical and osmotic fragility of foreign and effete cells (Young (1947)). Such action could be achieved by the removal of antisphering substance (Furchgott (1940); Furchgott and Ponder (1940); Furchgott and Ponder (1941)) by the spleen. There is, thus, evidence that sphering occurs during normal intravascular haemolysis. From these considerations it would appear that sphering is an essential prerequisite to lysis under all conditions.

The disc-sphere transformation takes place in a series of steps and Ponder (1948) gives a detailed discussion

of the shape changes for haemolysis by very dilute saponin solutions. It is doubtful whether the forms he describes occur in hypotonic haemolysis, though, in any case the stages would be passed too rapidly for observation.

Progressive reduction in the tonicity of the haemolysing system results in progressive swelling of the cell to spheroid shape. There is no increase in the surface area of the spheroid as compared with the biconcave disc; this is achieved by a progressive reduction in the major axis diameter. The reduction may amount to 8 per cent of the original cell diameter (Ponder (1933), Haden (1934)). The decrease in cell diameter is due to a mechanical constraint imposed by the surface structure. When the ratio of the major and minor axes becomes approximately 2:1, there seems to be a sudden change in the mechanical resistance and the spheroid becomes truly spherical. At this stage the cells have the appearance of translucent glistening spheres. This phenomenon produces the decreased opacity observed in a suspension of these cells. Up to this point the changes described are reversible; by increase in the tonicity of the system the cells revert to biconcave disc form. If swelling goes beyond this point the changes are irreversible and haemolysis takes place irrespective of increased tonicity.

In this final stage the cells lose their translucent appearance, and as dusky prolytic spheres they lose their haemoglobin within a very short time. After the liberation of haemoglobin the ghost cell returns to somewhere near the volume and shape of the normal erythrocyte.

1.8. The Escape of Haemoglobin from Erythrocytes.

From about 1910 and for the next 20 years there was considerable controversy as to whether the escape of haemoglobin from the erythrocytes was an "all or none" process. That the haemoglobin liberated from lysed cells is quantitatively proportional to the number of cells destroyed, was shown by Saslow (1929) and Parpart (1931). In 1947 Hendry obtained direct confirmation as follows. He showed that the Colour Index of residual cells, between zero and 82 per cent haemolysis is constant, within the limits of experimental error. This is a convincing demonstration that unhaemolysed cells, even in very hypotonic media, lose no haemoglobin. Had they done so the C.I. would have fallen progressively with increasing degree of haemolysis.

The release of haemoglobin is, for all practical purposes, instantaneous and is claimed to occur all over the cell surface (Parpart and Ballentine (1952)). Fricke (1935), assuming free permeability of the cell surface to haemoglobin,

calculated that 90 per cent of the pigment should leave the cell in 0.16 second. In experiments attempting to check this, Ponder and Marsland (1936) found that only with a high lysin concentration (1:1000 saponin) was this figure approached. Their value, obtained by extrapolation, was 0.197 second. They also state that in hypotonic systems the figure is much higher, 0.6 to 0.8 second and remains constant irrespective of tonicity. These observations are of great interest, indicating that haemoglobin escapes much more easily in presence of a lytic agent. This is not surprising since most active lysins, that is those which are effective in trace amounts, generally attack the cell surface in some, as yet unknown, manner. In hypotonic systems the degree of surface disorganisation is probably much less and is more likely to occur at the same, less strong, points in the structure irrespective of the tonicity.

1.9. The Properties of the Erythrocyte Ghost.

The erythrocyte does not break into fragments either to release haemoglobin or immediately after the loss of this pigment. This is substantiated by the presence, in haemolysates, of ghost cells which are haemoglobin free. Provided that the dilution of the blood is not extreme, say about 1:6, the ghost cells appear as biconcave discs, somewhat approaching

the shape of normal red cells (Teitel-Bernard, 1932). Even with a relatively high dilution of blood, the addition of sufficient sodium chloride to render the system isotonic, produces reversion to discoid form. Fricke and Curtis (1935) have shown that electrical impedance measurements on ghost or intact cells are the same. Ponder has observed that shrunken lysed cells have a high membrane resistance and that their osmotic behaviour is similar to that of intact cells. Thus, though during haemolysis the membranes may be permeable to haemoglobin and ions, there appears to be a reversion to semi-permeability after lysis. This is confirmed by Teorell (1952), who reports that ghost cells behave as perfect osmometers if 7.5 per cent of their total volume is considered to have non-solvent properties. Teorell has shown also that high concentration gradients of sodium and potassium can be maintained for long periods across the ghost cell membrane.

Electron microscope studies (Latta, 1952) indicate little difference in surface detail between cell ghosts from hypotonic saline and intact erythrocytes. The treatment of specimens for electron microscopy may well produce artefacts and discretion is essential in the interpretation of such observations.

In spite of a considerable amount of work there is

no real knowledge about the manner in which haemoglobin leaves the cell. Much information is required about the red cell surface ultra-structure in particular before this problem can be adequately tackled.

1.10. Factors Known to Affect Haemolysis.

(a) The Anticoagulant.

Whole blood is, essentially, a suspension of cells in aqueous solution. Though this solution, the plasma, is complex in its make-up, there is no reason to suppose that the physical laws governing the colligative properties of solutions do not apply. Deviation from ideal behaviour may be ascribed to interactions among the solute components. It follows, therefore, that the addition of a relatively impermeable solute to whole blood will increase the osmotic pressure difference across the erythrocyte membrane. This state favours cell shrinkage and obtains in the presence of excess anticoagulant. Since the anticoagulant function of citrate or oxalate is combination with calcium ions, it is possible, by avoiding the use of excess material, to reduce the osmotic increment to negligible proportions. This point is generally overlooked and concentrations of 0.2 to 0.3 per cent oxalate or citrate are commonly used (Guest and Siler (1934); Heller and Paul (1934); Ponder (1948)).

The normal calcium level in whole blood is about 6 mg./100 ml.; about half of this is diffusible and involved in the clotting process. Allowing 100 per cent excess anticoagulant, clotting is prevented by the addition of 17 mg. sodium oxalate or 20 mg. sodium citrate per 100 ml. blood. The respective osmotic increments are 0.05 and 0.09 atmospheres. Calcium citrate is unionised, though soluble, and therefore contributes further to the osmotic pressure. When 0.3 per cent sodium oxalate is used its osmotic increment amounts to 2.0 atmospheres while for 0.3 per cent sodium citrate the value is 1.2 atmospheres. Under these conditions the 12.5 and 7.5 per cent cell shrinkage observed by Guest and Siler is to be expected.

Heparin, having a molecular weight of the order of 1200 and being a very powerful anticoagulant, produces a negligible osmotic pressure increase. Up to 200 mg. per 100 ml. blood produce no change in cell volume (Guest and Siler, 1934). Heparin is therefore preferable, since the ratio, anticoagulant to blood, is not critical from the osmotic point of view. In addition, the view is put forward by Hunter et al. (1940) that certain protoplasmic properties of the erythrocyte, such as viscosity, are dependent on the presence of calcium ions in optimal concentration.

At an early stage in this investigation it was ob-

served that haemolysis by certain substances was affected by a change in calcium ion concentration. In view of this, heparin, in a concentration of 15 mg. per 100 ml. blood has been used throughout as the anticoagulant.

(b) The Condition of the Blood.

It is preferable that the erythrocytes should undergo the minimum of manipulation prior to experiment. In certain cases it may be necessary to wash the cells free from plasma, but their response can hardly be considered normal in view of the gross change in the suspending medium and the possible loss of surface and intracellular material. It is claimed that washing with isotonic sodium chloride has no effect on osmotic fragility (Saslow (1932); Monaghan and White (1936)). The latter authors report that washing with isotonic sucrose results in a measurable uptake of added protein by the washed cells. Confirmation that a suspending medium of low electrolyte content removes surface material from ghost cells has been provided by Waugh and Schmidt (1940). They found a progressive loss of up to 60 per cent lipid and 30 per cent protein from the cell surface.

With normal donors it is therefore preferable to use whole blood since the plasma solutes also endow the haemolysing system with a useful buffering capacity.

Whitby and Hynes (1935) demonstrated a fragility

difference between the cells of venous and oxygenated blood. This difference may be eliminated by oxygenation of venous blood. The point has been investigated by Hendry (1947), who has shown that the removal of carbon dioxide from venous blood has quantitatively the same effect on fragility as oxygenation. By using phosphate buffers of constant tonicity Hendry (1948a,b) has shown that there is no difference in fragility between oxygenated and venous blood at constant pH. By inhibition of carbonic anhydrase with sulphonilamide the same author demonstrated qualitatively that the decrease in fragility due to oxygenation is less marked. Here some change is inevitable since bicarbonate can release carbon dioxide spontaneously. This the relevant factor is the displacement of carbon dioxide, though indirectly through its effect on pH.

Hendry (1948b) has also shown, for partially haemolytic concentrations of buffers at constant tonicity, the pH has a pronounced effect upon the degree of haemolysis attained. If a hypotonic buffer solution giving 85 per cent haemolysis at pH 6.8 is taken as the datum, then, with the same tonicity, at pH 6.3 and 7.3, the corresponding values are 65 and 50 per cent haemolysis respectively. That is, resistance to haemolysis is a minimum at pH 6.8 and this resistance increases significantly to the acid side of 6.8

and increases markedly to the alkaline side.

It can be seen, therefore, that the pH effect is a major variable and for its reduction the blood must be fully oxygenated. In this state the erythrocytes have their maximum inherent resistance to haemolysis.

(c) Temperature.

The degree of haemolysis attained in any hypotonic haemolysing system is a function of the temperature of that system. This effect has been investigated by a number of workers and a variety of theories have been put forward to account for the observations.

It has been suggested by Ponder (1935) that the changes are due to the escape of osmotically active intracellular solute, but, quantitatively, this is not probable. Jacobs et al. (1936) believe that the increase in osmotic resistance with rise in temperature is due to the increased base - binding power of haemoglobin within the cell. The observations of Ham (1948), that temperatures up to 46°C. produce no demonstrable change in the morphology or fragility of the erythrocyte, suggest that the cause does not reside in the membrane itself.

Hendry (1949) has considered this question and draws attention to the fact that osmotic pressure, at constant concentration, is directly proportional to the absolute

temperature. This point is overlooked by most authors. The prime importance of this temperature factor was demonstrated by Hendry as follows. Three series of solutions, each serial member differing in concentration by 0.01 gm. sodium chloride per 100 ml., were equilibrated at three different temperatures. Specimens of blood from a single sample were distributed into each tube and, after 30 minutes, the percentage haemolysis in each tube was determined. When the percentage haemolysis was plotted against the concentration in gm. sodium chloride per 100 ml., three separate curves were obtained, corresponding to the three selected temperatures. When, on the other hand, the percentage haemolysis was plotted against the calculated osmotic pressure in atmospheres, all points lay on a single curve, showing that, at constant osmotic pressure, the degree of haemolysis was independent of the temperature.

Though other factors may be involved, the main one is the direct effect of temperature upon the osmotic pressure of the system.

This effect of temperature change applies to plasma solutes and liberated cell contents also. With normal blood and constant dilution the osmotic effect of these two factors is small and constant and therefore may be neglected.

1.11. Proposed Line of Research.

Existing knowledge on haemolysis in solutions of organic nitrogenous substances, such as ureas, urethanes and amides, is wholly qualitative in nature. It is, therefore, of interest to obtain quantitative data with a view to attempting to correlate molecular structure with haemolytic properties.

Malonamide is the only member of the above groups which, in isotonic concentration, induces haemolysis at a conveniently measurable rate at room temperature.

Since the behaviour of natural membranes is influenced by the presence of electrolytes, it is proposed to study the effects of ions upon haemolysis in hypotonic aqueous malonamide.

The effect of glycine and certain carbohydrates on malonamide-induced haemolysis will also be investigated.

Section 2.

Experimental Methods.

2.1. The Preparation of the Blood.

All experiments herein recorded were carried out on fresh normal human blood. Immediately after withdrawal by venepuncture the blood was transferred to a clean dry test tube containing approximately 1.5 mg. BDH heparin powder (95 I.U./mg.) per 10 ml. and mixed by repeated inversion. Within 10 minutes the blood was transferred to a tonometer and exposed to moist oxygen at room temperature and pressure. Oxygenation was maintained for 20 to 30 minutes prior to experiment. Except in the case indicated, no attempt was made to wash or otherwise manipulate the cells. All experiments were completed within eight hours of the withdrawal of the blood.

2.2. The Determination of Osmotic Pressures.

The osmotic pressure of a dilute solution at $t^{o}C.$ related to its freezing point depression by the equation

$$\pi = - \frac{L\,T}{\bar{V}_1 T_o^2} \cdot \Delta T$$

(Glasstone, 1947), where

π = Osmotic pressure of the solution

$\bar{V}_1$ = Partial molar volume of solvent.

= Molar volume, V' of solvent for dilute solutions.

L = Molar latent heat of fusion of solvent.

T = Absolute temperature at which osmotic pressure is measured.

$= (273 + t^{\circ}C)\ ^{\circ}K.$

T_o = Freezing point of pure solvent in $^{\circ}K.$

t = Freezing point depression due to added solute.

By substituting known values in the above equation, the osmotic pressure of a dilute solution at $t^{\circ}C.$ is found.

For aqueous solutions:

$L = 79.8 \times 18$ cals/mole

$V' = 18$ cc./mole

$T = 273\ ^{\circ}K$

and since, for dilute solutions these value are constant

$$t^{\circ}C = -\frac{79.8}{273} \cdot \frac{273 + t}{273} \cdot \Delta t \text{ cals./cc.}$$

The units are convertible to atmospheres as follows:

R = Universal gas constant $\approx$ 2 cals./deg.

$= 82.04$ cc. atmos./deg.

$\therefore$ 1 cal $\approx$ 41.02 cc. atmos.

$$\therefore \quad t^{\circ}C = -\frac{79.8 \times 41.02}{273} \cdot \frac{273 + t}{273} \cdot \Delta t \text{ atmos.}$$

$$\text{i.e.} \quad t^{\circ}C = -12.\Delta t. \frac{273 + t}{273} \text{ atmospheres at } t^{\circ}C.$$

The F.Pt. depression for serum is -0.560°, therefore the osmotic pressure of serum at $20^{\circ}C$ is

$$20^{\circ} = (-12)(-0.560)\frac{293}{273}$$

$= 7.2$ atmospheres.

On this basis the concentrations of sucrose and sodium chloride required for a given osmotic pressure and tonicity at 20°C. are shown below.

Osmotic Pressure. 20°C.	Tonicity. 20°C.	Concn. Sucrose. gm.% at 20°C	Concn. NaCl. gm.% at 20°C
7.2	1.000	9.345	0.9326
6.5	0.903	9.090	0.8478
5.0	0.695	7.045	0.6522
3.5	0.486	5.000	0.4565
1.5	0.208	2.138	0.1956
1.0	0.139	1.435	0.1304
0.5	0.070	0.733	0.0652

All osmotic pressures have been calculated on this basis from freezing point data given in International Critical Tables (1929) and Landolt-Börnstein Roth Tabellen (1912).

2.3. The Preparation of Haemolysing Solutions.

All non-electrolytes, except those of A.R. quality, were purified by recrystallisation or fractionation. In the case of solids good crystal form and a melting point within ± 3° of the published figure (Handbook of Chemistry and Physics (1949)) were taken as the criteria of purity. Liquids were redistilled and the fraction 3° either side of the published boiling point collected for use. The solid materials were dried to constant weight and stored in a vacuum desiccator over conc. sulphuric acid and caustic soda pellets.

The electrolytes, of A.R. quality, were used without further purification, due allowance being made for water of crystallisation. Lithium chloride was prepared in solution by the reaction of calculated amounts of lithium carbonate and dilute hydrochloric acid. The neutrality of the solution was checked by pH meter, made up to volume and standardised by chloride estimation.

The amino acids, all of the DL type, were recrystallised from water, dried and stored in vacuo over conc. sulphuric acid and caustic soda pellets.

All solutions were made up on a w/v per cent concentration basis, using anhydrous solute wherever possible. Where stable hydrates were used, allowance has been made for water of crystallisation.

2.4. The Haemolysing System.

The haemolysing systems consisted of 1 part fully oxygenated whole blood to 20 parts solution; the dilution of the blood is, therefore, 1:21. The systems, contained in glass vessels of suitable size, were maintained by theremostatic water bath to within $\pm$ 0.3° of the required experimental temperature.

2.5. Measurement of Degree of Haemolysis.

The unhaemolysed cells of a 3 ml. specimen of the

haemolysing system were centrifuged down and 2 ml. of the supernatant transferred to a 5" x $^{5}/_{8}$" test tube. The haemoglobin in the supernatant was then determined by the alkaline haematin method of Clegg and King (1942), the colour intensity being measured by the "Spekker" photoelectric absorptiometer, using a green filter, No.604, spectrum green and d = 1.00 cm.

The total haemoglobin was measured by treating an uncentrifuged 2 ml. portion of the system in the same way.

The degree of haemolysis of the specimen is given by

$$\text{Per cent Haemolysis} = \frac{\text{Spekker Reading Test}}{\text{Spekker Reading Total}} \times 100$$

2.6. Measurement of the Rate of Haemolysis.

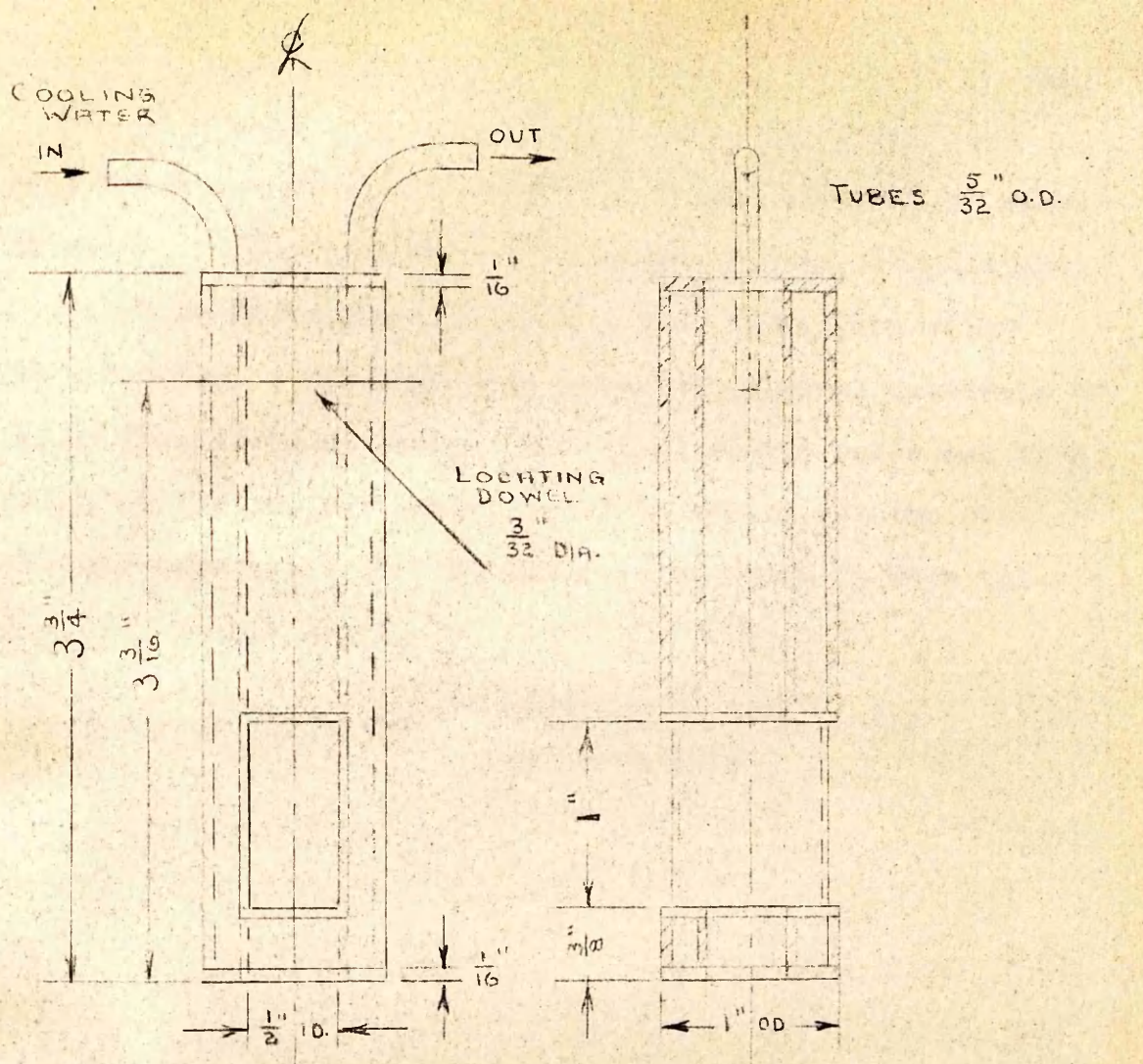
In slowly haemolysing systems the rate of haemolysis was obtained by estimating the degree of haemolysis in serial specimens of the system at suitable intervals of time. Three minutes was found to be the shortest practicable time interval. For a reading required at time "t" the haemolysing system was removed from the water bath and mixed by 3 inversions at time (t - 60 secs.). After return to the bath at (t - 50 secs.) a 3 ml. specimen was withdrawn by pipette, transferred to a 5" x $^{5}/_{8}$" centrifuge tube and placed in the centrifuge, an MSE minor model. At (t - 30 secs.), the centrifuge was started and the lever moved

to No.2 on the front scale. The lever was then moved to No.4 at (t - 25 secs.), to No.6 at (t - 20 secs.) and to No.7 at (t - 15 secs.). The centrifuge remained at full speed, 4,000 r.p.m. until (t + 30 secs.), when the power was cut off. With one specimen and a balance tube the machine stopped by (t + 80 secs.), when 2 ml. supernatant were withdrawn for haemoglobin estimation. It was thus possible to repeat the procedure every three minutes. For longer time intervals, though the procedure became easier to carry out, the time factor remains just as critical and the routine described for obtaining a specimen at time "t" was strictly adhered to throughout the work.

Since the rate in rapidly haemolysing systems cannot be measured as described above, a different procedure was adopted. 3 ml. specimens of the haemolysing solution were distributed in 4" x $\frac{1}{2}$" test tubes carried in a rack in the water bath at constant temperature. After the temperature equilibration period 0.15 ml. fully oxygenated blood was run into the tube under test from a 1.00 ml. graduated pipette. A stop watch was started at the instant the addition of blood was complete. The watch was stopped at the point of complete haemolysis and the time noted. The end point was obtained by holding the tube against a standard background and before a strong light source. Haemolysis was considered complete

when all trace of opacity had disappeared from the system. The standard background consisted of three broken lines $^{5}/_{8}$" apart made up of 30 0.2 mm. spots per inch. At complete haemolysis these spots stood out clearly through the haemolysate. This method of determining the rate of haemolysis is suitable only for systems in which complete lysis occurs within 3 to 5 minutes.

In systems requiring 6 to 10 mins. for complete haemolysis the Unicam S.P. 350 Model DG spectrophotometer was used. This instrument, using a wave length of 628 mu (red), was used to measure the rate of change in opacity of haemolysing systems. It was found that when the instrument had reached its working temperature the temperature within the adaptor socket was about 40 - 45°C. Since $\frac{1}{2}$" diameter test tubes were used to contain only 4 ml. haemolysing system the results were inaccurate due to over-heating. A hollow adaptor was therefore constructed as shown in the accompanying diagram (Fig.1.). Water at the desired temperature was drawn from the thermostat water bath through the cooler by vacuum pump. In this way constant temperature was maintained for the duration of the experiment. The instrument was set mechanically to read zero transmission through a 1:21 suspension of blood in isotonic sodium chloride. By increasing the light passing, a reading of 80 per



SPECIFICATION.

OUTER TUBE 1/16" BRASS
INNER TUBE 1/32" BRASS
APERTURE 1" × 1/2" × 1/32 COPPER.
CAPS 1/16" BRASS

ALL JOINTS SILVER SOLDERED.

SCALE F.S.

COOLER FOR UNICAM SPECTROPHOTOMETER.

FIG 1.

cent transmission was obtained for a 1:21 dilution of blood in water. This procedure was necessary since organic non-electrolyte haemolysates transmit more light than water haemolysates. Readings were taken at suitable intervals of time, from 15 to 30 secs. The final steady value was then taken as the 100 per cent haemolysis levels and the percentage haemolysis for the intervening readings were calculated on this basis.

$$\text{Per cent Haemolysis} = \frac{\text{Reading t secs.}}{\text{Reading 100\%}} \times 100$$

Section 3.

Results.

3.1. Haemolysis Induced by Hypotonic Malonamide Solutions.

(a) Delayed Haemolysis in Hypotonic Aqueous Malonamide.

If whole blood be diluted in the ratio 1:21 with slightly hypotonic malonamide solution (Π = 6.5 atmospheres at 20°C), 55 to 60 minutes are required for complete haemolysis (Fig.2). Of this period there is a zero haemolysing lag phase of 15 - 20 minutes, followed by the main lytic phase of about 20 minutes, after which the rate falls off as the 100 per cent lysis level is approached. The graph of the process is a sigmoid curve.

On reduction of the osmotic pressure of the malonamide solution to 5.0 atmospheres at 20°C. the lag phase is reduced to about 10 minutes. The lytic phase occupies about 10 minutes and complete haemolysis occurs in about 36 minutes.

Further reduction of the osmotic pressure of the solution to 3.5 atmos. brings the lag phase down to 5 minutes, the lytic phase requires about 7 minutes and haemolysis is complete in 20 to 22 minutes.

On bringing the osmotic pressure down to 2.5 atmos. there is no lag phase because, at this osmotic pressure, there is an immediate degree of haemolysis, approximately 10 per cent. The lytic phase occupies about 4 minutes and there is complete haemolysis in 10 minutes.

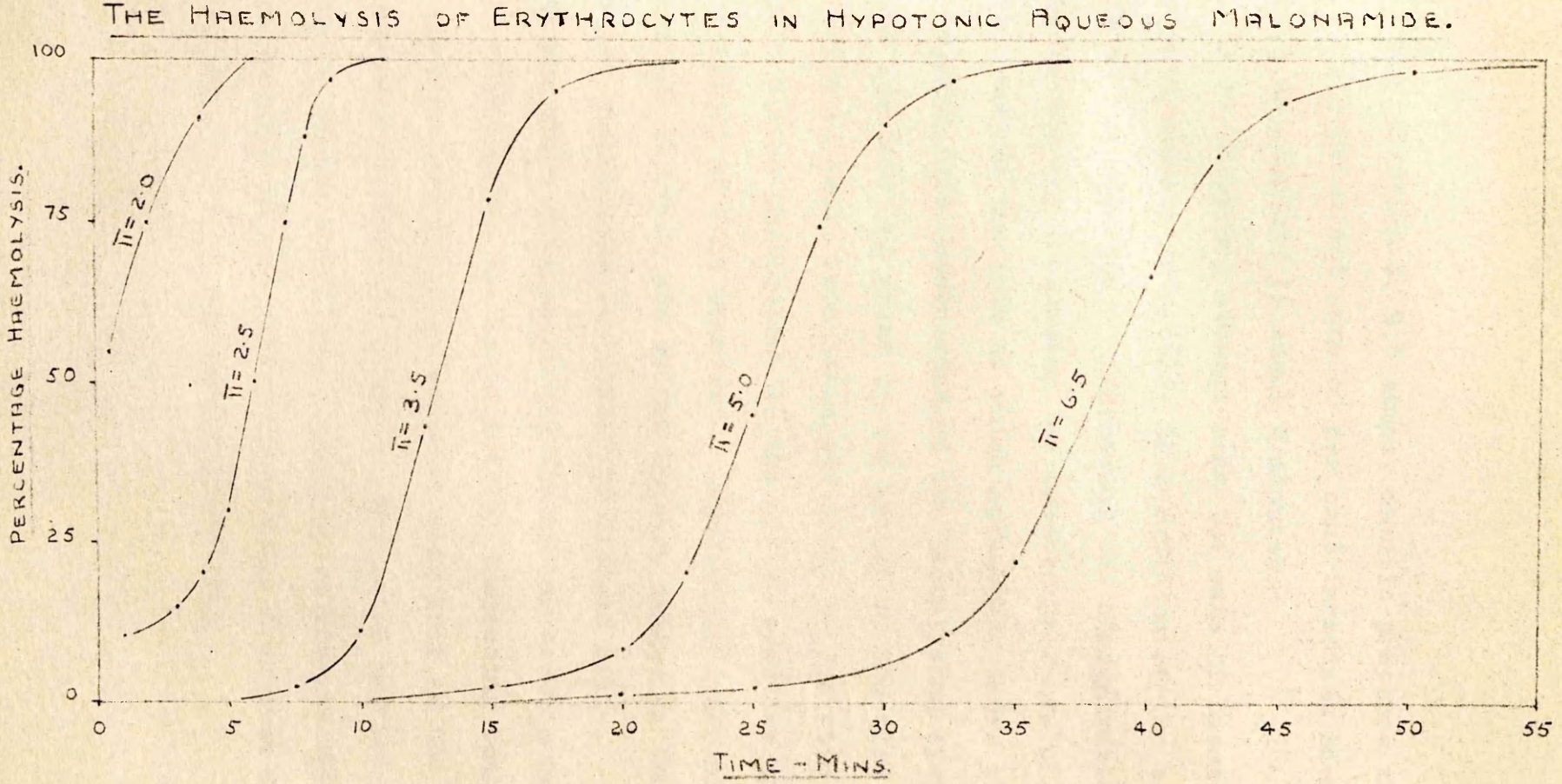

THE HAEMOLYSIS OF ERYTHROCYTES IN HYPOTONIC AQUEOUS MALONAMIDE.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
$\bar{\pi}$= 2·0
$\bar{\pi}$= 2·5
$\bar{\pi}$= 3·5
$\bar{\pi}$= 5·0
$\bar{\pi}$= 6·5
0
5
10
15
20
25
30
35
40
45
50
55
TIME - MINS.
THE EFFECT OF CHANGE IN OSMOTIC PRESSURE AT 20° C.

FIG. 2.

In a solution of 2.0 atmos. osmotic pressure there is, again, no lag phase and some 50 per cent immediate haemolysis. Haemolysis is complete in about 6 minutes.

It is therefore evident that the rate of haemolysis in hypotonic aqueous malonamide is a function of the osmotic pressure of the solution. Increasing the concentration of malonamide, thereby increasing the osmotic pressure of the solution, reduces the rate at which haemolysis takes place.

Microscopic examination of the haemolysing system indicates that the lag phase is the result of the time taken by the cells to swell from biconcave discs to prolytic spheres. Visual examination of the system provides macroscopic evidence of this process. Immediately after mixing the blood and solution the system appears opaque to the eye and has the characteristic "sheen" of discoid cells. With standing the sheen disappears and the system becomes translucent. The process is slowed down by increasing tonicity of the system. It is of interest to note that in the system of 3.5 atmospheres osmotic pressure, the cells require some 5 minutes to become spherical. This is so despite the fact that the tonicity of the system is slightly less than 0.5 and therefore conducive to immediate swelling.

(b) Osmotic Haemolysis in Malonamide Solutions.

With the present methods it has proved impossible to obtain reliable haemolysis curves of the osmotic fragility type. As Fig.2 indicates, the lag phase disappears completely with anything but the smallest initial degree of haemolysis. Above an initial value of about 30 per cent haemolysis it is not possible to distinguish between hypotonic osmotic haemolysis and haemolysis induced by the presence of malonamide in solution; this, of course, assuming that a difference does exist between the processes.

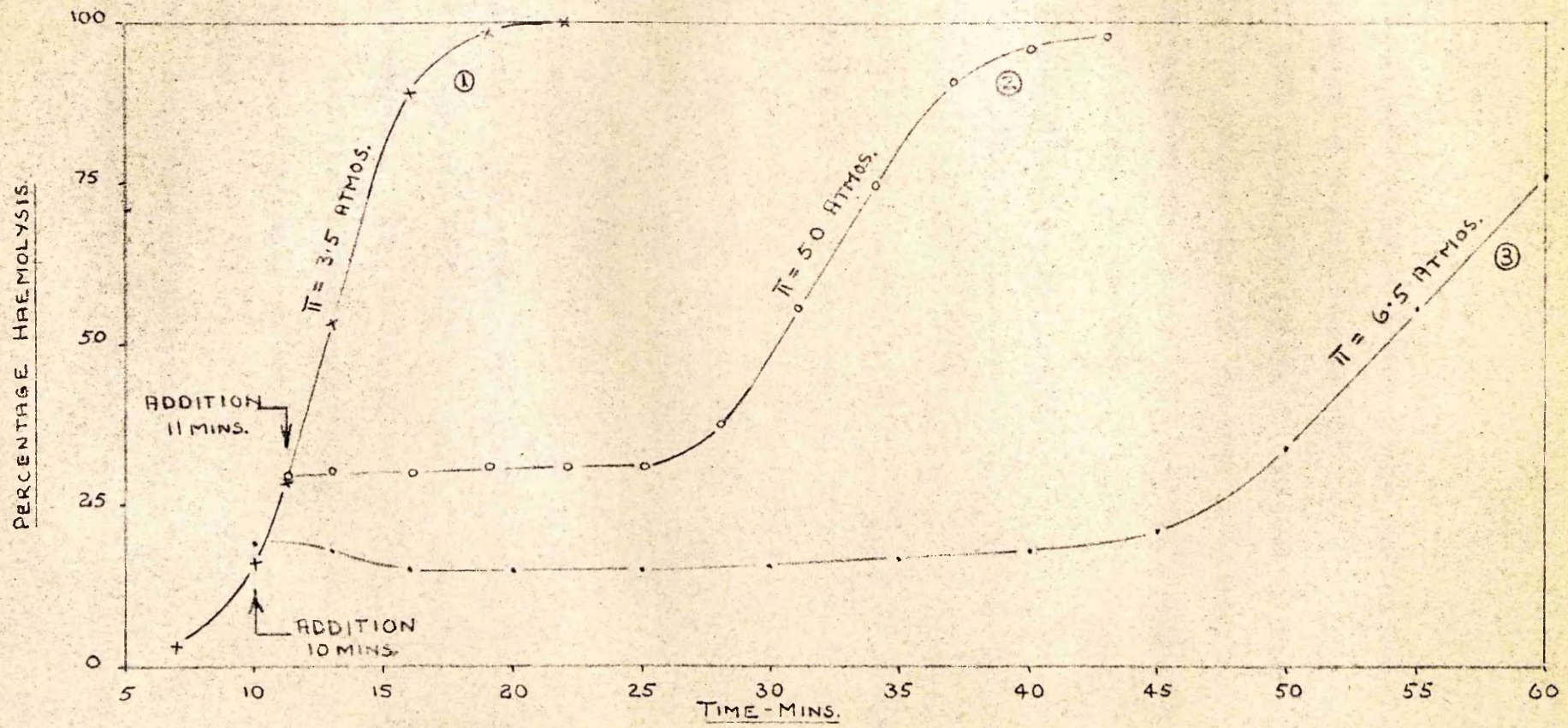
(c) The Effect of Adding Solid Solute During Haemolysis.

Since haemolysis in hypotonic aqueous malonamide is a function of the osmotic pressure of the solution, it is of interest to observe the effect of increasing the osmotic pressure during the lytic phase.

Fig.3.1. shows the haemolysis curve obtained with a solution of π = 3.5 atmospheres malonamide. Fig.3.2. shows the result of adding, during the haemolytic phase, sufficient solid malonamide to raise the osmotic pressure of the solution to 5.0 atmospheres. Haemolysis is arrested for about 15 minutes, at which point it restarts and proceeds at a slower rate to the 100 per cent value.

Fig.3.3. shows the effect of adding, during haemolysis, that quantity of solid malonamide which raises the osmotic pressure of the solution to 6.5 atmospheres.

THE EFFECT OF INCREASING THE OSMOTIC PRESSURE OF A HAEMOLYSING SYSTEM DURING THE LYTIC PHASE. TEMP. 20°C.



1. MALONAMIDE SOLN. Π = 3·5 ATMOS.
2. ——— " ——— + SOLID MALONAMIDE Π = 1·5 ATMOS.
3. ——— " ——— + SOLID MALONAMIDE Π = 3·0 ATMOS.

FIG. 3.

There is a proportionately longer delay before haemolysis restarts, again at a much slower rate.

In both cases haemolysis is checked by cell shrinkage due to the sudden increase in osmotic pressure. As with the earlier experiments, haemolysis restarts only when the critical cell volume is restored.

(d) The Permeability of Malonamide.

All existing evidence shows that osmotic swelling, which is governed by the relative extra- and intra-cellular osmotic pressure, is complete within a matter of seconds. This is the result of the movement of water, to which the cell membrane apparently offers no measurable resistance. It therefore follows that, in hypotonic solutions of malonamide, the lower the osmotic pressure, the greater the rapid initial degree of swelling.

This accounts for the decrease in the lag phase with decreasing osmotic pressure. In solutions of 3.5 atmospheres the degree of osmotic swelling is greater than in solutions of 6.5 atmospheres. The cells in the former system are nearer their critical volume than those in the latter and will, therefore, haemolyse sooner; the lag phase is, accordingly, shorter.

The occurrence of delayed haemolysis in aqueous malonamide, irrespective of the degree of hypotonicity, shows

that cell swelling continues beyond the point required for re-establishment of simple osmotic equilibrium. This continued swelling is the result of disturbance of the new equilibrium by the penetration of malonamide, which diffuses with its concentration gradient. With the increasing intracellular concentration of malonamide there is an associated influx of water, which restores osmotic equilibrium. Thus, at any instance in the process, the increased intra-cellular osmotic pressure due to malonamide is reduced by dilution of intra-cellular solute with incoming water. This excess water, in turn, induces cell swelling.

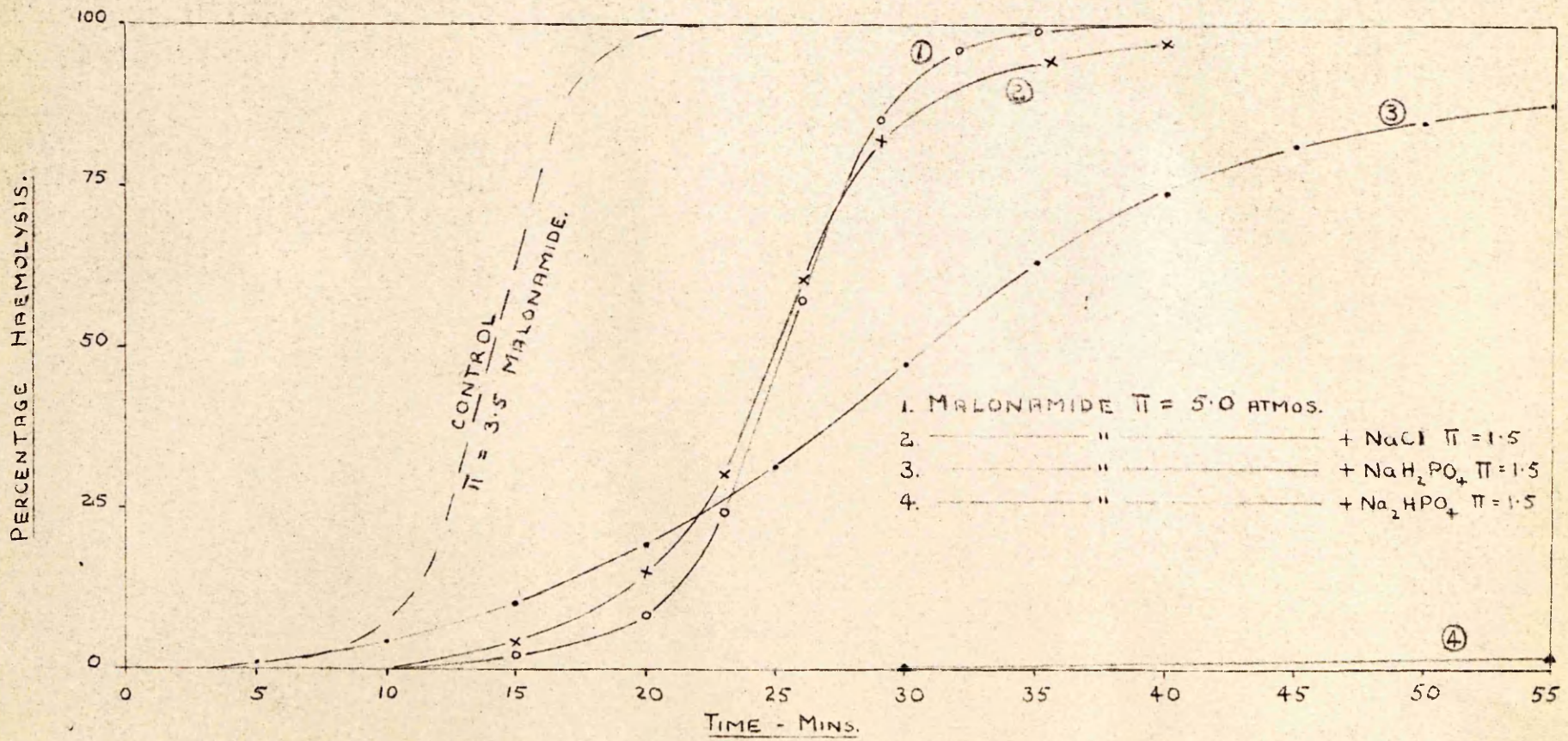
As the diffusion of malonamide continues, so also does the penetration of water; as a result the cells gradually attain their critical volume and undergo haemolysis. Since the cells are freely permeable to water, their permeability to malonamide is likely to be a limiting factor.

(e) The Effect of pH on Malonamide-induced Haemolysis.

Although malonamide has a weakly acid reaction in solution, the plasma buffers in the dilution used are adequate to bring the system to near neutrality. The pH of a π = 5.0 atmos. solution of malonamide is about 4.65, due mainly to dissolved carbon dioxide; when whole blood is added to a dilution of 1:21 the pH rises to about 7.25.

The Haemolysis of Erythrocytes in Buffered Hypotonic Aqueous Malonamide.

Temp. 20°C.



No.	pH Solution.	Initial pH System.	Final pH System
1.	4·64	7·08	7·24
2.	4·67	7·10	7·22
3.	4·46	5·61	5·93
4.	8·69	8·49	8·22

Fig. 4.

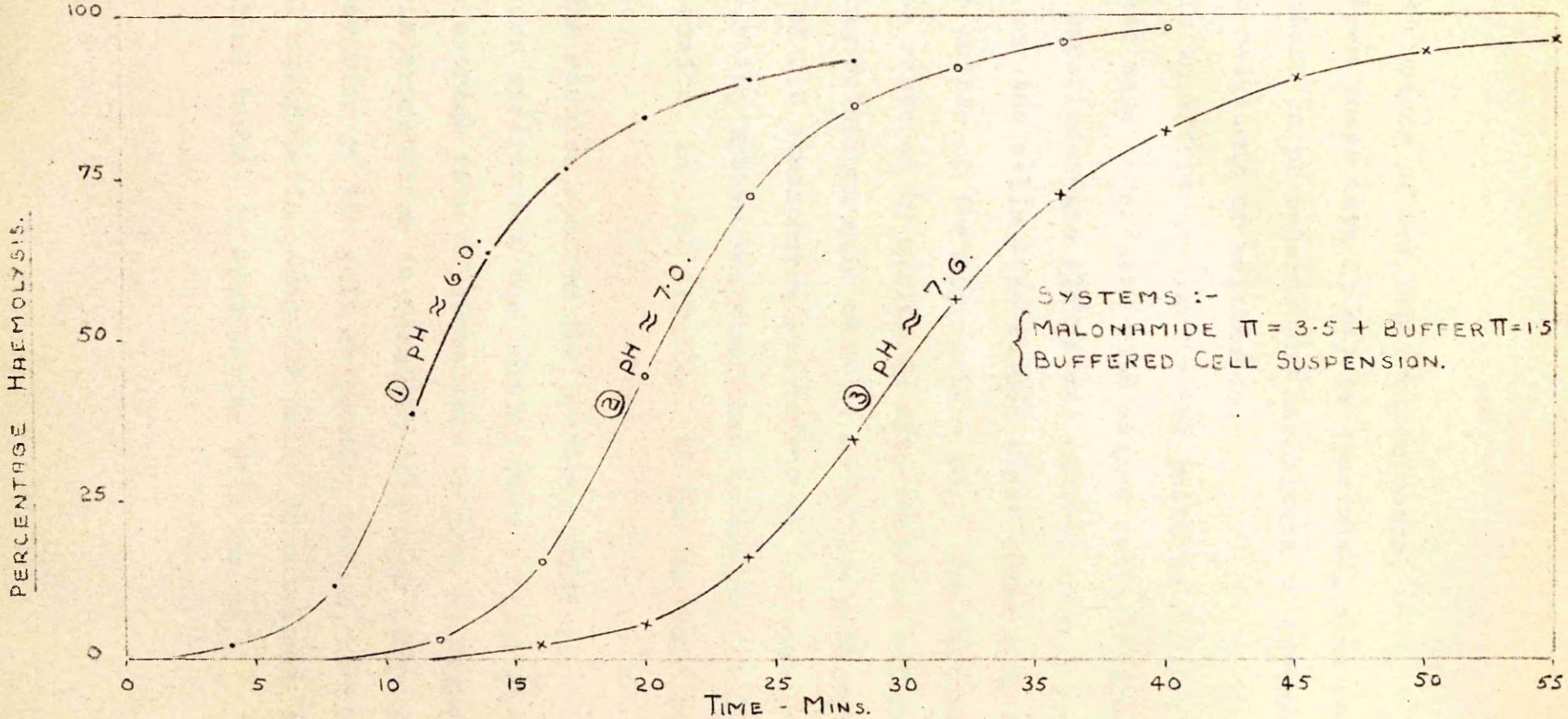
To test the effect of variation in pH, iso-osmotic quantities (π = 1.5 atmos.) of sodium dihydrogen phosphate, disodium hydrogen phosphate and sodium chloride were added to solutions of π = 3.5 atmos. malonamide. The total osmotic pressure of each solution is constant at 5.0 atmospheres and the osmotic contribution of the plasma solutes is also constant.

It can be seen from Fig.4 that there is only a slight difference in the behaviour of systems 1 and 2 which have constant osmotic pressure and pH. The presence of sodium chloride modifies the curve but slightly compared with sodium dihydrogen phosphate (Fig.4.3.). It should be noted, however, that the trend is similar to that induced by sodium chloride, only more pronounced; initial haemolysis is faster, while terminal haemolysis is slower. It is difficult to say whether the result is due to the drop of 1.5 pH units, the presence of different anions, or partly to each. At this point it may be recorded that globulin precipitation is a complicating factor in whole blood systems below pH 6.

There seems little doubt about the effect of disodium hydrogen phosphate (Fig.4.4.) since only some 2 per cent haemolysis was observed after one hour. In point of fact the interpretation is open to doubt here also. In addition to the 1 unit pH rise a divalent anion has been submitted for the

THE HAEMOLYSIS OF BUFFERED CELL SUSPENSIONS IN BUFFERED HYPOTONIC MALONAMIDE.

TEMP. 20°C.



Nº	pH SOLN.	INITIAL pH SYSTEM.	FINAL pH SYSTEM.
1.	5.97	6.08	6.07
2.	7.00	7.00	7.06
3.	7.92	7.71	7.53

FIG. 5.

monovalent anions of the other experiments.

From these data it may be concluded, with reservations, that increase in pH retards the haemolysis of erythrocytes in hypotonic solutions of malonamide.

In an effort to clarify the position further, some experiments were undertaken with washed cells (Fig.5.). For each experiment the plasma was removed from a specimen of blood and the cells were washed three times with isotonic phosphate buffer of the appropriate pH. The initial plasma volume was restored by making up with isotonic buffer. The haemolysing solutions were made up as before with malonamide solution of 3.5 atmospheres and buffer of 1.5 atmospheres. The haemolysing system was completed by adding the buffered cell suspension, in 1:21 dilution, to the buffered aqueous malonamide.

The results confirm the previous findings. Haemolysis occurs earlier and the initial rate is greater at low pH. The average rate of haemolysis is less at higher pH. Cautious interpretation is required here also since there is gross alteration of the cell suspending medium, considerable mechanical manipulation, and the ratio of univalent to divalent anions varies in association with the pH.

(f) The Effect of Change of Temperature on Haemolysis in Hypotonic Solutions of Malonamide.

In general, it may be stated that the higher the temperature the greater the degree of haemolysis in solutions of electrolytes (Jarisch (1921), Jacobs and Parpart (1931), Jacobs, Glassman and Parpart (1936), Hendry (1949)). It is also true that the higher the temperature the greater the rate of haemolysis in solutions of non-electrolytes (Masing (1914), Jacobs, Glassman and Parpart (1935), Hendry (1952)). The latter generalisation applies also to the rate of haemolysis in malonamide solutions.

The effect of change of temperature has been examined by adding normal oxygenated blood to solutions of malonamide and determining the rate of haemolysis at 5, 10, 15, 20, 25, 30 and 35°C. Three standard malonamide solutions have been used, viz. solutions of 3.5, 5.0 and 6.5 atmospheres. The results are shown graphically in Figures 6, 7 and 8.

Two conclusions can be drawn from this data:

(1) That at constant osmotic pressure, the rate of haemolysis increases steadily as the temperature is raised. This is in agreement with the general findings of other authors as mentioned above.

(2) That the effect of change of temperature becomes greater as the osmotic pressure of the malonamide

The Effect of Temperature Change on the Rate of Haemolysis at Constant Osmotic Pressure.

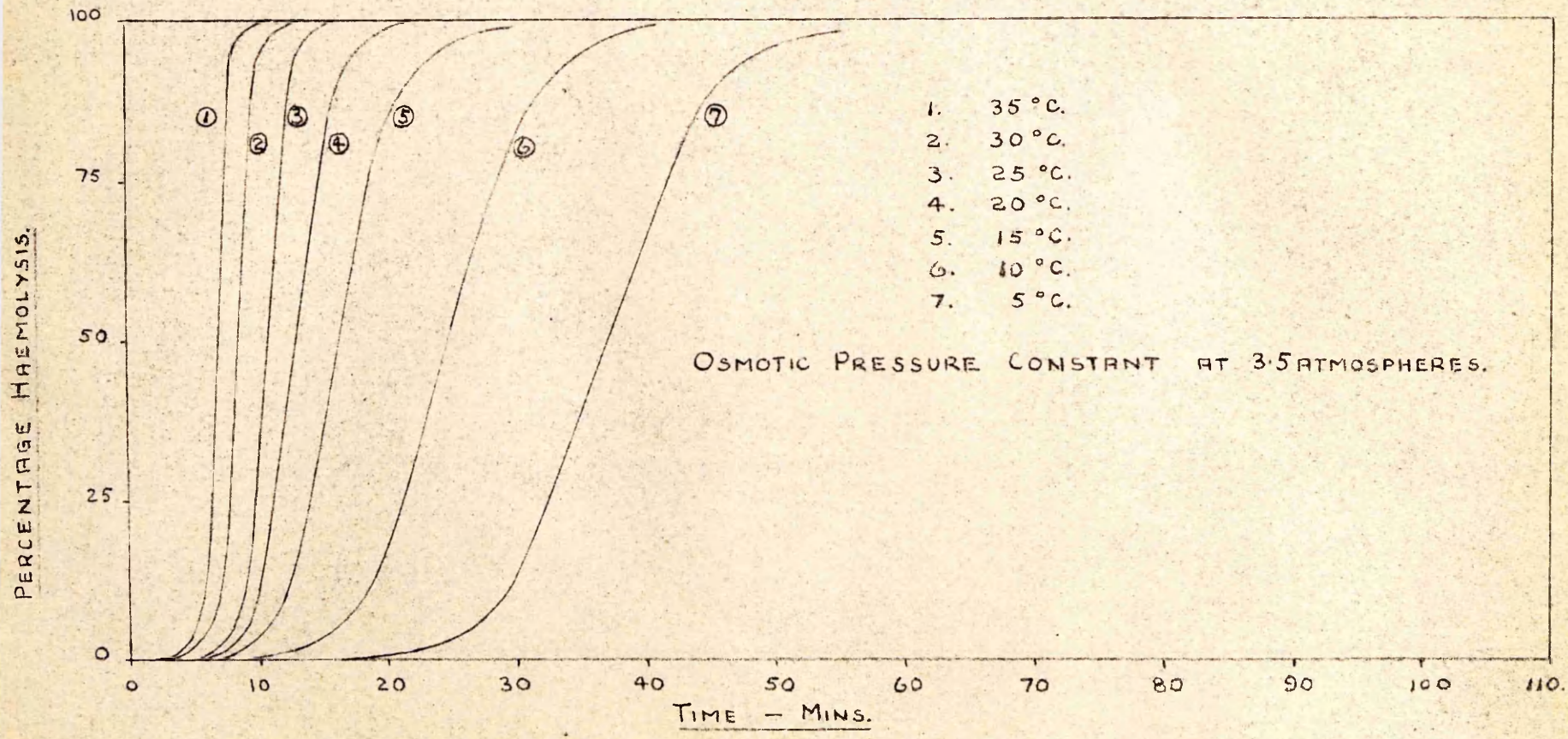
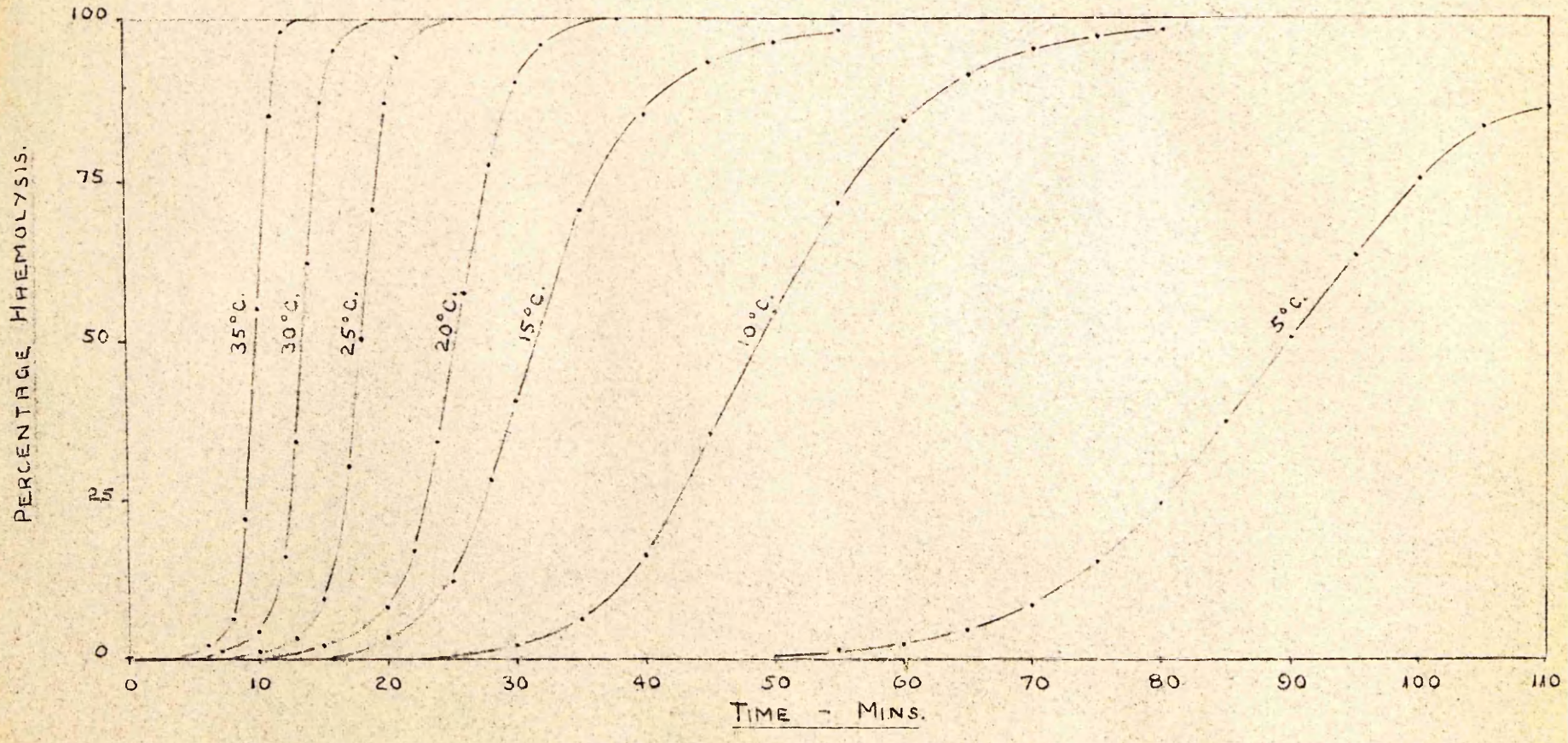


Fig. 6.

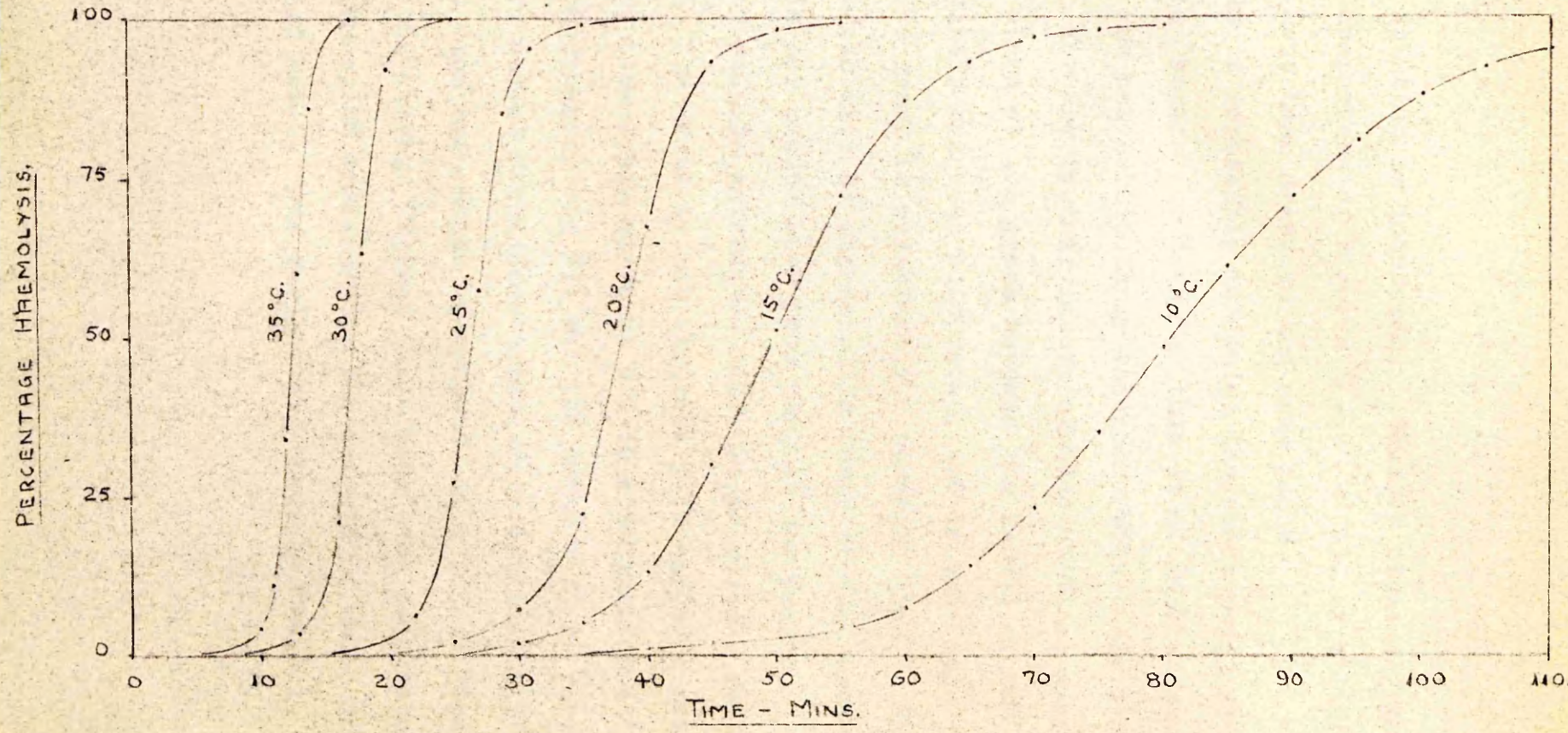
THE EFFECT OF TEMPERATURE CHANGE ON THE RATE OF HAEMOLYSIS AT CONSTANT OSMOTIC PRESSURE.



OSMOTIC PRESSURE CONSTANT AT 5·0 ATMOSPHERES.

FIG. 7.

THE EFFECT OF TEMPERATURE CHANGE ON THE RATE OF HAEMOLYSIS AT CONSTANT OSMOTIC PRESSURE.



OSMOTIC PRESSURE CONSTANT AT 6·5 ATMOSPHERES.

FIG 8.

solution is increased. This is obvious from a comparison of Figures 6 - 8 - all of which have been drawn to the same scale.

It is impossible to decide the exact point at which haemolysis "begins" or "ends"; this is of little importance since we are not particularly concerned with these indefinite points. For the further interpretation of these graphs, only the slope of the graph between the levels of 25% and 75% haemolysis has been considered, and in each case this part of the graph is practically a straight line. The rate of haemolysis will be expressed in terms of the percentage haemolysis per minute over this part of the graphs. A certain error will be introduced since it is obvious that in some cases the graph is not absolutely linear over this range (e.g., the haemolysis curve at $5^{o}C$ in a malonamide solution of 5.0 atmospheres, Fig.7), but the error will be very small.

To reduce fortuitous error, calculations will be made, not from the single experiments depicted in Figs. 6 - 8, but from the average figures which have been calculated from a large number of experiments, the details of which need not be recorded here. The results are shown in Table 1.

Table 1.

The Rate of Haemolysis, between 25% and 75% lysis, of Normal Erythrocytes in Solutions of Malonamide.

(a) Malonamide: osmotic pressure = 3.5 atmospheres.

Temperature	5°C	10°C	15°C	20°C	25°C	30°C	35°C
Number of Experiments	3	4	6	14	3	6	5
% Haemolysis per minute	4.5	6.5	9.9	14.2	23.2	32.5	39.1

(b) Malonamide: osmotic pressure = 5.0 atmospheres.

Temperature	5°C	10°C	15°C	20°C	25°C	30°C	35°C
Number of Experiments	2	3	4	6	2	3	2
% Haemolysis per minute	2.1	3.5	5.9	10.0	15.5	25.2	37.2

(c) Malonamide: osmotic pressure = 6.5 atmospheres.

Temperature	5°C	10°C	15°C	20°C	25°C	30°C	35°C
Number of Experiments	-	2	2	4	2	3	2
% Haemolysis per minute	-	2.1	3.6	7.6	12.7	16.9	33.9

From these figures can be calculated the Q_{10} values over five temperature ranges, namely, 5-15, 10-20, 15-25, 20-30 and 25-35°C. It is at this point that the assumption, that the rate of haemolysis is linear between 25 and 75 per cent haemolysis, becomes important. The values of Q_{10} are shown in Table 2.

Table 2.

Calculated values of Q_{10}.

Osmotic Pressure	3.5 atmos.	5.0 atmos.	6.5 atmos.
Temperature Range	Q_{10}		
5 - 15°C	2.2	2.8	-
10 - 20°C	2.2	2.9	3.6
15 - 25°C	2.3	2.6	3.5
20 - 30°C	2.3	2.5	2.2
25 - 35°C	1.7	2.4	2.7

The rate of haemolysis increases two to three-fold for each 10° rise in temperature.

Another method of expressing the influence of temperature on the rate of a reaction is the equation of Arrhenius -

$$V = Ae^{-E/RT} \quad \ldots\ldots\ldots \quad (1)$$

or, in its more usual form,

$$\log_e V = \log_e A - \frac{E}{RT} \qquad \ldots\ldots\ldots \quad (2)$$

or $$\log_{10} V = \log_{10} A - \frac{E}{2.303RT} \qquad \ldots\ldots\ldots \quad (3)$$

where:-

V = the rate of the reaction, in this case the percentage haemolysis per minute.

E = energy of activation.

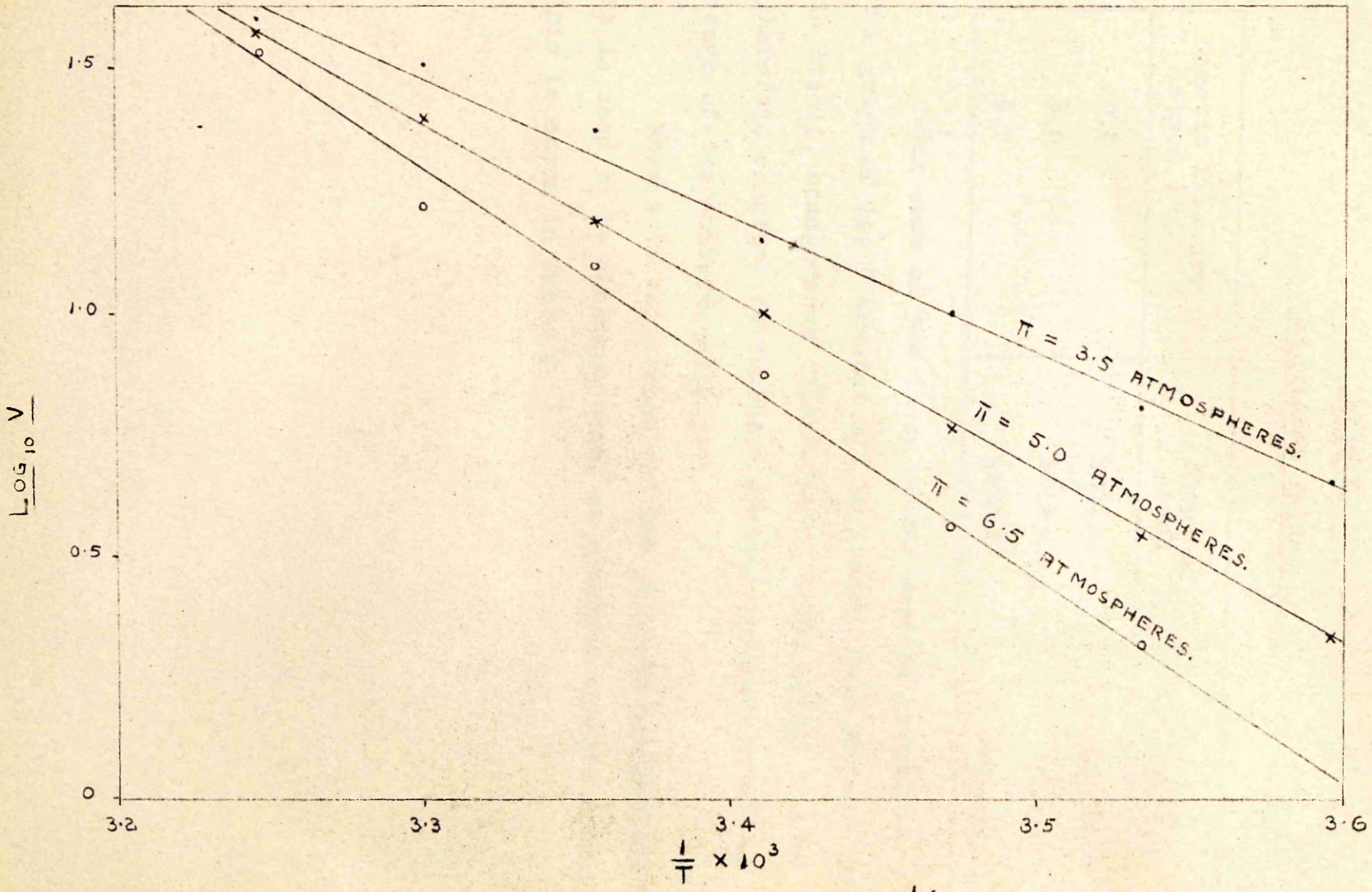
R = the gas constant.

A = a constant for the process under defined conditions.

e = the base of natural logarithms.

This equation requires that the plot of $\log_e V$ or log V against 1/T shall be a straight line, a relationship which holds for a great number of physical and chemical processes and which has been found to hold in the present instance. Fig.9 shows the relationship between log V and 1/T in the three cases described above, namely, when cells are suspended in solutions of malonamide of 3.5, 5.0 and 6.5 atmospheres osmotic pressure.

Furthermore, the slope of the graph gives a direct measure of the value of $-E/2.303R$ and if the slope is calculated, the value of E may be obtained. The values of E are:-



GRAPH - LOG V AGAINST 1/T

FIG. 9.

Table 3.

Calculated Values of E.

Osmotic Pressure (atmos.)	Slope of graph	E (cals.)
3.5	2830.0	13.000
5.0	3675.0	16.900
6.5	4350.0	20.000

For each of the three chosen osmotic pressures, since the graph of log V against 1/T is linear, each process is, in itself, homogeneous (Hinshelwood, 1940, p.45). E is therefore constant at constant osmotic pressure over the range of temperatures employed.

When A is calculated for the separate temperatures, it is seen to be constant also, at constant osmotic pressure. This is shown in Table 4.

Table 4.

Calculated Values of A.

Temperature at which A is calculated.	Values of A		
	π = 3.5 (atmos.)	π = 5.0 (atmos.)	π = 6.5 (atmos.)
5°	6.3×10^{10}	3.7×10^{13}	-
10°	6.4×10^{10}	3.5×10^{13}	4.6×10^{15}
15°	6.3×10^{10}	3.5×10^{13}	4.4×10^{15}
20°	6.1×10^{10}	3.7×10^{13}	5.0×10^{15}
25°	6.9×10^{10}	3.5×10^{13}	4.7×10^{15}
30°	6.7×10^{10}	3.5×10^{13}	3.6×10^{15}
35°	5.7×10^{10}	3.2×10^{13}	4.2×10^{15}

These results show that the effect of temperature change, between 5° and 35°C, on the haemolysis of erythrocytes in aqueous malonamide at constant osmotic pressure, is adequately described by the exponential equation. It must be recorded at this point that the exponential term of the equation is concerned only with the effect of change of temperature on the process. The term, A, covers all other variables and its interpretation is therefore much more difficult.

Since both E and A vary with solution osmotic pressure,

it is of interest to graph their relationships.

Figure 10a shows that E is a linear function of the osmotic pressure of the haemolysing solution and has, on extrapolation to zero solution osmotic pressure, a limiting value of 4,600 calories. This decrease in activation energy with decreasing osmotic pressure is in accord with the fact that haemolysis occurs more rapidly at low osmotic pressures.

Log A is also a linear function of osmotic pressure (Fig.10b) and has the limiting value 5.3 on extrapolation to zero solution osmotic pressure. That A should vary with the osmotic pressure implies that there is a fundamental difference between the processes being measured at different osmotic pressures. It is difficult to account for the occurrence of such a fundamental difference between malonamide-induced haemolysis at 3.5 atmospheres and malonamide-induced haemolysis at 6.5 atmospheres osmotic pressure.

In spite of these defects the equation provides at least a useful way of summarising a considerable body of data.

The extrapolated values of E and log A are amenable to physical interpretation. They are the values which should be obtained when haemolysis takes place in a system of whole blood diluted 1:21 with distilled water. Using the method described for rapidly haemolysing systems, the energy

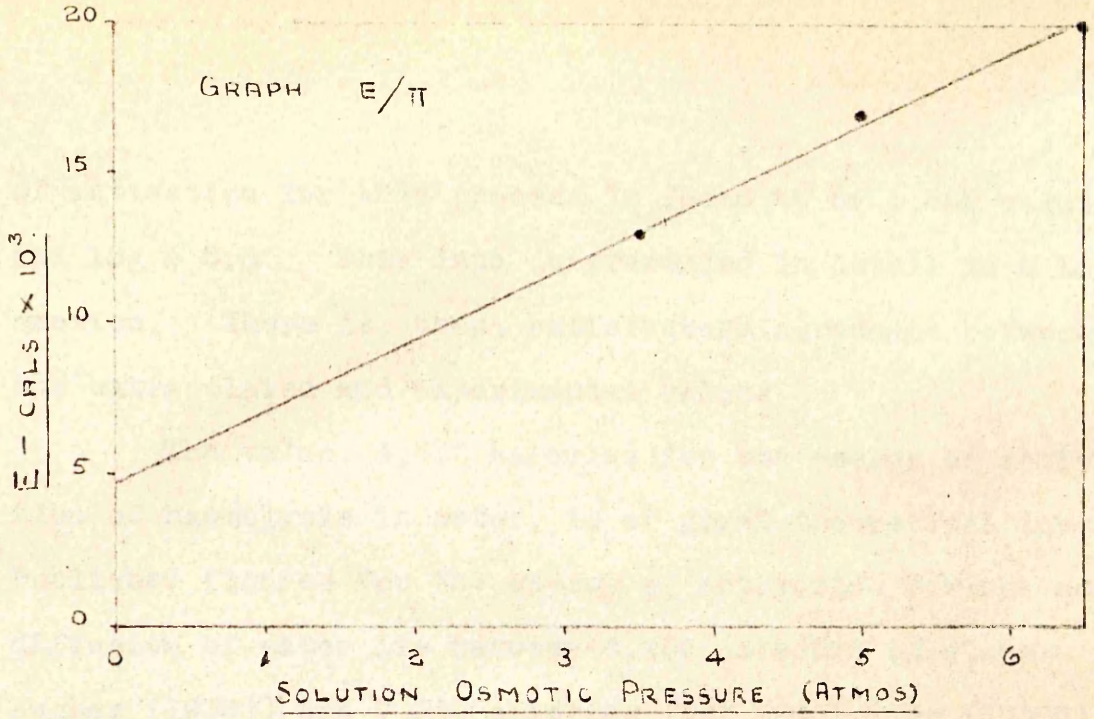


FIG. 10a.

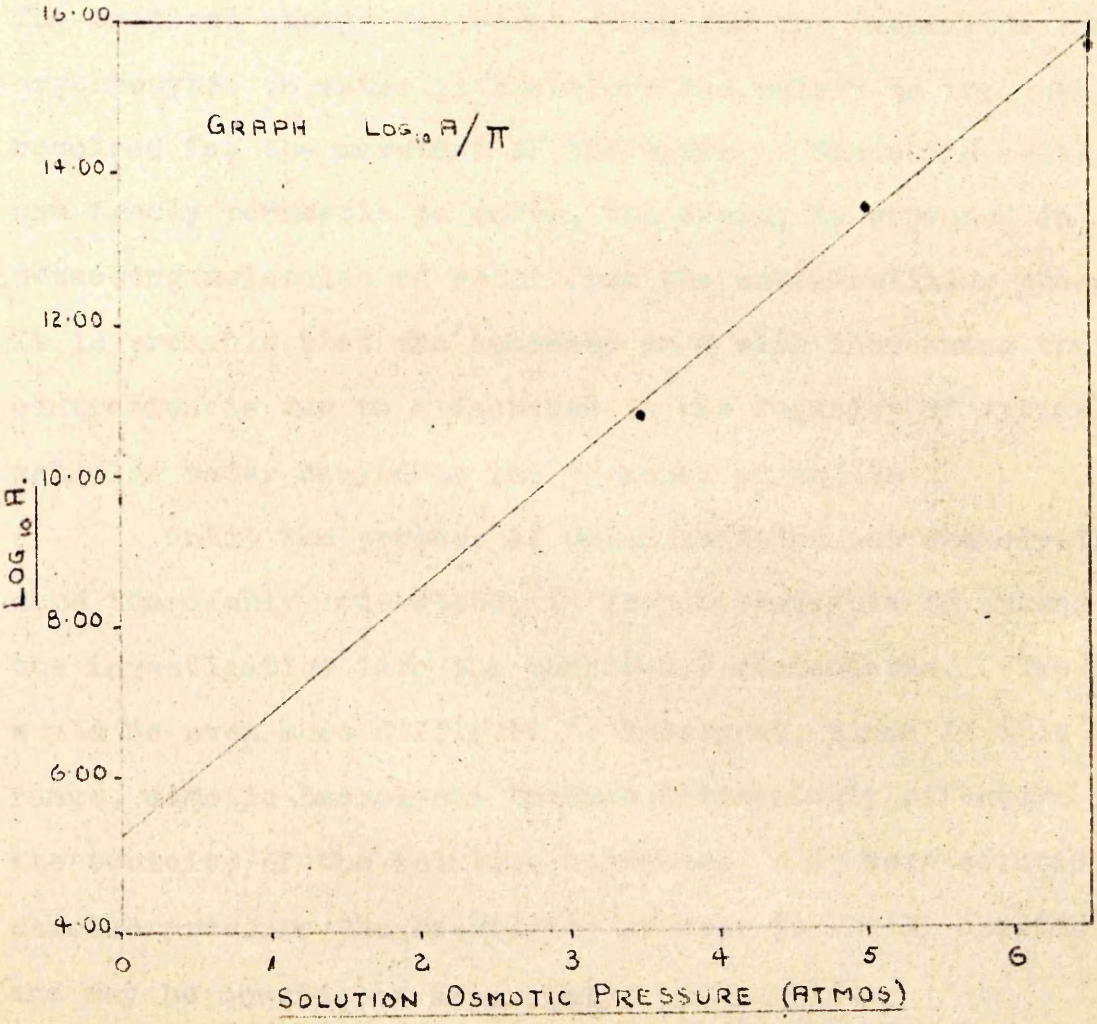


FIG. 10b.

of activation for this process is found to be 4,550 calories and log A 5.8. This data is presented in detail in a later section. There is, thus, satisfactory agreement between the extrapolated and experimental values.

The value, 4,550 calories for the energy of activation of haemolysis in water, is of great theoretical interest. Published figures for the energy of activation for the self-diffusion of water lie between 4,400 calories (Moelwyn-Hughes (1933)) and 5,300 calories (Orr and Butler (1935)). The critical energy increment found for the haemolysis of erythrocytes in water is therefore due solely to the energy required for the movement of the water. Since the cells are freely permeable to water, the energy is expended in detaching molecules of water from the extra-cellular phase. It is probable that the increase in E with increasing concentration is due to a decrease in the fugacity of extra-cellular water caused by the presence of solute.

Until the process of malonamide-induced haemolysis is more thoroughly understood, it is not desirable to extend the investigation into the zone 0-3.5 atmospheres. The data would be even more difficult to interpret, since in this range, osmotic haemolysis becomes increasingly effective as the tonicity of the solution decreases. At zero solution osmotic pressure the haemolytic process is wholly osmotic and may be considered separately.

3.2. The Effects of Ions on Malonamide-induced Haemolysis.

(a) Alkali-metal Cations.

The effect of ions on haemolysis is not reported in the literature. Their action on malonamide-induced haemolysis therefore, merits investigation.

The presence of small, osmotically equivalent concentrations (π = 0.5 atmosphere) of the chloride of sodium, potassium, caesium and rubidium, accelerate haemolysis in malonamide solutions of 3.5 atmospheres osmotic pressure. Lithium chloride, under the same conditions, causes inhibition (Fig.11).

This unexpected finding justifies a detailed experimental example, for which sodium chloride has been chosed.

The haemolysing solution contains 1.49 gm./100 ml. malonamide and 0.0652 gm./100 ml. sodium chloride. The experiment is carried out at 20°C., at which temperature the osmotic pressure due to malonamide is 3.5 atmospheres, and that due to sodium chloride is 0.5 atmosphere. The total osmotic pressure of the solution is therefore 4.0 atmospheres, compared with 3.5 atmospheres in the control. Haemolysis is, nevertheless, more rapid in the system of higher osmotic pressure.

20 ml. malonamide solution, of 3.5 atmospheres osmotic pressure, is placed in a 60 ml. test-tube fitted with a ground

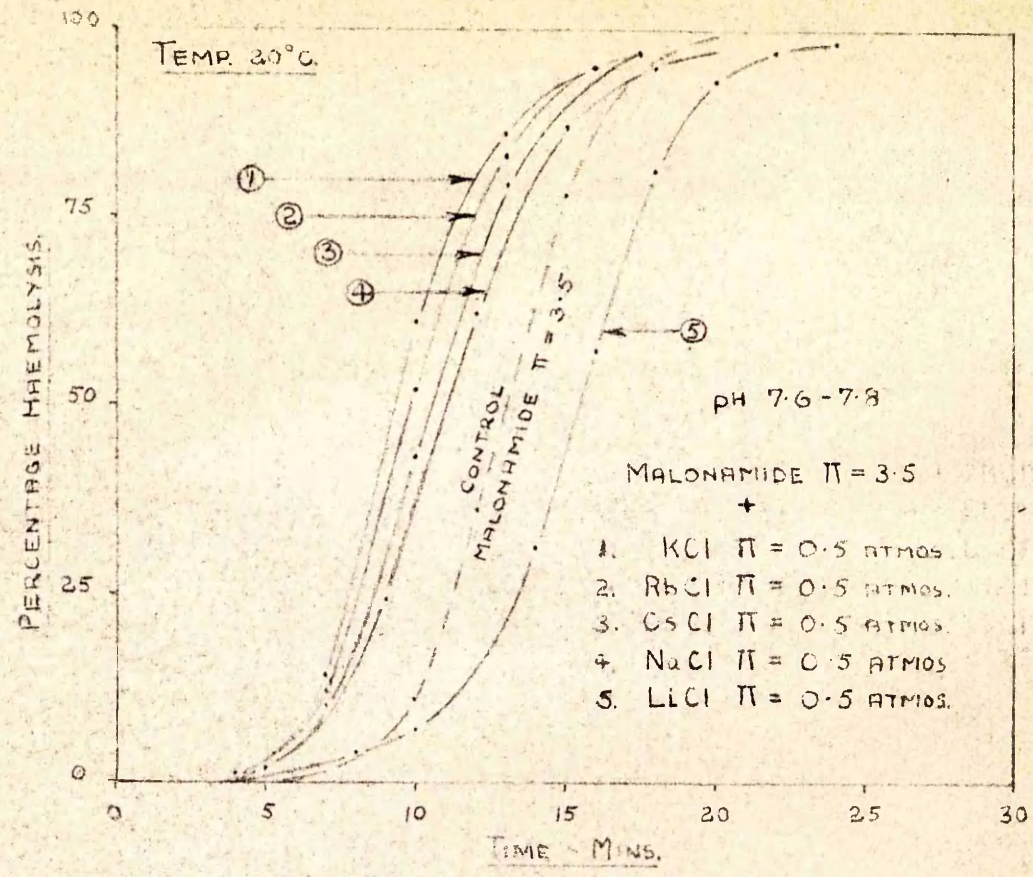

TEMP. 20°C.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
5
10
15
20
25
30
TIME - MINS.
CONTROL
MALONAMIDE Π = 3·5
pH 7·6 - 7·8
MALONAMIDE Π = 3·5
+
1. KCl Π = 0·5 ATMOS.
2. RbCl Π = 0·5 ATMOS.
3. CsCl Π = 0·5 ATMOS.
4. NaCl Π = 0·5 ATMOS
5. LiCl Π = 0·5 ATMOS.

FIG. 11.

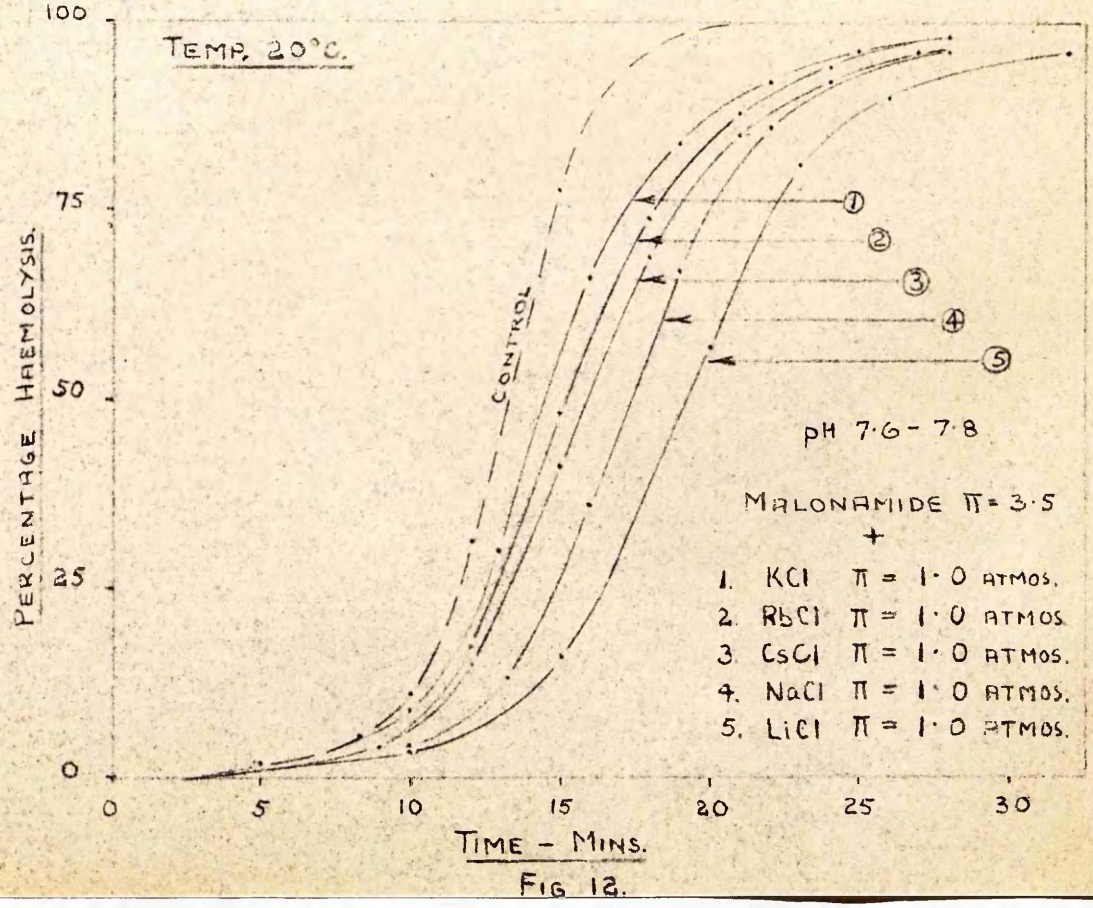

TEMP. 20°C.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
5
10
15
20
25
30
TIME - MINS.
CONTROL
pH 7·6 - 7·8
MALONAMIDE Π = 3·5
+
1. KCl Π = 1·0 ATMOS.
2. RbCl Π = 1·0 ATMOS.
3. CsCl Π = 1·0 ATMOS.
4. NaCl Π = 1·0 ATMOS.
5. LiCl Π = 1·0 ATMOS.

FIG 12.

glass stopper. To a similar tube is added 20 ml. of a solution containing malonamide equivalent to 3.5 atmospheres and sodium chloride equivalent to 0.5 atmosphere. The tubes are placed in a water bath at 20°C. To each is added 1.0 ml. of normal, heparinised, fully oxygenated blood, at zero time. The tubes are removed from the water bath, the contents mixed by gentle inversion, and returned to the bath. At 4 minute intervals, the contents are mixed and 3 ml. specimens are removed from each system for the determination of degree of haemolysis. The results of an experiment of this type are tabulated below.

Table 5.

Accelerated malonamide-induced haemolysis in presence of 0.5 atmosphere sodium chloride.

Time (mins)	Control %H. Malonamide (π = 3.5 atmos.)	Test % H. Malonamide + NaCl (π = 3.5 + = 0.5 atmos.)
4	0	1
8	9	20
12	50	72
16	91	93
20	99	98

The remaining members of the series, with the exception of lithium chloride, have similar effects, as shown in Fig.11. It is difficult to account for the fact that one member of the series inhibits haemolysis, while the remaining members, in

osmotically equivalent concentrations, cause acceleration of haemolysis.

The dilution of whole blood with an electrolyte-free solution causes a decrease in the lateral cohesion which exists between the molecules of the cell membrane (Davson and Danielli (1952, p.308)). Permeability will therefore be greater than normal even in isotonic non-electrolytes. The permeability will be increased further by the swelling which occurs in hypotonic media. Since the effect of ions on the membrane depends on their charge and concentration, an increase in concentration or an exchange of monovalent ions for divalent ions should reduce permeability and, therefore, the rate of haemolysis.

That haemolysis is accelerated shows the cells to be cation permeable at this concentration of electrolyte (π = 0.5 atmosphere). The concentration of ions is too low to produce a measurable change in the intermolecular cohesive forces of the membrane. Electrolyte as well as malonamide diffuse into the cell and therefore a greater volume of water must enter to restore osmotic equilibrium. The degree of swelling in this system is, accordingly, greater than that of the control and the cells haemolyse sooner than those of the control.

With a higher concentration of electrolyte, osmotically equivalent to 1.0 atmosphere, no acceleration of haemolysis is observed (Fig.12). This may be explained on the basis that there is sufficient electrolyte present in the system to restore cation impermeability. In this system malonamide only is entering the cell. A smaller volume of water is then required to move in for the restoration of osmotic equilibrium. There is, therefore, a lesser degree of swelling and haemolysis takes longer than the control.

In presence of electrolyte osmotically equivalent to 1.5 atmospheres (Fig.13), the degree of inhibition is increased further since, with cation impermeability restored and a higher extra-cellular osmotic pressure, less water is required to diffuse into the cell to establish osmotic equilibrium. The degree of swelling is less and the cells haemolyse more slowly.

The order of decreasing inhibitory effect of the cations is,

$$Li^+ > Na^+ > Cs^+ > Rb^+ > K^+.$$

It is noteworthy that this order is maintained over the range of concentrations employed, whether haemolysis is accelerated or inhibited. The series, as such, cannot be correlated with any of the physical properties of the ions.

THE HAEMOLYSIS OF ERYTHROCYTES IN HYPOTONIC MALONAMIDE IN PRESENCE OF OSMOTICALLY EQUIVALENT CONCENTRATIONS OF ALKALI METAL SALTS.

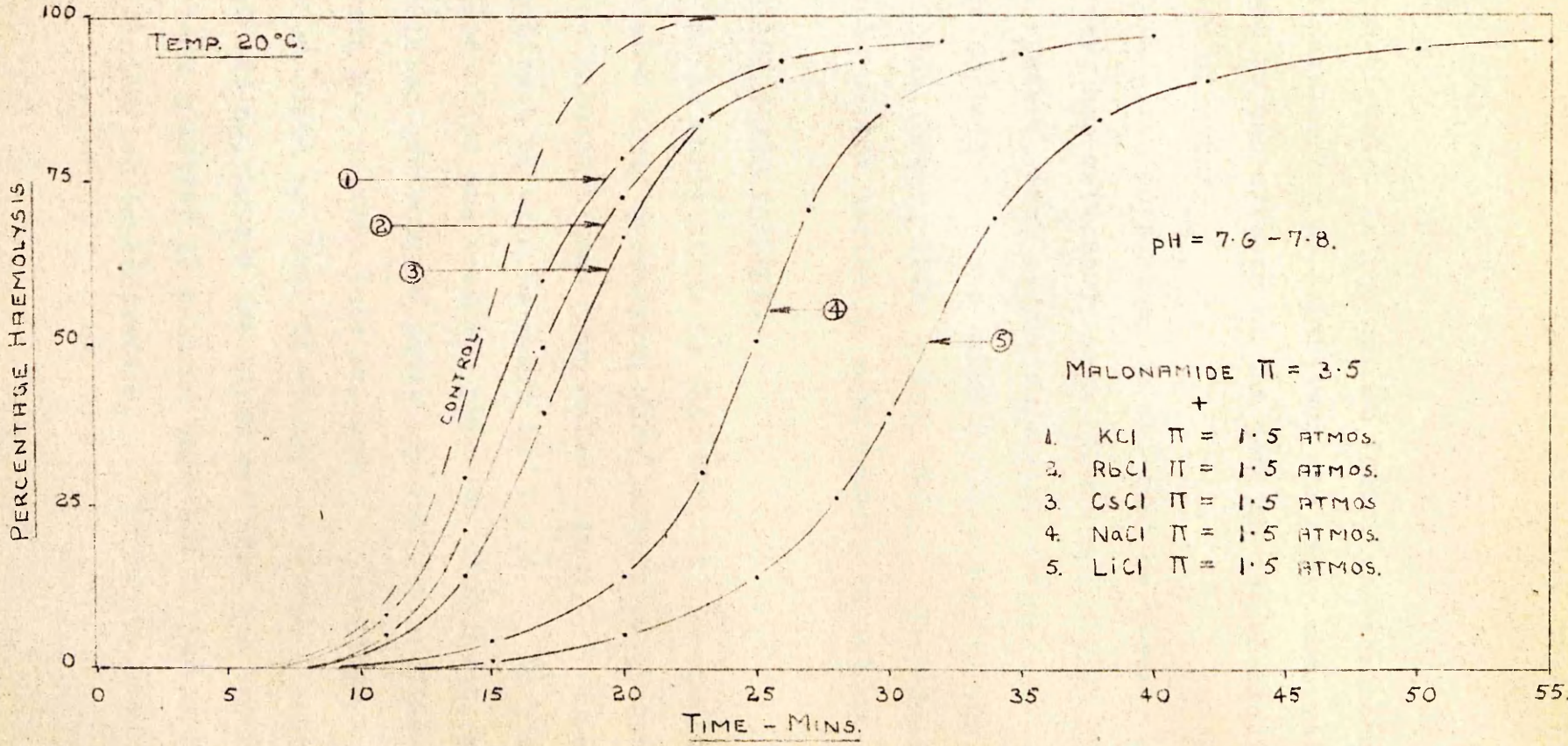


FIG. 13.

Since flow of water is the common factor whether haemolysis is accelerated or inhibited, the order observed may be related to the effects of the ions on the rheological properties of water.

This cation series, in the same or closely related order, has effects in many physiological and physicochemical systems, such as the degree of dispersion of lyophilic colloids and the hydration and dehydration of cell colloids (Höber, 1945, p.293, 299). The reason for the effects of the series in such systems is unknown.

(b) Alkaline earth cations.

At an early stage in this work it was observed that oxalated blood haemolysed more quickly in hypotonic malonamide solutions than heparinised blood. Experimental evidence of this is shown in Fig.14. In graph 1 heparinised blood was treated, prior to experiment, with the calculated quantity of potassium oxalate required to precipitate the calcion ions present. In graph 2 the heparinised blood was used directly. Graph 3 shows the effect of adding, before the blood was used for experiment, a sufficient quantity of calcium chloride to double the normal calcium ion concentration. It can be seen that

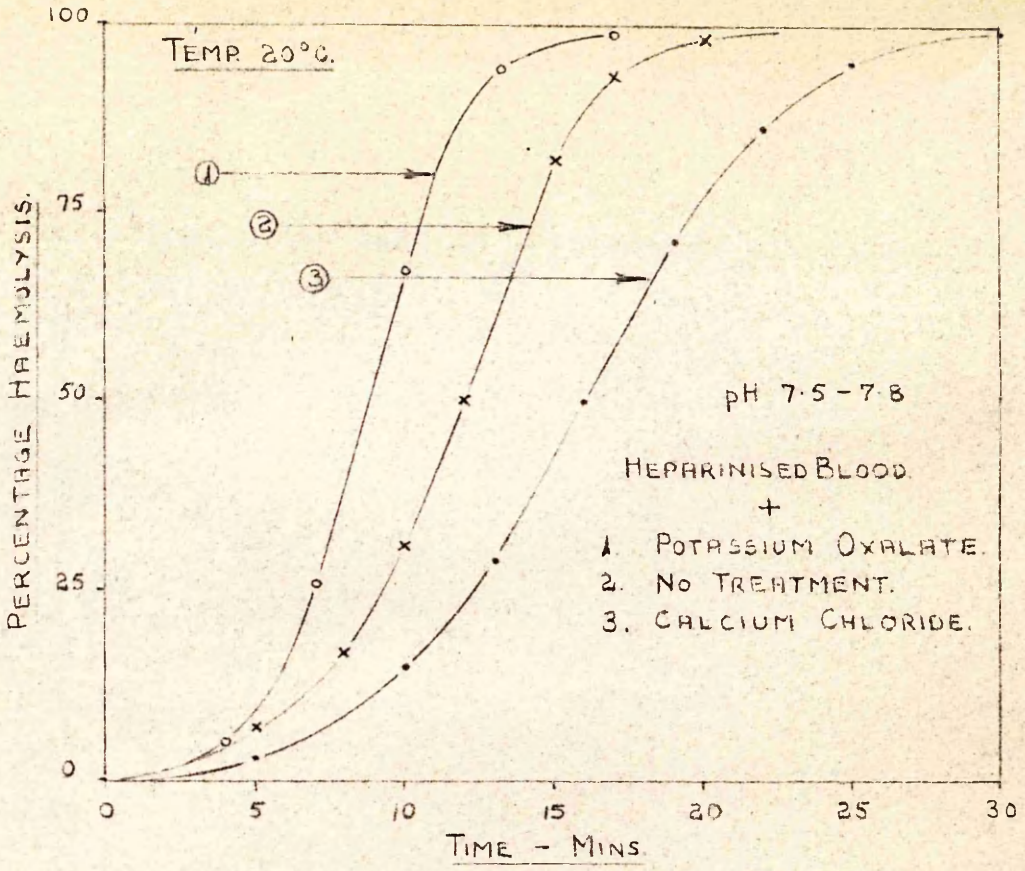

TEMP. 20°C.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
5
10
15
20
25
30
TIME - MINS.
pH 7·5 - 7·8
HEPARINISED BLOOD
+
1. POTASSIUM OXALATE.
2. NO TREATMENT.
3. CALCIUM CHLORIDE.
①
②
③

FIG. 14.

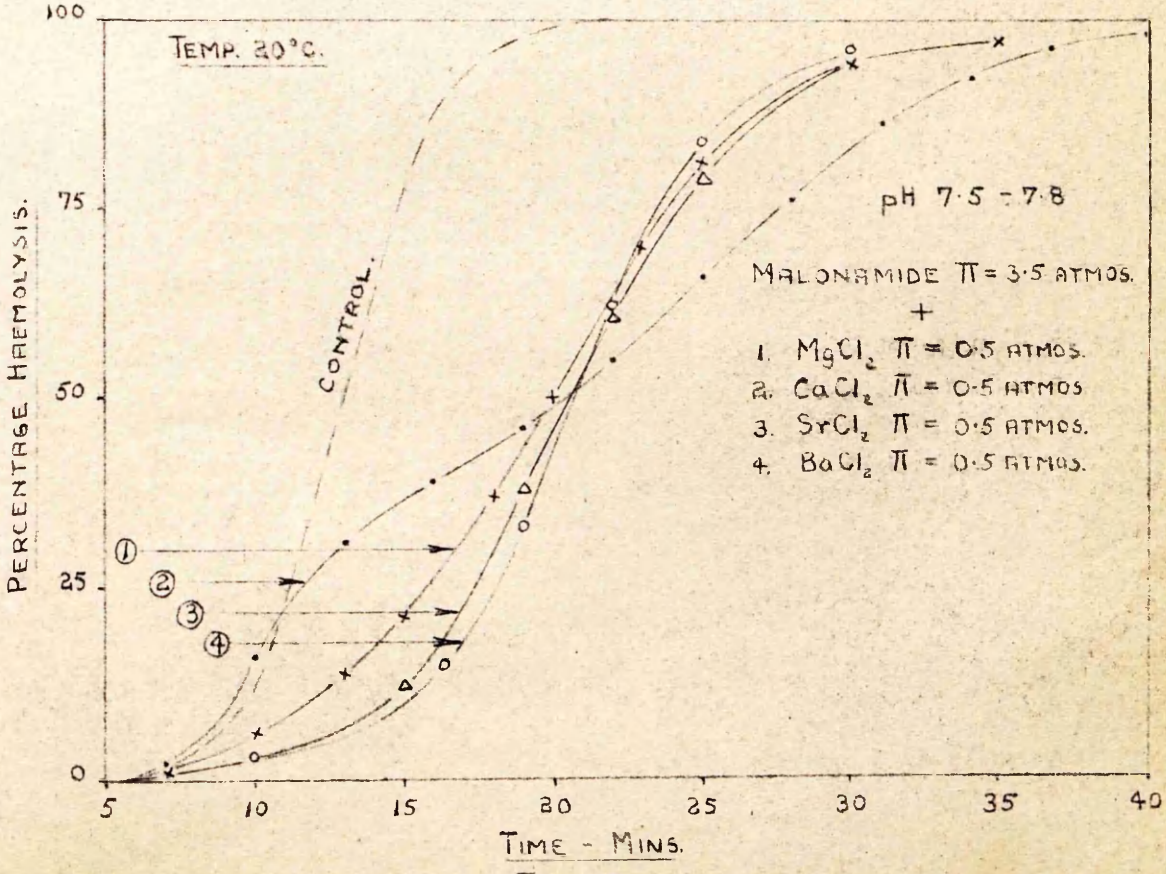

TEMP. 20°C.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
5
10
15
20
25
30
35
40
TIME - MINS.
CONTROL.
pH 7·5 - 7·8
MALONAMIDE Π = 3·5 ATMOS.
+
1. MgCl₂ Π = 0·5 ATMOS.
2. CaCl₂ Π = 0·5 ATMOS
3. SrCl₂ Π = 0·5 ATMOS.
4. BaCl₂ Π = 0·5 ATMOS.
①
②
③
④

FIG. 15.

variation in the calcium ion concentration has a pronounced effect upon the rate of haemolysis in hypotonic aqueous malonamide.

Fig.15 shows the effect of the addition of small, osmotically equivalent concentrations (π = 0.5 atmos.) of the alkaline earth chlorides, on the rate of haemolysis in malonamide solutions of 3.5 atmospheres osmotic pressure. In this series of cations calcium appears to have a unique action in producing a biphasic haemolysis curve and marked inhibition. The remaining ions of the series also produce a degree of inhibition and are closely related to their effects. The order of effectiveness for inhibition is,

$$Ca^{++} > Mg^{++} > Sr^{++} > Ba^{++}.$$

Increasing the concentration of these salts to 1.0 atmosphere (Fig. 16) serves to emphasise the above observations. The degree of inhibition is considerable and in the same order as before. At this concentration the degree of haemolysis attained in presence of calcium ions appears to reach a limiting value, at which it remains, independent of the time of exposure to lytic conditions.

EFFECT OF ALKALINE EARTH CHLORIDES ON MALONAMIDE-INDUCED HAEMOLYSIS, AT CONSTANT OSMOTIC PRESSURE.

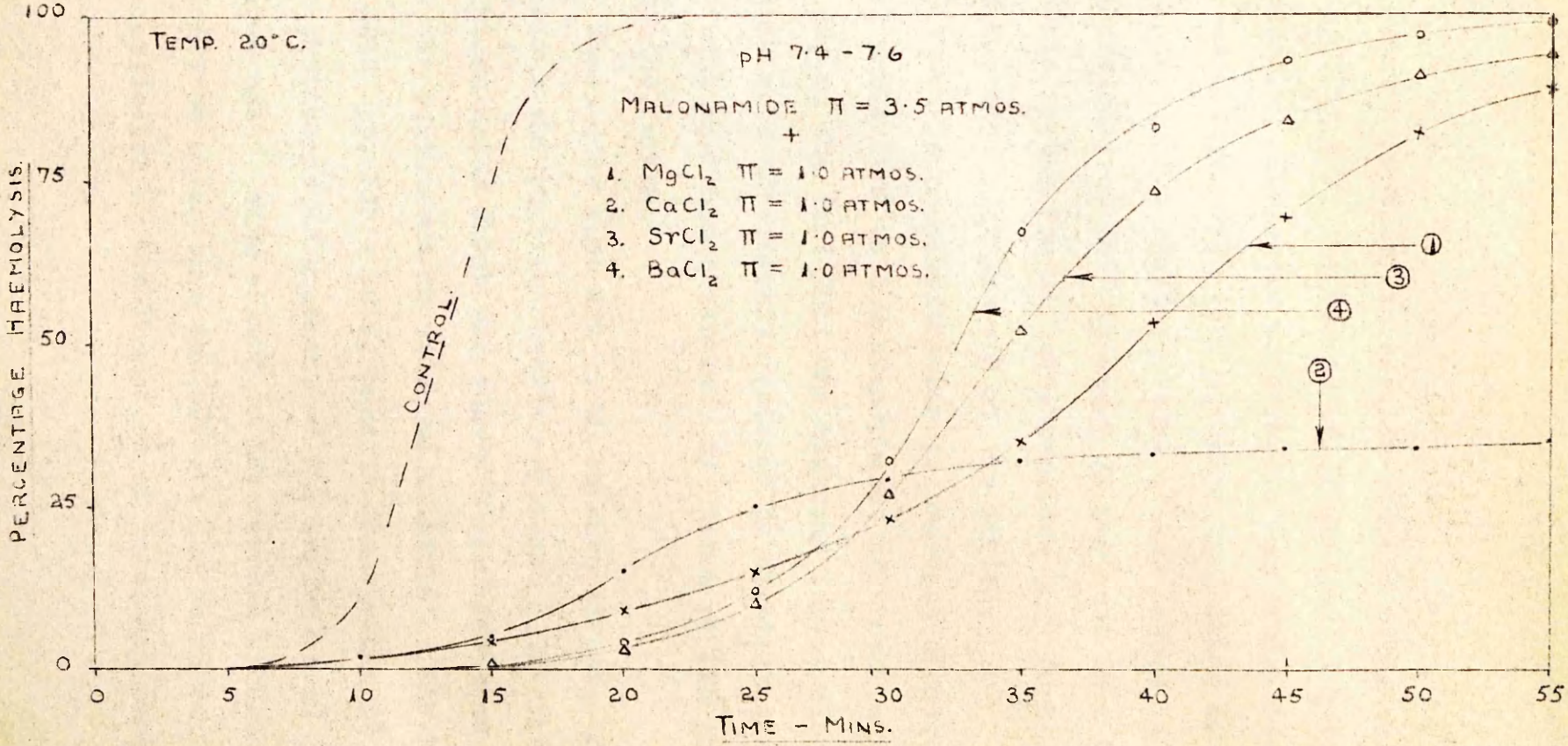


FIG 16.

With a further increase in osmotic pressure to 1.5 atmos. (Fig.17), the degree of inhibition is very much greater. The order of capacity for inhibition is the same as before, with very striking inhibition by calcium ions. The limiting value of the degree of haemolysis in presence of Ca^{++} is about 14 per cent in 2 hours. Leaving this system for 24 hours produces only some 20 per cent haemolysis at the end of the period.

There is no acceleration in presence of concentrations, equivalent to 0.5 atmosphere, of these salts. This is due to the fact that the membrane inter-molecular adhesion is more effectively restored by divalent cations compared with monovalent cations, by virtue of the greater change on the former.

These ions also are known to have effects on physiological and physico-chemical systems. The range of conditions under which the observations have been made and the variations in order of activity of the ions (Höber, 1945, pp. 303 - 307), make it inadvisable to claim that any particular arrangement of the series is an established one. With regard to the results presented here, it may be noted that the effects due to these ions may be complicated by the formation of insoluble phosphates

EFFECT OF ALKALINE EARTH CHLORIDES ON MALONAMIDE-INDUCED HAEMOLYSIS AT CONSTANT OSMOTIC PRESSURE.

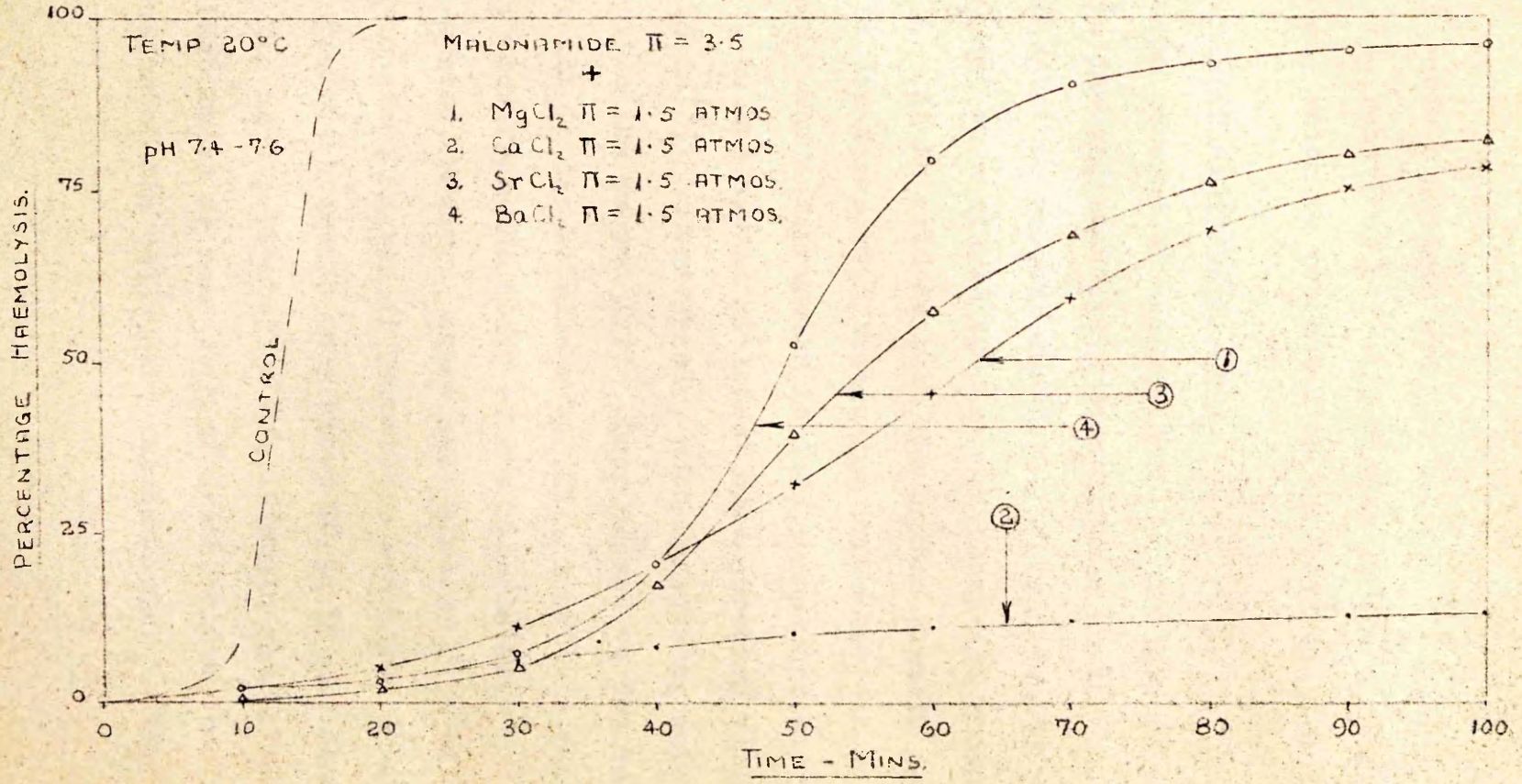


FIG 17.

with cell surface anionic groups.

The behaviour of calcium ions merits a detailed examination, which is presented in the following section.

(c) The Action of Calcium Ions.

The potency of calcium ions for inhibition of malonamide induced haemolysis made it seem worth while to investigate the process further. The results of an experiment on a single specimen of blood are shown in Fig. 18. It is evident that the double sigmoid is tending to disappear with decreasing concentration of calcium ions. The interruption is less pronounced and nearer the origin in the system of lowest concentration with respect to Ca^{++}. Conversely, with increasing calcium concentration the second lytic phase ceases to become operative and in the extreme case (Fig. 18.5) the rate is uniformly slow from the start of the experiment. In the last system, however, there must also be a levelling off to a constant degree of haemolysis since the final reading after 24 hours is only about 20 to 25 per cent.

The data presented in Fig.18 would appear to suggest that there is some form of union between the cells, or components of the cells, and calcium ions. The shapes

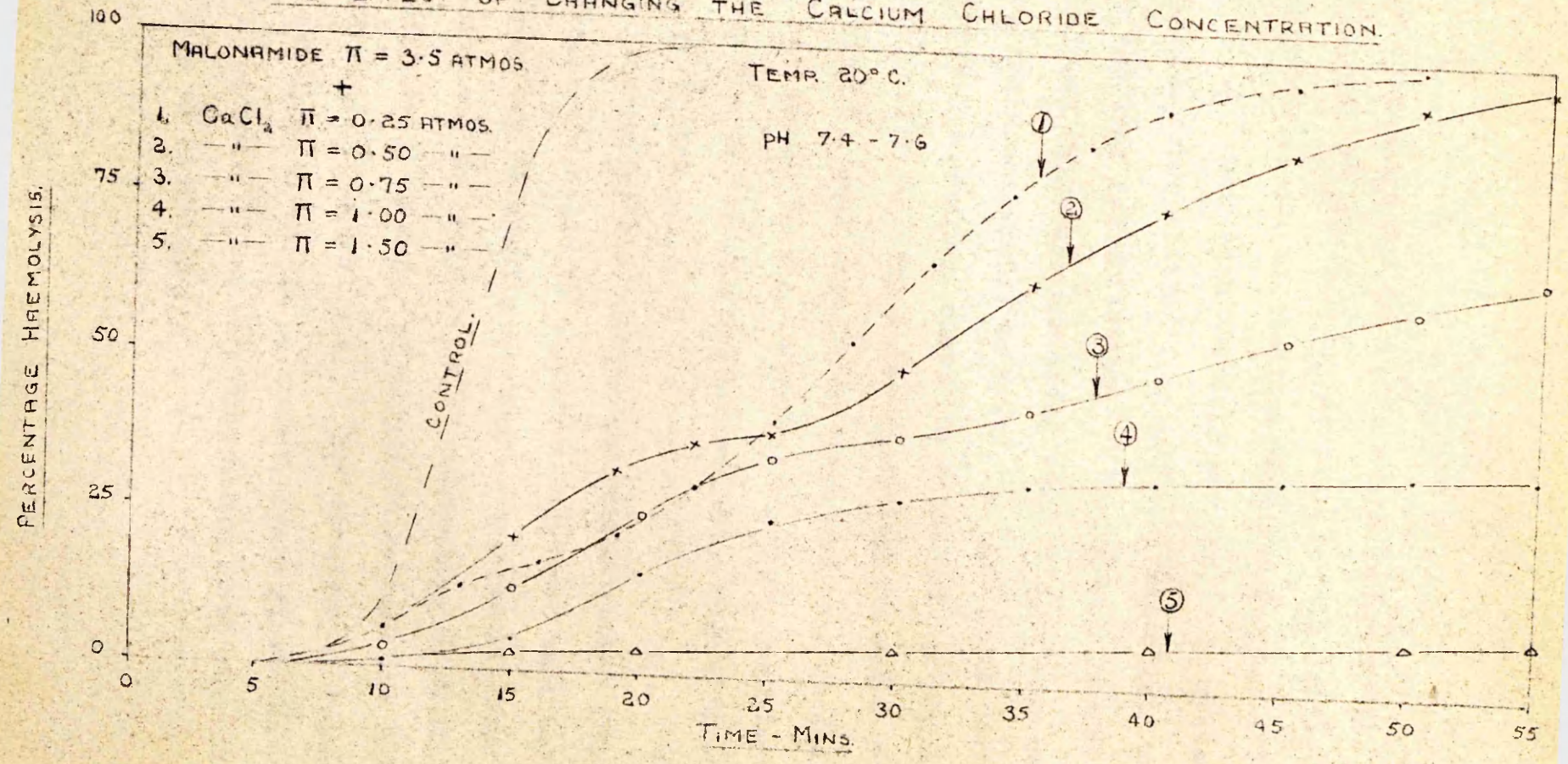

THE EFFECT OF CHANGING THE CALCIUM CHLORIDE CONCENTRATION.
MALONAMIDE π = 3·5 ATMOS
+
1. CaCl2 π = 0·25 ATMOS.
2. —"— π = 0·50 —"—
3. —"— π = 0·75 —"—
4. —"— π = 1·00 —"—
5. —"— π = 1·50 —"—
TEMP. 20° C.
pH 7·4 - 7·6
CONTROL.
PERCENTAGE HAEMOLYSIS.
TIME - MINS.
0
25
50
75
100
5
10
15
20
25
30
35
40
45
50
55

FIG. 18.

of the curves are due to a pronounced change in the order of magnitude of the rate of haemolysis; the latter is probably the result of the time required for the effective union of Ca^{++} ions with the cell surface. In systems of low calcium concentration (Fig.18. 1,2) haemolysis occurs before the ions are effective. During this period the unhaemolysed cells are combining with calcium ions which enhance their resistance to lysis. The upper portion of the curve, therefore, shows the haemolysis of these resistant cells. The intervening episode shows the haemolysis of a cell population which varies from wholly normal resistance to that degree of enhanced resistance corresponding to the calcium ion concentration of the particular system.

Increasing the calcium ion concentration (Fig.18. 3,4) enables the cells to acquire a degree of enhanced resistance before malonamide induced haemolysis can occur. This resistance to lysis is still increasing, as indicated by the difference in order of magnitude between the initial and final rates of haemolysis. The intervening episode, between the initial and final states, again shows the haemolysis of cells which vary from moderate to high in their degree of resistance.

In Fig.18. 5, the calcium ion concentration confers an initially high degree of resistance on the cells. The resistance is still incomplete since the cells continue to haemolyse, though extremely slowly.

The data is strongly suggestive of a form of interaction between cells and calcium ions resulting in increased resistance to haemolysis.

Fig.19. 1 shows the result of adding to a system of 3.5 atmos. malonamide, during the lytic phase, a quantity of calcium chloride equivalent to 0.5 atmos. osmotic pressure. The initial lag is due to the slight increase in osmotic pressure, from 3.5 to 4.0 atmospheres. The form of the post addition curve is the same as that obtained with the system in which this concentration of calcium chloride is present initially. As before, the shape is due to the time lag between the addition of calcium ions and their effective combination with the cells.

When excess calcium ions, initially present in the system, are removed by precipitation with the calculated quantity of potassium oxalate, there is a striking change in the haemolysis curve (Fig.19. 4). The very great increase in haemolysis suggests that calcium is reversibly combined with the cells and its complete removal causes irreversible damage to the cell surface. The shape of the post oxalate addition curve is due to the slowing down of calcium oxalate

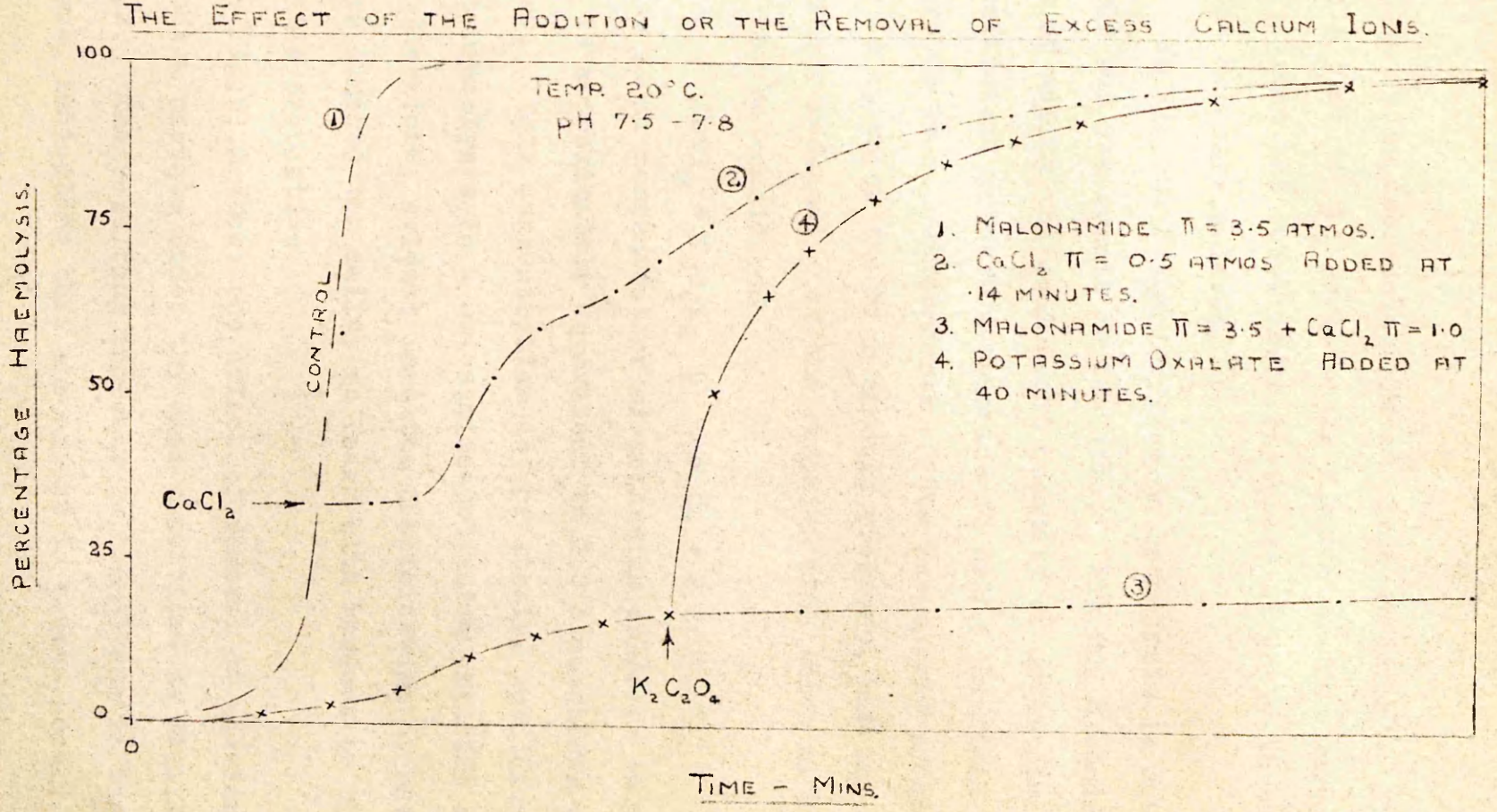

THE EFFECT OF THE ADDITION OR THE REMOVAL OF EXCESS CALCIUM IONS.
TEMP. 20°C.
pH 7·5 - 7·8
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
CONTROL
CaCl₂
K₂C₂O₄
1. MALONAMIDE Π = 3·5 ATMOS.
2. CaCl₂ Π = 0·5 ATMOS ADDED AT 14 MINUTES.
3. MALONAMIDE Π = 3·5 + CaCl₂ Π = 1·0
4. POTASSIUM OXALATE ADDED AT 40 MINUTES.
TIME - MINS.

FIG. 19.

formation, with decreasing oxalate concentration. It is probable that if the added oxalate concentration were sufficiently great the curve would rise more directly to the 100 per cent haemolysis level.

In Fig.20. 2 the increase in haemolysis is not quite so rapid but the result is the same. Fig.20. 3 shows the effect of adding the equivalent quantity of potassium oxalate, plus sufficient potassium chloride to bring the final osmotic pressure up to 7.0 atmospheres. The haemolytic process continues in spite of the high osmotic pressure, but the rate is considerably reduced. If the reaction with which we are concerned may be considered,

$$CaCl_2 + C_2O_4K_2 = C_2O_4Ca + 2\ KCl$$

then the final concentration of potassium chloride in the system is approximately equivalent to 3.5 atmospheres osmotic pressure. This concentration is, of itself, sufficient to prevent haemolysis in a malonamide-potassium chloride system. It is, therefore, evident that the precipitation of calcium ions has damaged the cells, at least with respect to their haemolytic properties.

Fig.21.2 shows the effect of removal of calcium ions with sodium oxalate under the same conditions as Figs.19.4 and 20.2. The resulting increase in haemolysis is the same as before, indicating that the effect is caused solely by

THE EFFECT OF REMOVING THE INHIBITING CALCIUM IONS AND CONCURRENTLY RAISING THE OSMOTIC PRESSURE.

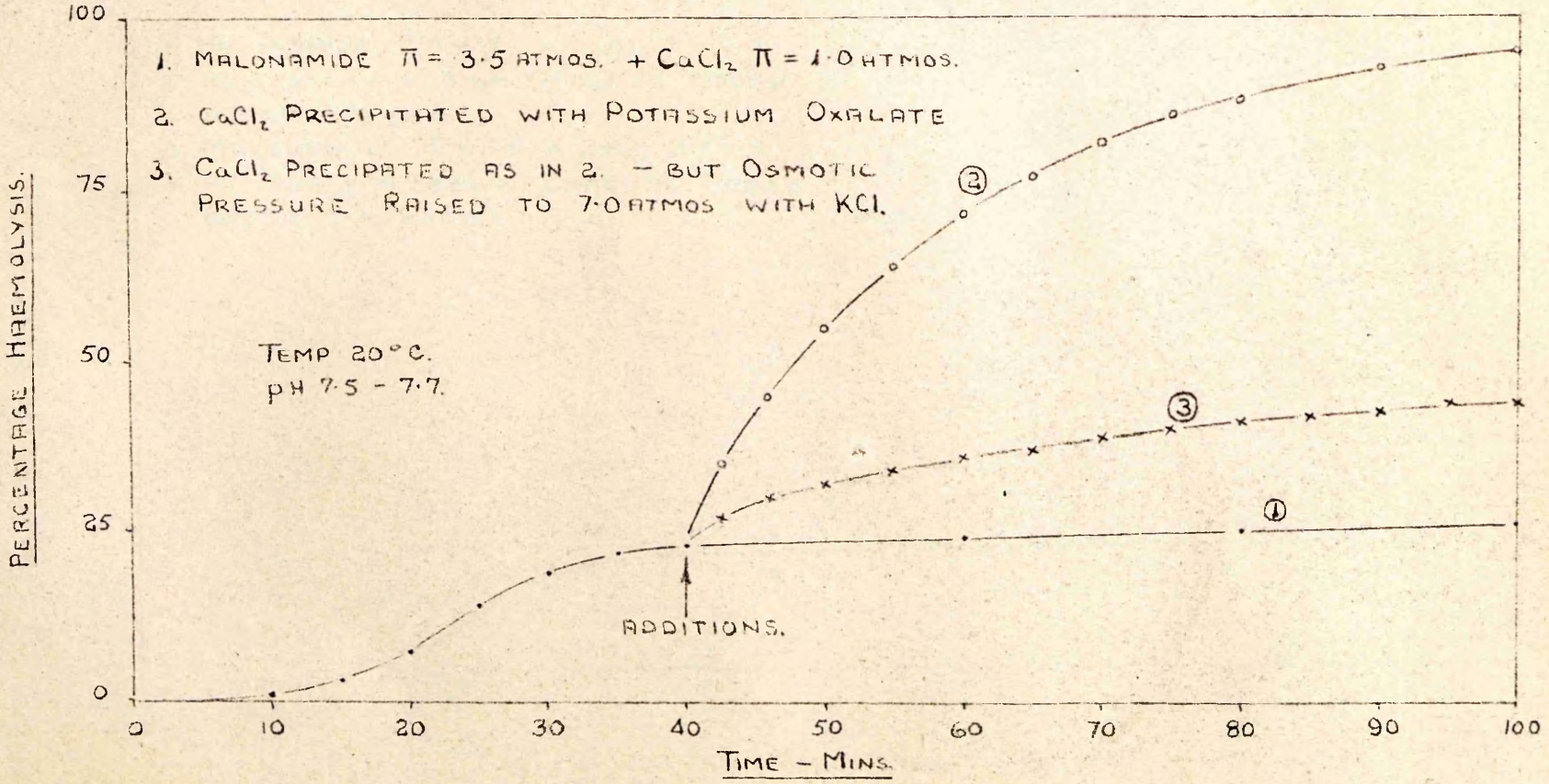


FIG. 20.

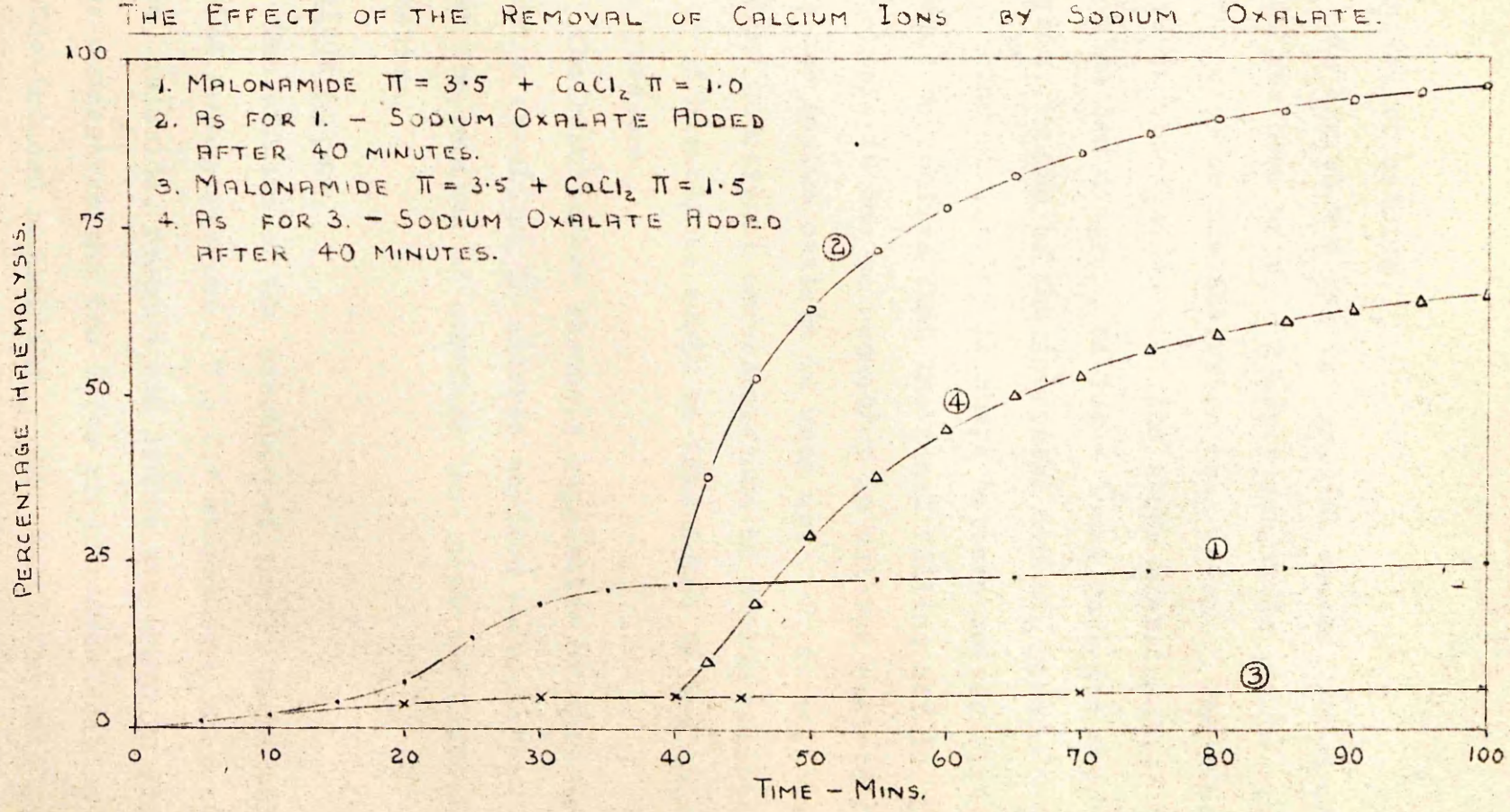

THE EFFECT OF THE REMOVAL OF CALCIUM IONS BY SODIUM OXALATE.
1. MALONAMIDE π = 3·5 + CaCl₂ π = 1·0
2. AS FOR 1. – SODIUM OXALATE ADDED AFTER 40 MINUTES.
3. MALONAMIDE π = 3·5 + CaCl₂ π = 1·5
4. AS FOR 3. – SODIUM OXALATE ADDED AFTER 40 MINUTES.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
10
20
30
40
50
60
70
80
90
100
TIME – MINS.
①
②
③
④

FIG. 21.

the removal of calcium.

In Fig.21.4 a greater initial concentration of calcium chloride has been used. A correspondingly greater quantity of sodium oxalate has therefore been added. The same type of curve is obtained as with the other experiments; its position on the diagram, having a lower rate and degree of haemolysis, is due to the increased osmotic pressure of the system. The similarity in shape between curves 21.2 and 21.4 tends to confirm that the final falling off in the rate of haemolysis is due to reduction in calcium oxalate formation as the sodium oxalate is used up. It is pointless to use higher additional concentrations of sodium oxalate, since the rate of haemolysis would be diminished by the increased osmotic pressure.

The results are strongly suggestive of cell-calcium combination resulting in altered surface characteristics and that the removal of combined ions produces surface disorganisation.

(d) Halide Anions.

The effects of the presence of small, osmotically equivalent concentrations (π = 0.5 atmosphere) of potassium fluoride, chloride, bromide and iodide are shown in Figure 22. At these concentrations the salts produce acceleration of malonamide-induced haemolysis. The reason for this is the

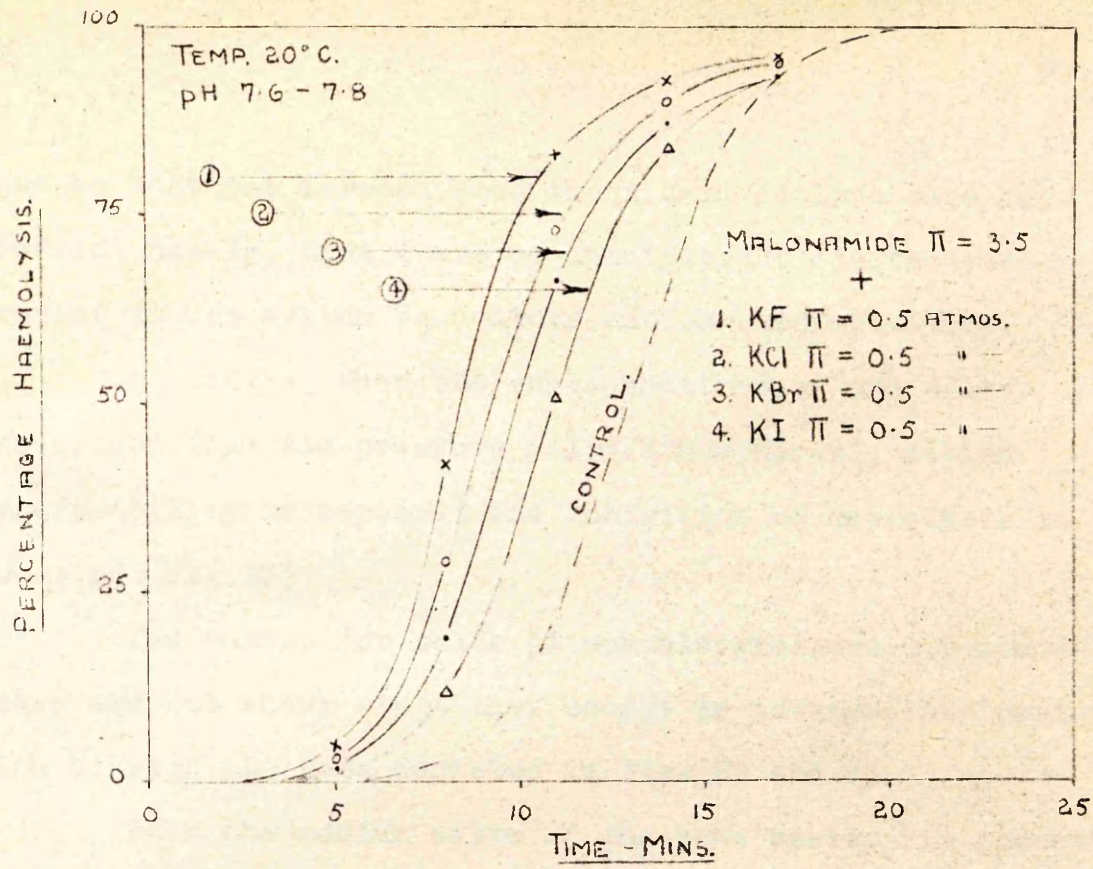

TEMP. 20° C.
pH 7·6 - 7·8
MALONAMIDE Π = 3·5
+
1. KF Π = 0·5 ATMOS.
2. KCl Π = 0·5 "
3. KBr Π = 0·5 "
4. KI Π = 0·5 "
CONTROL.
PERCENTAGE HAEMOLYSIS.
TIME - MINS.
0
25
50
75
100
0
5
10
15
20
25

FIG 22.

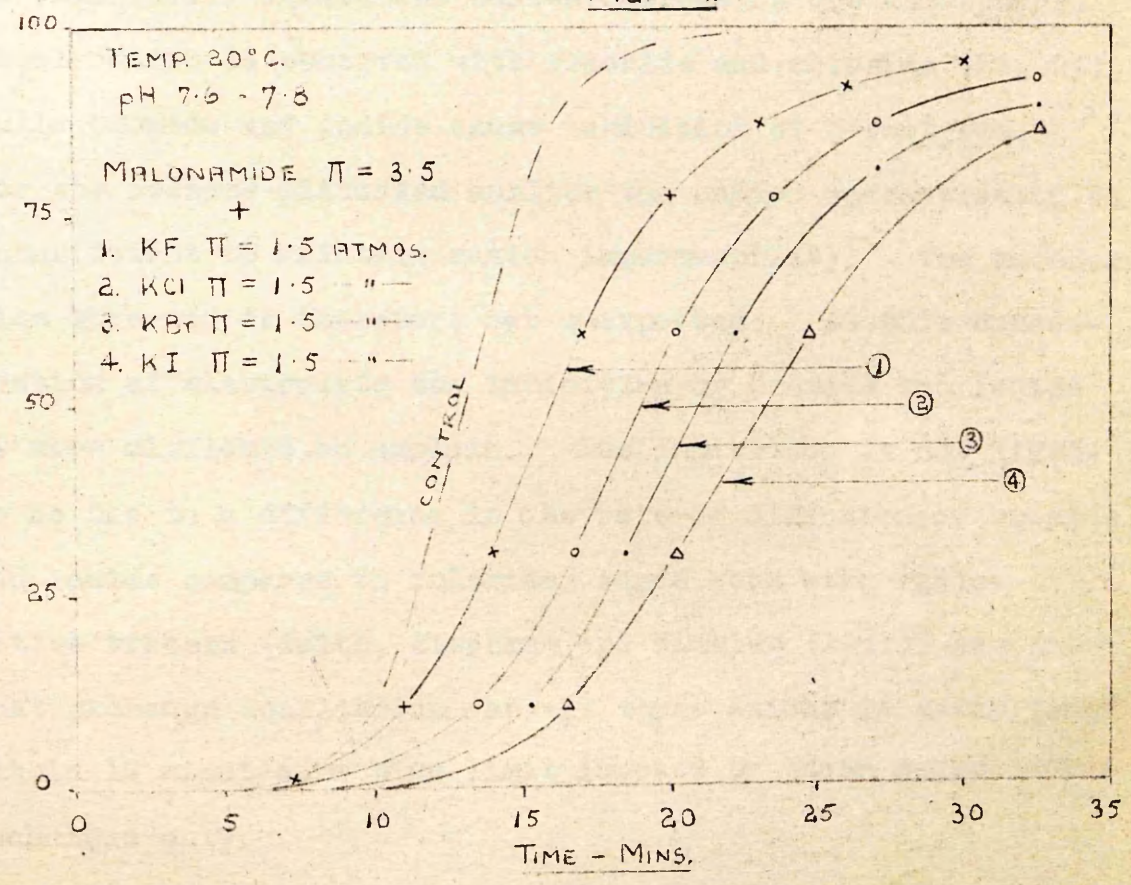

TEMP. 20° C.
pH 7·6 - 7·8
MALONAMIDE Π = 3·5
+
1. KF Π = 1·5 ATMOS.
2. KCl Π = 1·5 "
3. KBr Π = 1·5 "
4. KI Π = 1·5 "
CONTROL
PERCENTAGE HAEMOLYSIS.
TIME - MINS.
0
25
50
75
100
0
5
10
15
20
25
30
35

FIG. 23.

same as that put forward when the alkali cations were considered, namely, that there is insufficient electrolyte present in the system to restore cation impermeability.

As before, when the concentrations of the salts are raised (osmotic pressure = 1.5 atmospheres), cation impermeability is restored and inhibition of haemolysis is observed (Fig.23).

The curves for salts of osmotic pressure 1.0 atmosphere are not shown since they occupy an intermediate position between the sets depicted in Figs.22 and 23.

When the sodium salts of the same series are present in osmotically equivalent concentrations of 0.5 atmosphere, acceleration is observed with fluoride and chloride (Fig.24), while bromide and iodide cause inhibition of haemolysis. For the reasons discussed earlier the cation concentration is insufficient to maintain cation impermeability. The acceleration observed is therefore not unexpected. At this concentration of electrolyte the inhibition by bromide and iodide is more difficult to explain. The inhibition is not likely to be due to a difference in the rate of diffusion of bromide and iodide compared to chloride, since work with radioactive tracers (Smith, Eisenman and Winkler (1941)) has shown that exchange equilibrium for all three anions is established within 10 minutes, a time limit imposed by their manipulative technique only.

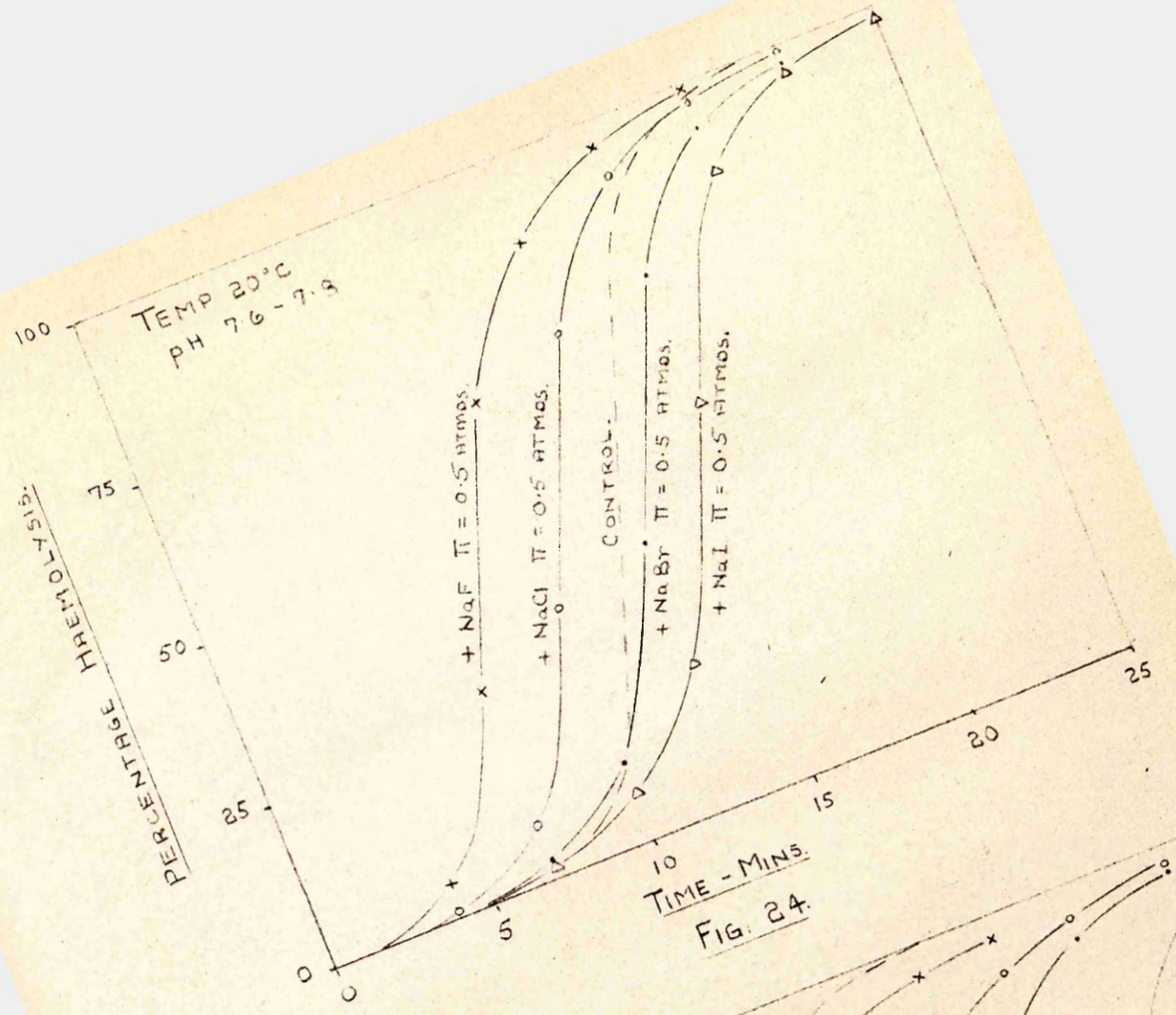

TEMP 20°C
PH 7.6 - 7.8
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
5
10
15
20
25
TIME - MINS.
+ NaF Π = 0.5 ATMOS.
+ NaCl Π = 0.5 ATMOS.
CONTROL
+ NaBr Π = 0.5 ATMOS.
+ NaI Π = 0.5 ATMOS.

FIG. 24.

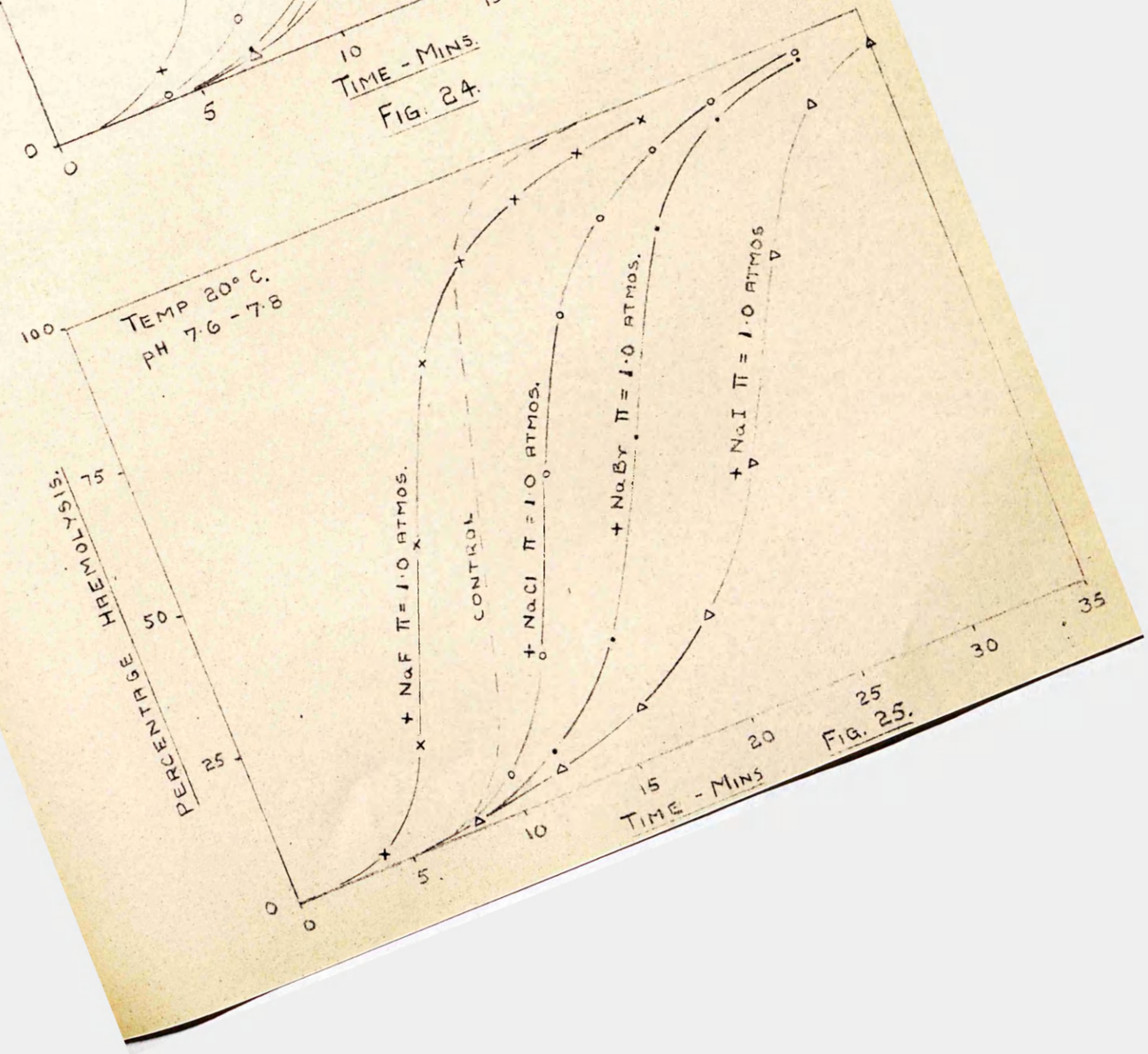

TEMP 20° C.
PH 7.6 - 7.8
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
5
10
15
20
25
30
35
TIME - MINS
+ NaF Π = 1.0 ATMOS.
CONTROL
+ NaCl Π = 1.0 ATMOS.
+ NaBr Π = 1.0 ATMOS.
+ NaI Π = 1.0 ATMOS

FIG. 25.

On comparing Figs.22 and 24, potassium and sodium salts respectively, it becomes apparent that the change of cation is partly responsible for the inhibition observed. The "spread" of the curves is much greater with the sodium salts, although the order of anion effect remains the same. The reason for this difference is not at present understood.

The sodium salts, in osmotically equivalent concentrations (osmotic pressure = 1.0 atmosphere), with the exception of fluoride, produce inhibition of malonamide-induced haemolysis (Fig.25). The order of anion effect remains the same.

A further increase in the osmotic pressure of the sodium salts to 1.5 atmospheres, Fig.26, shows marked inhibition by all except fluoride. When Fig.26 is compared with Fig.23 - the potassium salts in osmotically equivalent concentrations - the difference due to change of cation becomes very apparent. The difference in "spread" between the two sets of curves amounts almost to a different order of magnitude of effects. Furthermore, it may be noted that potassium fluoride has a greater inhibitory effect than sodium fluoride. No explanation can be put forward, at present, for this observation. The position of sodium fluoride as intrinsically haemolytic is in agreement with the observations of Hendry (1948).

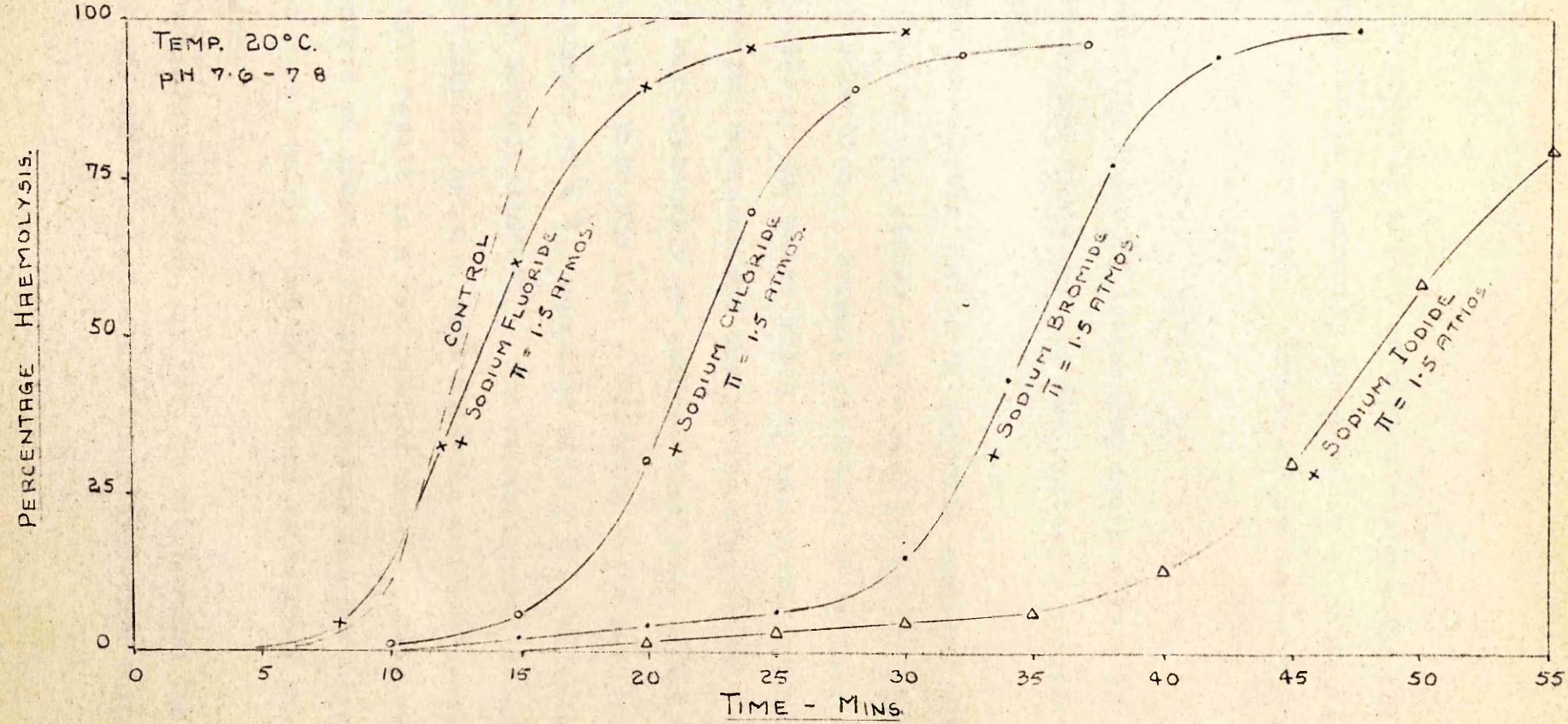

THE EFFECT OF HALIDE ANIONS ON MALONAMIDE - INDUCED HAEMOLYSIS.
TEMP. 20°C.
pH 7·6 - 7·8
CONTROL
+ SODIUM FLUORIDE π = 1·5 ATMOS.
+ SODIUM CHLORIDE π = 1·5 ATMOS.
+ SODIUM BROMIDE π = 1·5 ATMOS.
+ SODIUM IODIDE π = 1·5 ATMOS.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
5
10
15
20
25
30
35
40
45
50
55
TIME - MINS.

FIG. 26.

The order of effect of the anions remains the same throughout; it is apparently independent of cation and electrolyte concentration. Expressed as decreasing anionic inhibition, the order is,

$$I^- > Br^- > Cl^- > F^-$$

This is also the order of decreasing ionic volume of the anion series, and forms part of the lyotropic series (Höber, 1945, p.302).

The haemolytic action of fluoride compared with the other members of the series may be related to its high degree of primary solvation. Primary solvation is defined by Bockris (1949) as the water which is associated with an ion in its brownian movement and during its electrolytic transport. It may reasonably be assured that such water will penetrate cells with the ion. Fluoride has a mean primary solvation number of 4 ± 1 compared with 1 ± 1 for chloride (Conway and Bockris (1954)). The establishment of an exchange equilibrium between cell chloride and extra-cellular fluoride will result in a net gain of water. Cell swelling will therefore be greater in such systems and haemolysis will occur more readily than in systems containing the other halide anions.

No explanation can, as yet, be put forward for the effects of the other anions since their hydration numbers

are the same as chloride. The order of this anion series also remains unexplained, but will be discussed in a later section.

(e) Oxy-anions and Thiocyanate.

In view of the apparent importance of ion-water interaction on malonamide-induced haemolysis, it is of theoretical interest to observe the effects of oxy-anions on this process. These ions have the ability to form hydrogen bonds with adjacent water molecules and are therefore likely to have specific effects (Conway and Bockris (1954)). Although the oxy-acids are themselves unstable, their alkali-metal salts are quite stable under the conditions of these experiments (Sidgwick, 1950, p.1226).

The effects, on malonamide-induced haemolysis, of the presence of certain potassium salts in osmotically equivalent concentrations of 1.5 atmospheres, are shown in Figure 27.

The action of potassium chlorate is rather unexpected, in that it causes haemolysis to occur more quickly than in the control. From the point of view that the concentration of cation is sufficient to restore cation impermeability, as discussed in the earlier sections, it appears that chlorate ion is intrinsically more haemolytic than fluoride. This is clearly shown when Figs.23 and 27 are compared; though cation and osmotic pressure are the same in both systems, the

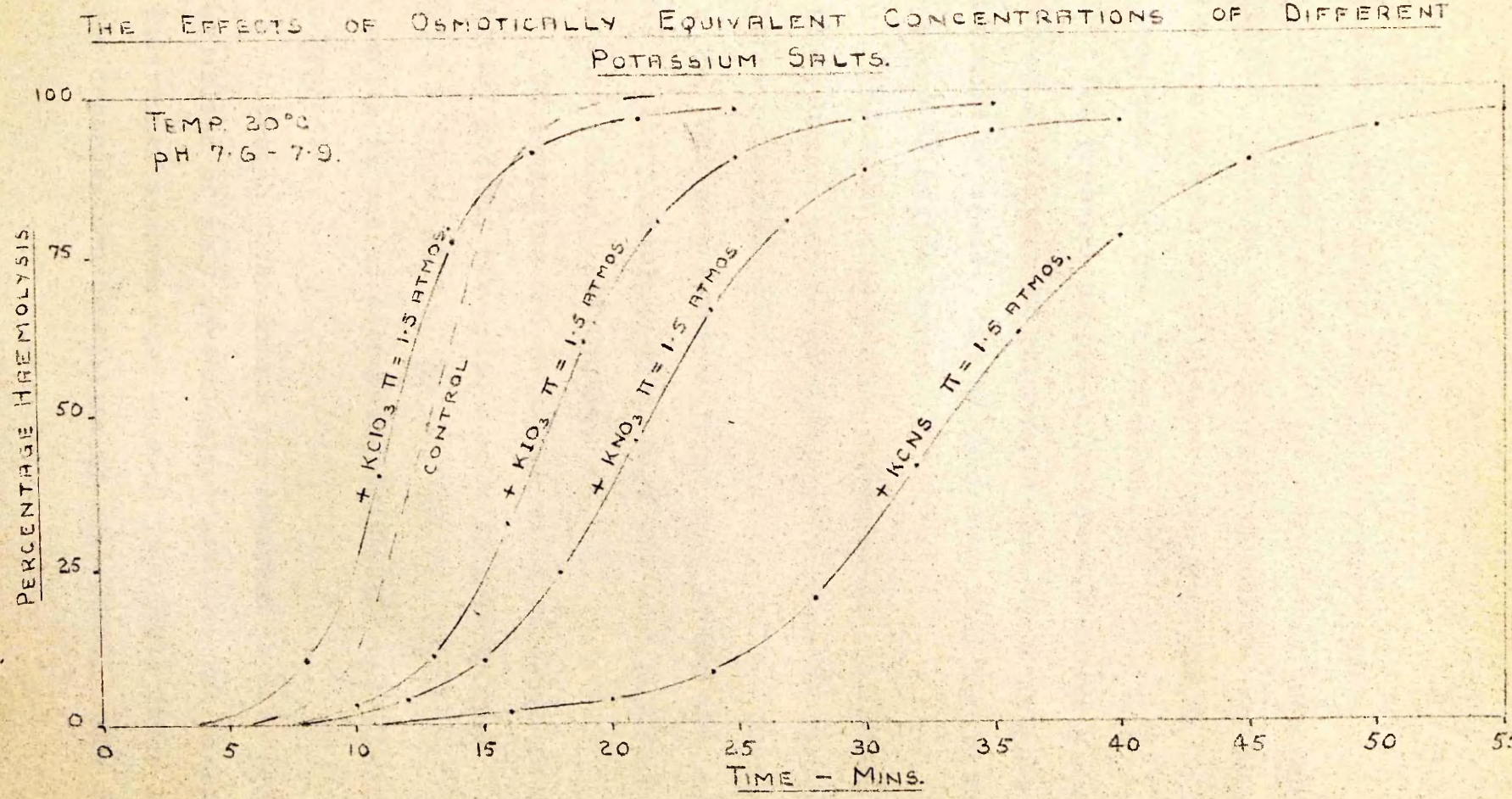

THE EFFECTS OF OSMOTICALLY EQUIVALENT CONCENTRATIONS OF DIFFERENT POTASSIUM SALTS.
TEMP. 20°C
PH 7·6 - 7·9.
PERCENTAGE HAEMOLYSIS
100
75
50
25
0
+ KClO3 Π = 1·5 ATMOS.
CONTROL
+ KIO3 Π = 1·5 ATMOS.
+ KNO3 Π = 1·5 ATMOS.
+ KCNS Π = 1·5 ATMOS.
0
5
10
15
20
25
30
35
40
45
50
55
TIME - MINS.

FIG. 27.

degree of inhibition in each is very different.

Potassium iodate, on the other hand, does produce inhibition, but, on comparing Figs.23 and 27, the degree of inhibition is seen to be greater than that due to fluoride and less than that due to chloride.

The presence of potassium nitrate causes a smaller degree of inhibition of malonamide-induced haemolysis than that caused by iodide, but greater than that due to the other oxy-anions or halide anions.

Potassium thiocyanate, osmotically equivalent to 1.5 atmospheres, causes considerable inhibition compared with the other potassium salts. This action is not unexpected since SCN^- occupies a terminal position in the lyotropic series. Its physiological effects are well reported in the literature (Höber, 1945, p.290).

On arranging the potassium salts in order of decreasing inhibitory action, the anion series is seen to be,

$$SCN^- > I^- > NO_3^- > Br^- > Cl^- > IO_3^- > F^- > ClO_3^-.$$

This series is one of decreasing inhibition with increasing ionic hydration.

Although these anions are reported to have effects on the behaviour of many physiological systems, the positions of the individual members vary so much that their order is obviously dependent on conditions of experiment. The reasons

for the series and its variations are not understood (Höber, 1945, p.302), but appear to be related to the hydration properties of the anions.

3.3. The Effect of Certain Non-electrolytes on Malonamide-induced Haemolysis.

Since the rate of haemolysis induced by malonamide depends on the osmotic pressure of the solution, the effect of increasing the osmotic pressure, by the addition of a second non-electrolyte, has been observed. For this purpose certain carbohydrates and polyhydric alcohols have been chosen. The rate of penetration of these substances into the erythrocyte varies greatly, from very rapid, in the case of glycerol, to zero in the case of sucrose; these substances do not react with malonamide under the conditions of experiment.

Since the rates of haemolysis of erythrocytes in hypotonic glucose solutions change greatly with temperature (Hendry (1951, 1954)), all experiments were carried out in the water bath at 20 $\pm$ 0.3°C. The control system contains malonamide osmotically equivalent to 3.5 atmospheres; the test systems contain the same concentration of malonamide, together with hexose in concentration equivalent to the stated osmotic pressure.

Figure 28 shows haemolysis curves obtained from

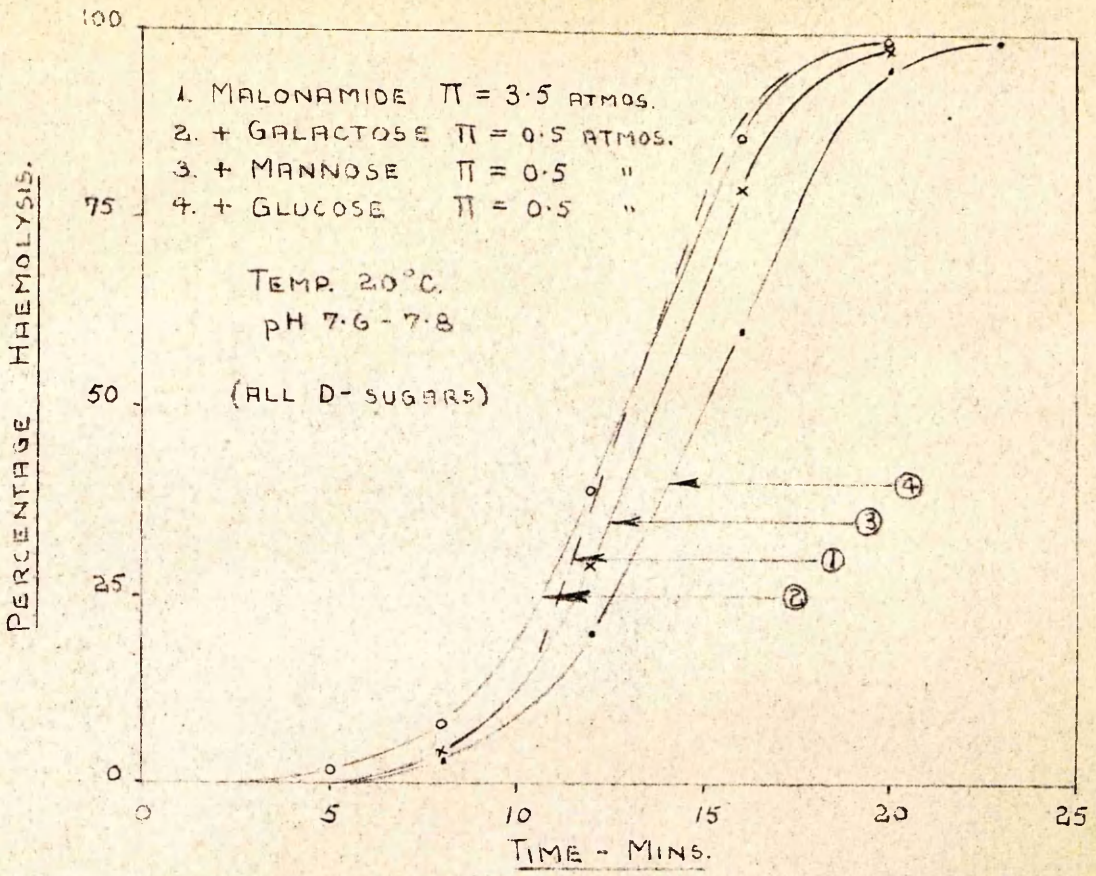

1. MALONAMIDE π = 3·5 ATMOS.
2. + GALACTOSE π = 0·5 ATMOS.
3. + MANNOSE π = 0·5 "
4. + GLUCOSE π = 0·5 "
TEMP. 20°C.
pH 7·6 - 7·8
(ALL D-SUGARS)
PERCENTAGE HAEMOLYSIS.
TIME - MINS.
0
25
50
75
100
5
10
15
20
25
①
②
③
④

FIG. 28.

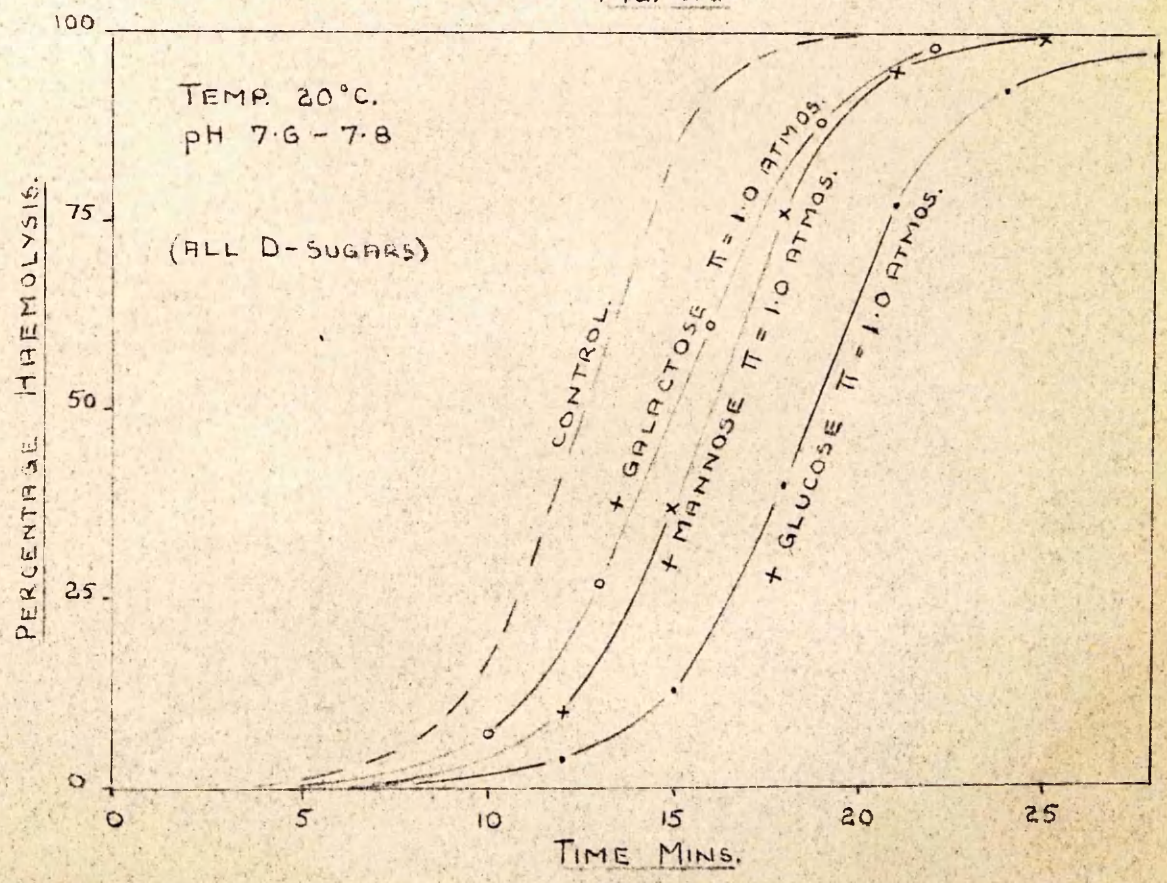

TEMP. 20°C.
pH 7·6 - 7·8
(ALL D-SUGARS)
CONTROL.
+ GALACTOSE π = 1·0 ATMOS.
+ MANNOSE π = 1·0 ATMOS.
+ GLUCOSE π = 1·0 ATMOS.
PERCENTAGE HAEMOLYSIS.
TIME MINS.
0
25
50
75
100
5
10
15
20
25

FIG. 29.

systems containing malonamide 3.5 atmospheres and glucose, galactose or mannose 0.5 atmosphere. The hexoses cause a slight inhibition of malonamide haemolysis, glucose being the most effective inhibitor.

On increasing the osmotic pressure of the hexoses to 1.0 atmosphere (Fig.29), the inhibition is greater and the sugars inhibit in the same order.

In Fig.30 are shown the effects of increasing the osmotic pressure of the hexoses to 1.5 atmospheres. The total solution osmotic pressure is now 5.0 atmospheres, and a second control, malonamide, 3.5 + 1.5 atmospheres, is included for comparison. Galactose, mannose and malonamide, in osmotically equivalent concentrations, have approximately the same inhibitory effect, compared with 3.5 atmospheres malonamide. Glucose remains the most effective inhibitor of the group.

These observations may be explained in terms of the rates of penetration of the different solutes. Galactose, mannose and malonamide apparently penetrate the cells at similar rates; they are present in the same molar concentrations (0.0625 M) and cause approximately the same degree of inhibition. Glucose, on the other hand, causes a much greater degree of inhibition although present in the same molar concentration as the others. This is due to the fact that

THE EFFECT OF PENETRATING CARBOHYDRATES ON MALONAMIDE-INDUCED HAEMOLYSIS.

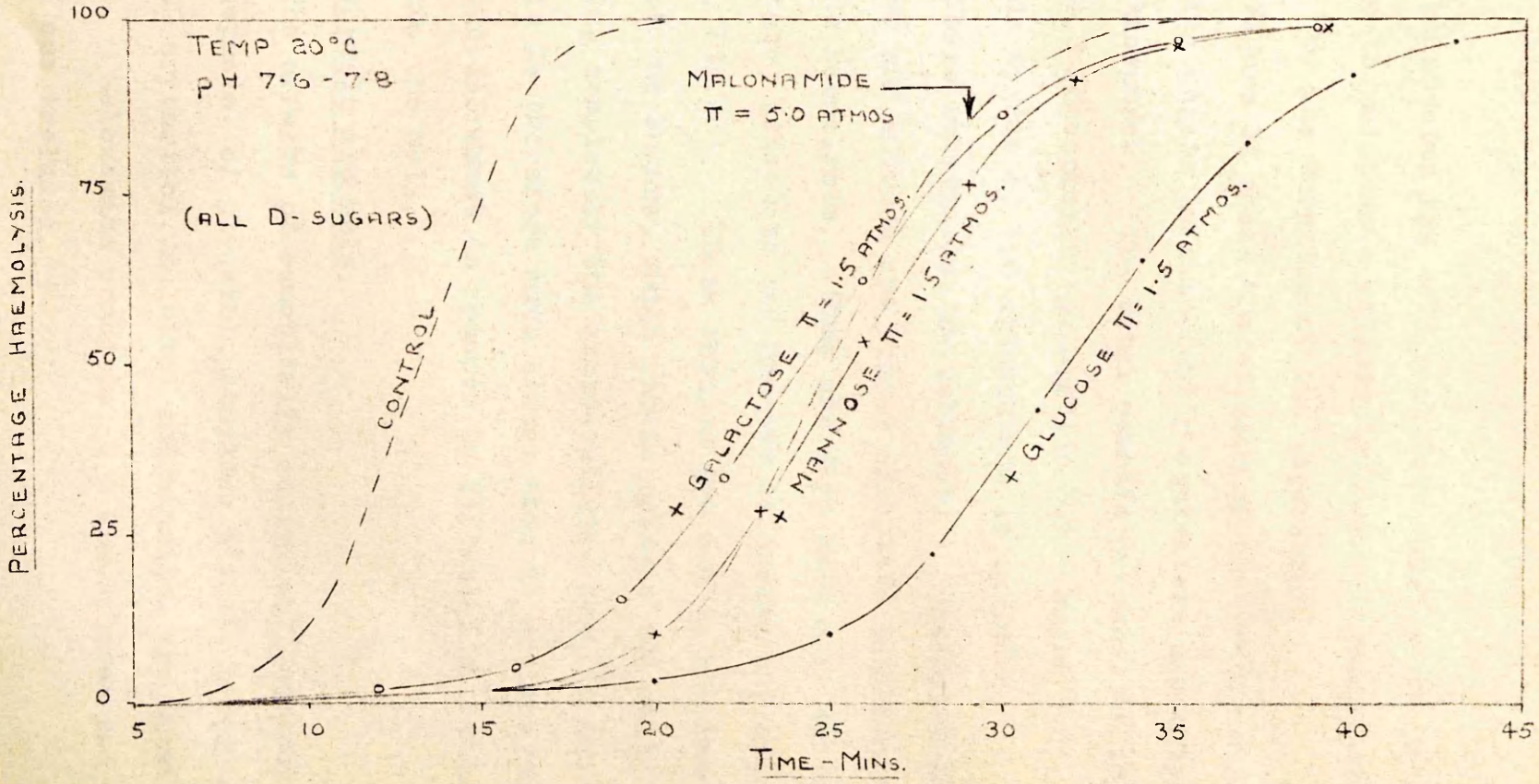


FIG. 30.

glucose penetrates the cell much less readily and is therefore able to maintain a relatively higher external osmotic pressure for the duration of the experiment.

Figure 31 shows the effects of the presence of non-penetrating sugars in osmotically equivalent concentrations of 0.5 atmosphere. The total osmotic pressure of the solution is 4.0 atmospheres (malonamide 3.5 + sugar 0.5), and a malonamide system of 4.0 atmospheres is included.

These substances, D-arabinose, -methyl-D-glucoside, D-fructose and sucrose are highly effective inhibitors of malonamide haemolysis. They are very much more effective than glucose, galactose and mannose in equivalent concentration (cf. Fig.28). These results are due to the impermeability of the sugars, which remain outside the cell, thereby maintaining completely the extra-cellular osmotic increment. Haemolysis is therefore much slower than in systems where the osmotic increment is reduced by diffusion of inhibitory solute into the cell.

Polyhydric Alcohols.

The effects of osmotically equivalent concentrations (0.5 atmosphere) of glycerol, ethylene glycol, propylene glycol, DL erythritol, dulcitol and mannitol are shown in Figure 32. Malonamide controls, 3.5 atmospheres and 3.5 + 0.5 are included.

THE EFFECT OF NON-PENETRTING CARBOHYDRATES ON MALONAMIDE-INDUCED HAEMOLYSIS.

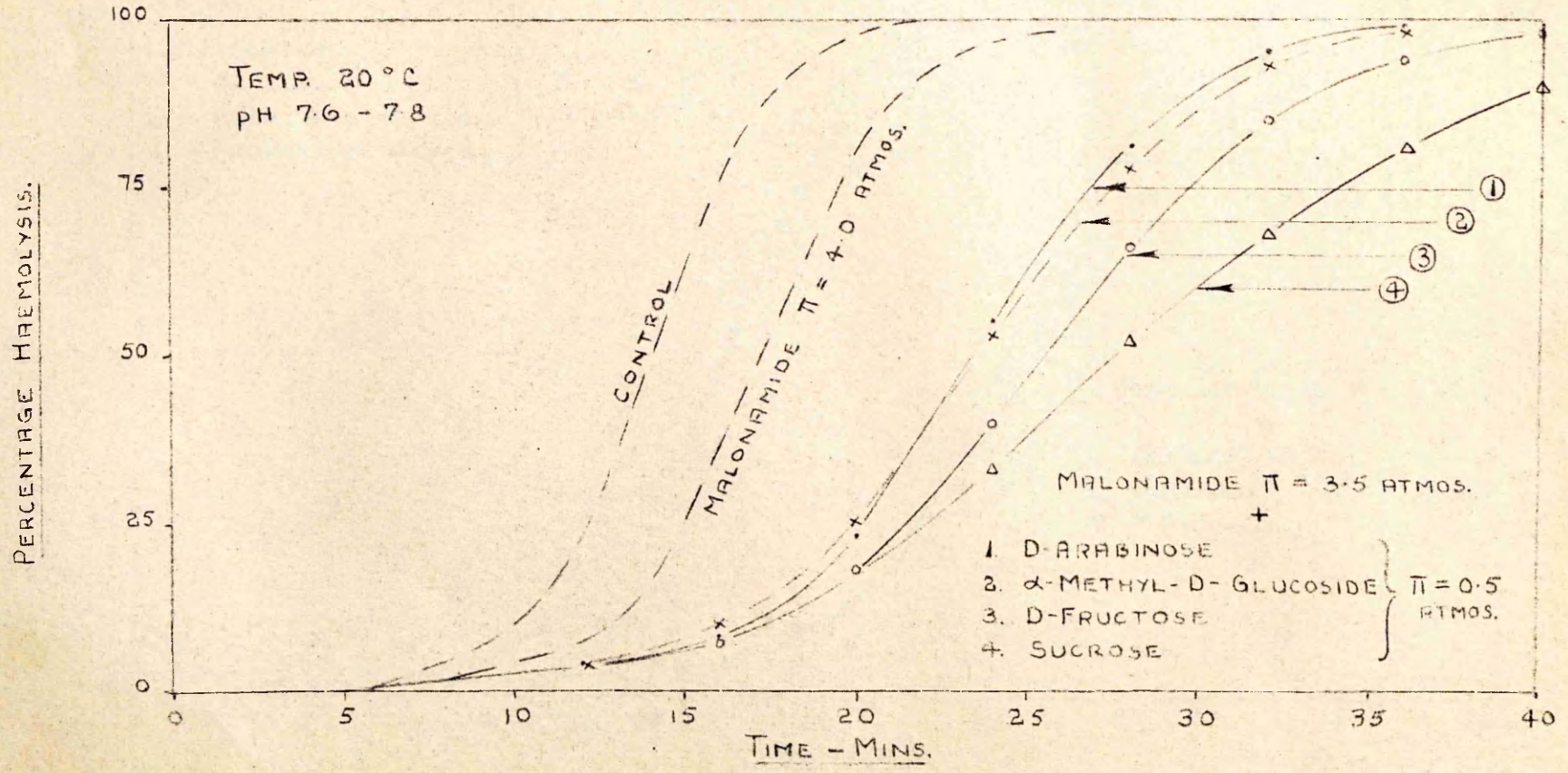


FIG. 31.

THE EFFECTS OF POLYHYDRIC ALCOHOLS ON MALONAMIDE - INDUCED HAEMOLYSIS.

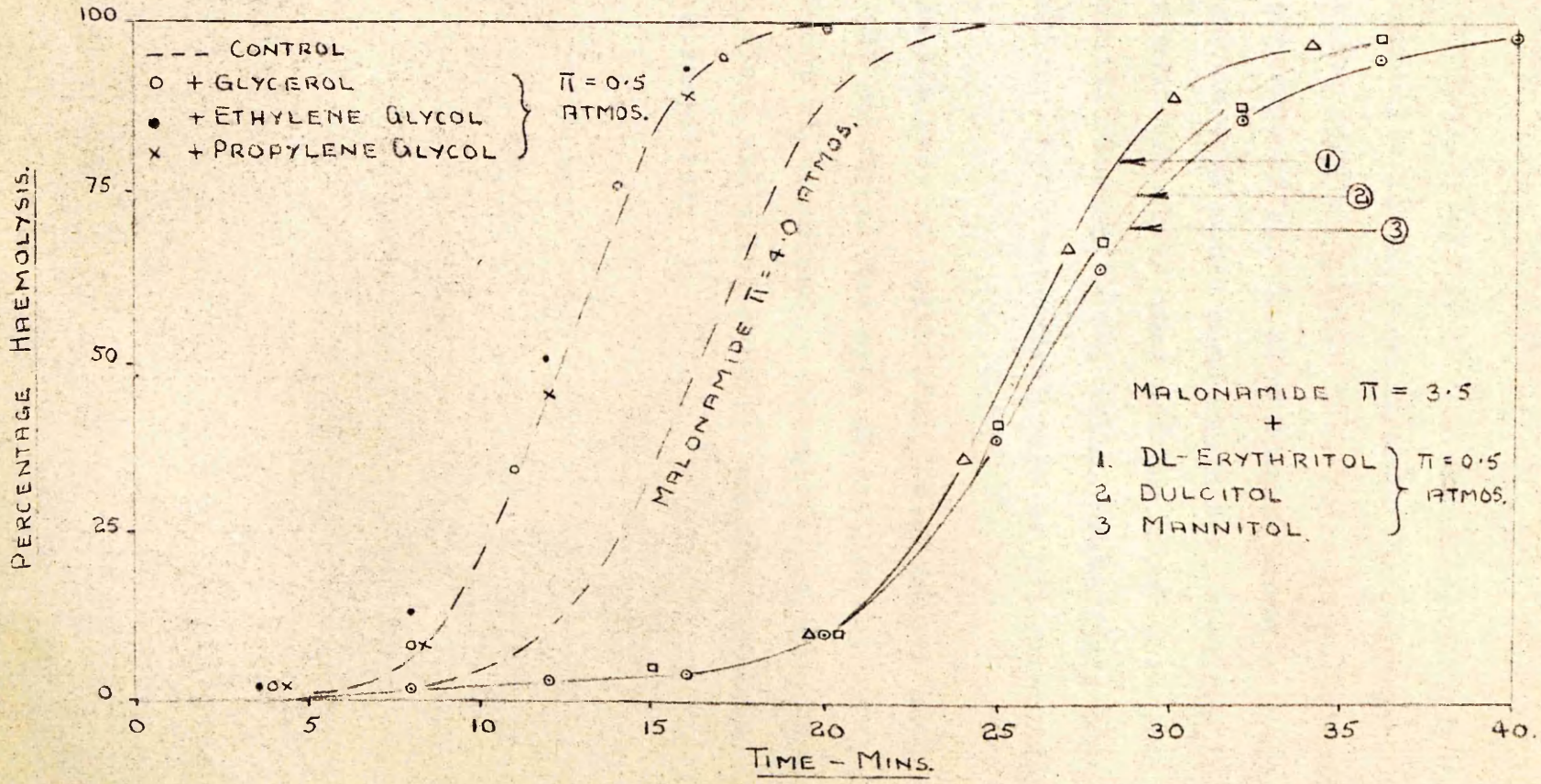


FIG. 32.

Glycerol, ethylene glycol and propylene glycol systems haemolyse in the same time as the control. They penetrate the cell so quickly that there is no external osmotic pressure effect to slow down malonamide haemolysis. Their presence does cause more rapid cell swelling in the first few minutes, while they and the water necessary for osmotic equilibrium enter the cell. Haemolysis therefore starts earlier, but its overall rate is not materially affected.

In the case of erythritol, dulcitol and mannitol, these do not penetrate the cell at all, as far as the duration of experiment is concerned. Haemolysis is therefore greatly inhibited since the extra-cellular osmotic increment is maintained completely.

3.4. The Effect of Temperature Change on Selected Inhibitors of Malonamide-induced Haemolysis.

(a) Phlorhidzin Inhibition.

Phlorhidzin, in 0.1 gm./100 ml. concentration, is a very effective inhibitor of malonamide-induced haemolysis (see Fig.32a). Its presence, in a haemolysing system of malonamide 3.5 atmospheres, greatly prolongs the duration of haemolysis. As shown by the graphs, the inhibition is considerably altered by temperature change.

This effect cannot be due to the change in pH, for, although the pH is less in presence of phlorhidzin than in the control, it has been shown earlier (p.37) that a decrease in pH increases the rate of malonamide-induced haemolysis. Nor is the inhibition due to osmotic pressure, since even assuming the three phenolic groups of the molecule to be completely ionised, which is unlikely, the osmotic pressure would approximate only to 0.13 atmosphere. It does not seem at all likely that an interrupted phosphorylation process is responsible for the inhibition, but it may be that phlorhidzin in some way alters the permeability of the membrane.

(b) Glycine Inhibition.

Glycine, in concentration osmotically equivalent to 0.5 atmosphere, causes inhibition of haemolysis induced by 3.5 atmospheres malonamide. This is shown in Figure 33.

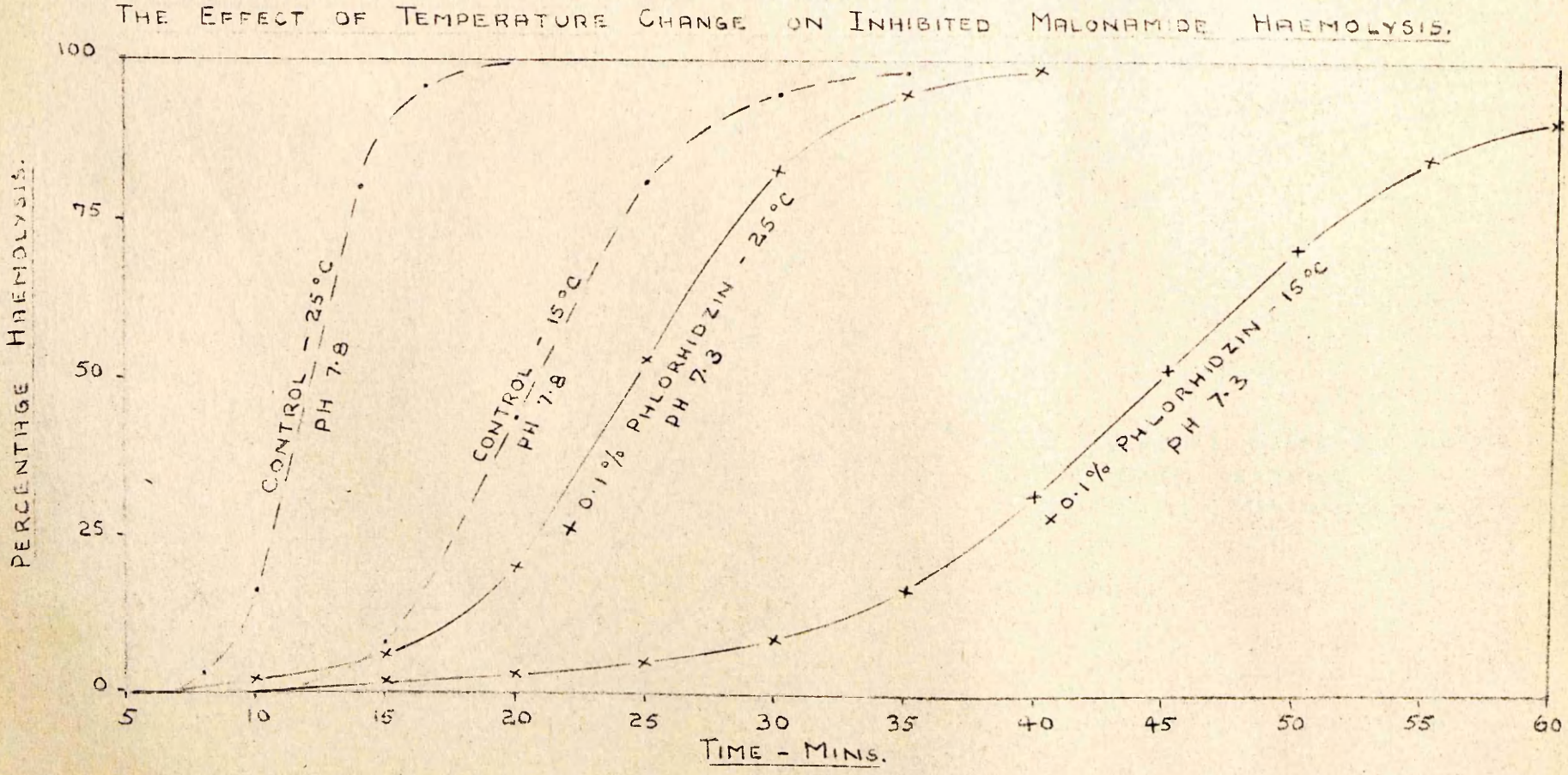

THE EFFECT OF TEMPERATURE CHANGE ON INHIBITED MALONAMIDE HAEMOLYSIS.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
5
10
15
20
25
30
35
40
45
50
55
60
TIME - MINS.
CONTROL - 25°C
PH 7.8
CONTROL - 15°C
PH 7.8
0.1% PHLORHIDZIN - 25°C
PH 7.3
0.1% PHLORHIDZIN - 15°C
PH 7.3

FIG. 32.a.

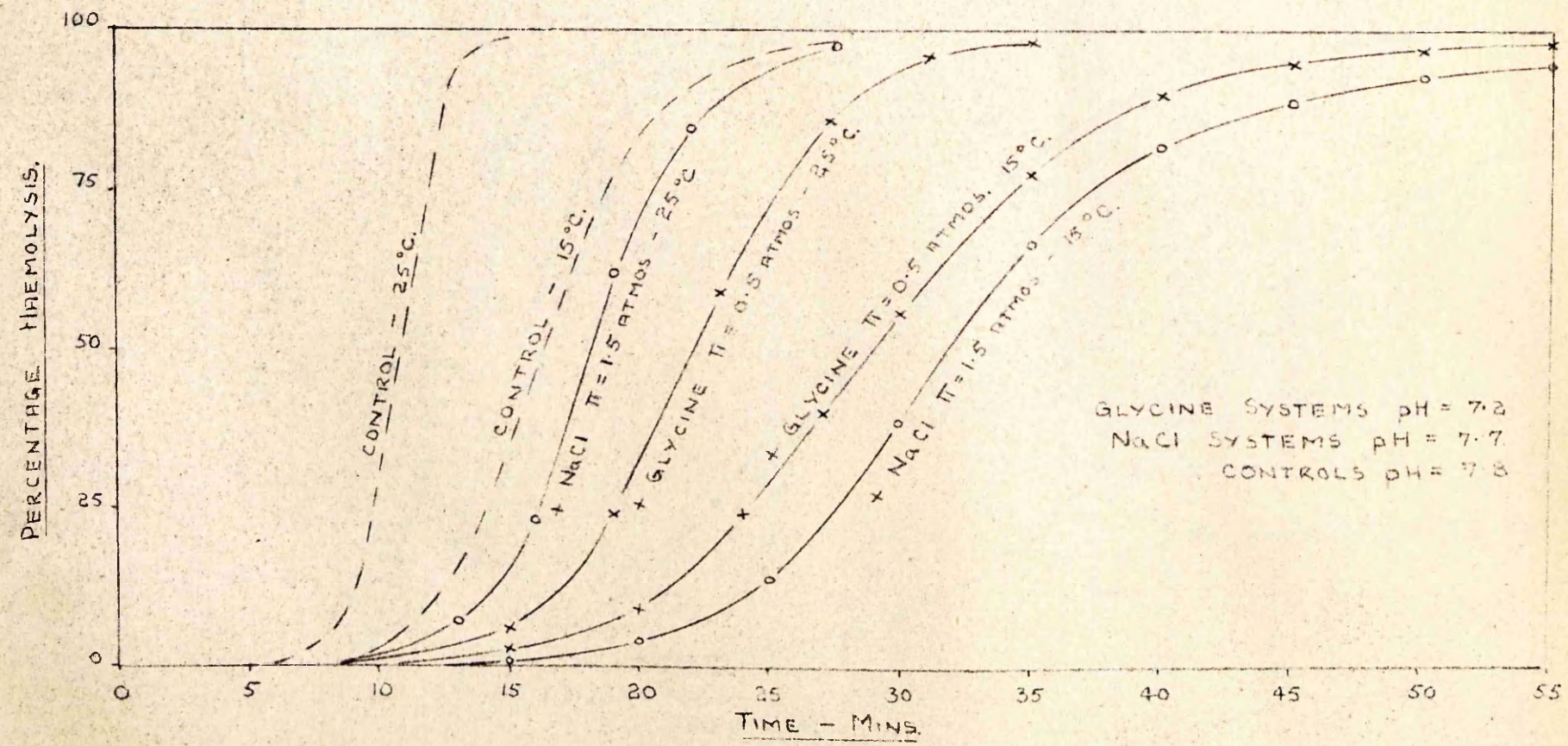

THE EFFECT OF TEMPERATURE CHANGE ON INHIBITED MALONAMIDE HAEMOLYSIS.
PERCENTAGE HAEMOLYSIS.
100
75
50
25
0
0
5
10
15
20
25
30
35
40
45
50
55
TIME - MINS.
CONTROL - 25°C.
CONTROL - 15°C.
NaCl π = 1·5 ATMOS - 25°C
GLYCINE π = 0·5 ATMOS - 25°C
GLYCINE π = 0·5 ATMOS. 15°C.
NaCl π = 1·5 ATMOS - 15°C.
GLYCINE SYSTEMS pH = 7·2
NaCl SYSTEMS pH = 7·7
CONTROLS pH = 7·8

FIG. 33.

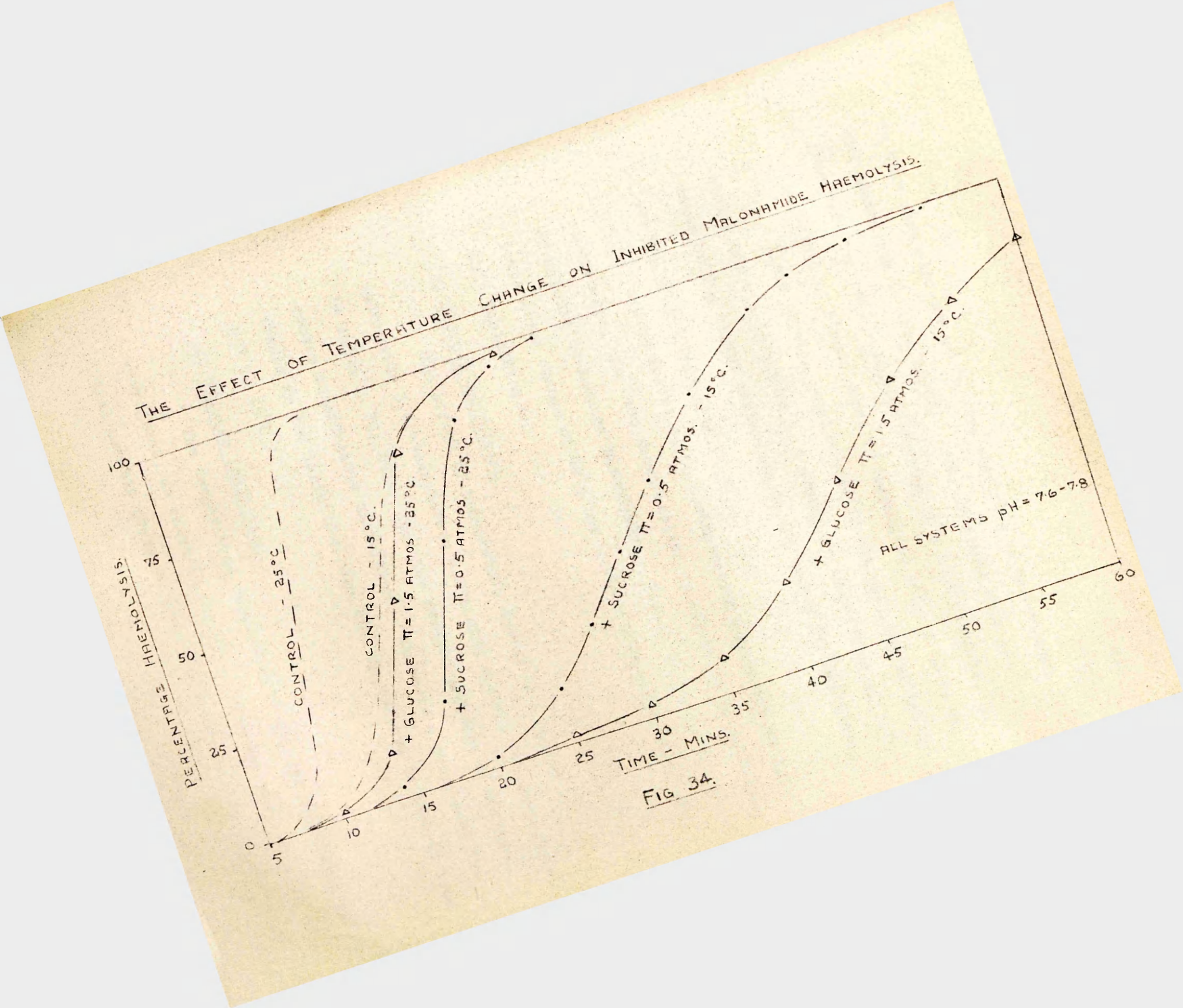

THE EFFECT OF TEMPERATURE CHANGE ON INHIBITED MALONAMIDE HAEMOLYSIS.
PERCENTAGE HAEMOLYSIS.
TIME - MINS.
CONTROL - 25°C.
CONTROL - 15°C.
+ GLUCOSE π = 1·5 ATMOS - 25°C.
+ SUCROSE π = 0·5 ATMOS - 25°C.
+ SUCROSE π = 0·5 ATMOS. - 15°C.
+ GLUCOSE π = 1·5 ATMOS. - 15°C.
ALL SYSTEMS PH = 7·6-7·8
0
5
10
15
20
25
30
35
40
45
50
55
60
25
50
75
100

FIG 34.

In this case also the pH is less than that of the control and haemolysis is less rapid. This substance does not penetrate the cell - aqueous glycine of 3.5 atmospheres, in 20 parts to 1 of blood, shows no haemolysis after 6 hours at 37°C. Since glycine remains outside the cell its effect is wholly osmotic in nature. A change in temperature of 10°C makes only a slight difference to the degree of inhibition.

(c) Sodium Chloride Inhibition.

Sodium chloride, in concentration equivalent to 1.5 atmospheres, has been shown earlier (p.50) to be an inhibitor of malonamide-induced haemolysis. Figure 33 shows that a temperature change of 10°C has quite a pronounced effect on this inhibition.

(d) Sucrose Inhibition.

The effect of temperature change on a system of malonamide 3.5 atmospheres, containing sucrose 0.5 atmosphere, is shown in Fig.34. This low osmotic pressure of sucrose causes considerable inhibition. There is a pronounced increase in the inhibition when the temperature is lowered from 25° to 15°C.

(e) Glucose Inhibition.

The temperature dependence of glucose inhibition is very great, as shown in Fig.34. Although added to give a final osmotic pressure three times that of the sucrose used

in the previous system, glucose produces a smaller degree of inhibition at 25°C.

(f) The Application of the Arrhenius Equation to Inhibited Malonamide Haemolysis.

Since the logarithm of the rate of haemolysis in these inhibited systems was found to give a straight line when plotted against 1/T, the Arrhenius equation has been applied to the data. The question of interpretation remains open, but the activation energy provides a quantitative assessment of the effect of temperature on a given process. The behaviour of the inhibitors may then be more accurately compared.

The methods are the same as those used in Section 3.1, and, as before, the rate of haemolysis is expressed as percentage haemolysis per minute. The average results from a series of experiments are tabulated below.

Table 6

The Effect of Temperature Change on the Rate of Inhibited Malonamide Haemolysis.

Inhibitor used at π, atmospheres	per cent haemolysis per minute		
	15°	20°	25°
Phlorhidzin ≈ 0.1	3.9	5.1	7.4
Glycine = 0.5	5.3	7.2	9.5
Sucrose = 0.5	5.2	8.3	12.7
Sodium Chloride = 1.5	5.8	9.8	13.2
Glucose = 1.5	4.0	7.3	13.3

From this data the values of E and A, for these inhibited systems, were found. These are shown in Table 7.

Table 7.

Experimental Values of E and Calculated Values of A.

Inhibitor used at π, atmospheres		E. cals.	A (calc. 20°C)
Phlorhidzin	$\approx$ 0.1	11,200	1.0×10^{9}
Glycine	= 0.5	10,200	2.5×10^{8}
Sucrose	= 0.5	15,700	3.6×10^{13}
Sodium Chloride	= 1.5	13,500	9.1×10^{10}
Glucose	= 1.5	21,200	3.7×10^{16}

The corresponding values for the control system, malonamide 3.5 atmospheres, are E = 13,000 cals., and $A = 6.1 \times 10^{10}$. The values of E for the systems containing phlorhidzin or glycine do not mean that haemolysis occurs more quickly than in the control. This is not so, as shown by Figs.32 and 33. It does mean that the rate of haemolysis, as measured by the slope of the graph, is less sensitive to temperature change when these concentrations of phlorhidzin or glycine are present. This is due to the fact that the exponential term of the equation deals with the effect of temperature change only.

In the same way, in the presence of sodium chloride

1.5 atmospheres, the system is influenced to the same extent as the control, by temperature change. With sucrose 0.5 atmosphere or glucose 1.5 atmospheres the systems are influenced, to a greater extent than the control, by a change in temperature.

This information is conveniently summarised by comparing the Q_{10} values for the inhibited systems. These are tabulated below.

Table 8.

Q_{10} Values for Inhibited Haemolysis.

Inhibitor used at π, atmospheres.		Q_{10} (15°-25°)
No inhibitor		2.3
Phlorhidzin	$\approx$ 0.1	1.9
Glycine	= 0.5	1.8
Sucrose	= 0.5	2.5
Sodium Chloride	= 1.5	2.3
Glucose	= 1.5	3.3

It is not proposed to discuss the significance of the values for A until the final section, when they will be considered in relation to the values given in the next section.

It will be noticed that the Q_{10} values and the energies of activation do not wholly represent the extent of the

inhibition produced by these substances. This is due to their derivation from the slopes of the graphs. No account is taken, therefore, of differences in the lag phase or the initial slow rate of haemolysis. Thus, in Figure 32 (0.1 per cent phlorhidzin), lowering the temperature from 25° to 15°C not only reduces the velocity of haemolysis, as measured by the change in slope of the graph, but also practically doubles the time required to reach 10 per cent haemolysis. There is a similar effect with sucrose and also, to a lesser extent, with glycine and sodium chloride. That the effects of temperature upon the slope of the graph and on the duration of the lag phase are related is shown by the fact that glycine, with the smallest Q_{10}, has the smallest difference in times required for 10 per cent haemolysis at 25° and 15°C. For sucrose, with a larger Q_{10}, this difference is also larger. These effects, both on the lag phase and on the rate of haemolysis, are related to the inability of the inhibitors, phlorhidzin, glycine, sucrose and sodium chloride, to penetrate the cell.

The presence of glucose, of osmotic pressure 1.5 atmospheres, greatly increases the temperature dependence of the haemolysing system. This is undoubtedly related to the very great effect of temperature on glucose penetration (Hendry (1951)). A small change in temperature will cause

glucose to penetrate at a very different rate. At 25°C glucose penetrates the cell very rapidly, the inflow of water causes rapid swelling and haemolysis occurs quickly (Fig.34). At 15°C glucose penetrates very slowly, and swelling due to water entry is also therefore slower; since most of the glucose remains outside the cell, the extracellular osmotic pressure is higher, reducing further the rate of haemolysis and increasing the duration of the lag phase. The inhibitory action of glucose depends therefore almost wholly on the temperature.

It is possible that the lack of temperature dependence shown by glycine and phlorhidzin inhibition could mean some type of combination with the cell membrane, which results in reduced permeability.

3.5. Haemolysis in Solutions of Rapidly Penetrating Substances.

It is not possible, with the present methods, to obtain haemolysis curves for substances which induce rapid lysis. It was found, however, that the times required for complete haemolysis at constant temperature and osmotic pressure were reproducible and tended to be characteristic for a given substance. For solutions in which haemolysis is complete within 60 seconds the time is reproducible to within ± 2 seconds. For haemolysis times up to 180 seconds the time is reproducible to within ± 10 seconds. Above a haemolysis time of 180 seconds the variaion is greater - mainly due to the difficulty in deciding when complete haemolysis is reached. For haemolysis times approaching or exceeding 5 minutes it is necessary to use the Unicam spectrophotometer. For each substance the times required for complete haemolysis at 10°, 15°, 20° and 25° have been obtained; except for those substances which penetrate very rapidly these measurements are given for solutions of 3.5 and 6.5 atmospheres osmotic pressure. Each value quoted in the tables is an average of 12 readings made on 4 different specimens of normal blood.

The times to 100 per cent haemolysis in solutions of rapidly penetrating substances are given in Table 9.

Table 9.

Times to 100% Haemolysis for Water and Solutions of Rapidly Penetrating Substances.

(Osmotic Pressure = 6.5 atmospheres).

Substance	Time in secs. to 100% Haemolysis			
	10°	15°	20°	25°
Water	22	20	17	14
Formamide	85	57	59	46
Acetamide	24	22	17	16
Propionamide	30	26	21	18
n-Butyramide	26	23	19	17
Succinimide	120	100	87	72
Nicotinamide	67	53	40	33
Methyl Carbamate	23	21	19	17
Ethyl Carbamate	21	20	17	16
N-Methyl Urethane	22	21	19	17
N-Ethyl Urethane	20	19	17	16
Methyl Alcohol	27	22	18	15
Ethyl Alcohol	22	20	16	15
n-Propyl Alcohol	24	22	18	15
n-Butyl Alcohol	25	22	18	16
Ethyl Acetate	25	21	18	15
Diethyl Ether	20	18	15	14
Acetone	20	18	15	14

Table 9 shows that the simple aliphatic amides, except formamide, together with the urethanes, penetrate the cell at much the same rate as water. Formamide, succinimide and nicotinamide slow down the rate of haemolysis compared to water, showing that these substances penetrate the cell more slowly than the others. Ether and acetone cause very

slightly more rapid haemolysis, which is not unexpected in the light of the known effects of lipid solvents on the cell membrane.

In Tables 10 and 11 are given the times to 100 per cent haemolysis in solutions of 6.5 and 3.5 atmospheres respectively, for the ureas and substituted ureas.

Table 10.

Times to 100% Haemolysis in Solutions of 6.5 atmospheres Ureas and Substituted Ureas.

Substance	Time in secs. to 100% Haemolysis			
	10°	15°	20°	25°
Urea	23	20	17	16
Methyl Urea	51	42	30	24
Ethyl Urea	182	102	67	43
Ethylene Urea	200	116	79	48
1.1.Diethyl Urea	37	30	23	17
1.3.Diethyl Urea	170	88	60	37
1.3.Dimethyl Urea	60	43	32	26
N-Acetyl-N'-Methyl Urea	30	23	17	15
Thiourea	300	200	150	107
Methyl Thiourea	128	76	52	34
Ethyl Thiourea	49	38	27	21
1.3.Diethyl Thiourea	32	26	21	18

Table 11.

Times to 100% Haemolysis in Solutions of 3.5 atmospheres Ureas and Substituted Ureas.

Substance	Time in secs. to 100% Haemolysis			
	10°	15°	20°	25°
Urea	24	20	17	15
Methyl Urea	34	27	23	19
Ethyl Urea	72	52	35	25
Ethylene Urea	118	79	52	37
1.1.Diethyl Urea	41	31	25	20
1.3.Diethyl Urea	71	51	38	23
1.3.Dimethyl Urea	46	35	29	23
N-Acethyl-N'-Methyl Urea	31	25	22	19
Thiourea	130	97	76	57
Methyl Thiourea	63	48	35	27
Ethyl Thiourea	40	30	25	20
1.3.Diethyl Thiourea	35	29	24	20

Tables 10 and 11 show that the presence of substituent groups and their location in the molecule greatly modify the haemolytic properties of urea.

When the times given in Table 10 for haemolysis at 20°C are considered, it is seen that the introduction of a methyl group into urea practically doubles the time required for complete haemolysis. A second methyl group, in the 3 position, makes no great difference to the time of haemolysis, and when one of the two methyl groups is exchanged for an acetyl group, the time required for complete haemolysis is restored to the original value for urea.

The introduction of an ethyl group into the urea molecule increases the time required for complete haemolysis by a factor of four. A second ethyl group on the same nitrogen atom reduces the time to near that of urea, but, when this group is on the second nitrogen atom, the time for haemolysis is again four times that for urea. The time for haemolysis in ethylene urea, the molecule of which is a five-member ring system, does not differ materially from the corresponding times for ethyl and 1.3.diethyl urea.

Thiourea requires about eleven times longer to cause haemolysis than an osmotically equivalent solution of urea. The introduction of alkyl substituent groups into the thio-urea molecule greatly reduces the time required for complete haemolysis. This is the opposite effect from that observed with urea.

Table 12 gives the times for complete haemolysis in solutions of 3.5 and 6.5 atmospheres polyhydric alcohols, also haemolysis times for 0.05 and 0.10 gm./100 ml. phlor-hidzin.

When the corresponding figures for n-propyl alcohol are compared with those of Table 12, it is seen that the introduction of a second hydroxyl group into the propane molecule prolongs the time required for haemolysis. A third hydroxyl group further prolongs this time. It may be noted

that the relative positions of the hydroxyl groups are very significant. This is seen when the figures for propylene glycol and trimethylene glycol are compared.

Table 12.

Times to 100% Haemolysis in Solutions of Polyhydric Alcohols and Phlorhidzin.

Substance	Time in secs. to 100% Haemolysis			
	10°	15°	20°	25°
(a) $\pi = 6.5$				
Ethylene Glycol	47	35	27	20
Propylene Glycol	51	37	27	21
Trimethylene Glycol	70	50	36	27
Glycerol	250	194	158	125
(b) $\pi = 3.5$				
Ethylene Glycol	29	23	20	16
Propylene Glycol	30	24	21	18
Trimethylene Glycol	42	34	27	21
Glycerol	107	92	77	66
(c) Phlorhidzin				
0.1 gm./100 ml.	63	51	43	35
0.05 gm./100 ml.	38	32	28	24

The time for complete haemolysis is greatly prolonged - compared to water - in presence of 0.1 or 0.05 gm./100 ml. phlorhidzin. Although this substance does not penetrate the cell, its osmotic pressure contribution to the system is too small to account for the effect. The pH change

caused by phlorhidzin - a drop from 7.8 to 7.5 - also seems inadequate to explain the effect. Interrupted phosphorylation seems unlikely to take place in such a short time and the time also seems inadequate to allow membrane permeability to be altered by a form of combination with phlorhidzin.

The time to 100 per cent haemolysis is convertible to the rate of haemolysis, expressed as per cent haemolysis per minute, by the formula

$$V = \frac{100}{\text{time in secs. to 100\% haemolysis}} \times 60$$

It was found that when these rates of haemolysis were plotted against 1/T, a straight line relationship held, indicating that the data conformed to the Arrhenius law. By the same methods as those described in the earlier sections, values have been found both for E, the energy of activation, and for A, the non-exponential term of the equation. These are presented in Tables 13 and 14.

Table 13.

Values of E and A for Water and Solutions (π = 6.5) of Rapidly Penetrating Substances.

Substance	E(cals)	A (20°)
Water only	4,500	6.7×10^6
Formamide	7,000	1.3×10^8
Acetamide	4,000	1.0×10^6
Propionamide	5,300	1.2×10^7
n-Butyramide	4,600	3.1×10^6
Succinimide	5,500	1.3×10^7
Nicotinamide	8,300	2.5×10^9
Methyl Carbamate	3,200	1.9×10^5
Ethyl Carbamate	3,500	3.8×10^5
N-Methyl Urethane	3,300	2.4×10^5
N-Ethyl Urethane	3,500	3.8×10^5
Methyl Alcohol	6,600	1.8×10^8
Ethyl Alcohol	5,600	2.8×10^7
n-Propyl Alcohol	5,900	4.6×10^7
n-Butyl Alcohol	5,400	1.6×10^7
Ethyl Acetate	5,800	3.8×10^7
Diethyl Ether	4,800	5.9×10^6
Acetone	4,900	7.2×10^6

Table 14.

Values of E and A for Ureas, Polyhydric Alcohols and Phlorhidzin Solutions.

Substance	π = 6.5 atmos.		π = 3.5 atmos.	
	E(cals)	A(20°C)	E(cals)	A(20°C)
Urea	4,400	2.3×10^{6}	4,600	3.4×10^{6}
Methyl Urea	9,100	1.4×10^{10}	6,600	1.5×10^{8}
Ethyl Urea	16,000	7.1×10^{15}	11,000	6.3×10^{11}
Ethylene Urea	16,100	7.9×10^{15}	11,400	8.8×10^{11}
1.1.Diethyl Urea	8,600	8.2×10^{9}	7,500	7.9×10^{9}
1.3.Diethyl Urea	16,400	3.2×10^{16}	9,800	5.3×10^{10}
1.3.Dimethyl Urea	9,000	1.2×10^{10}	7,200	3.9×10^{8}
N Acetyl N' Methyl Urea	9,100	2.9×10^{10}	5,500	1.7×10^{7}
Thiourea	12,000	1.1×10^{12}	8,900	4.5×10^{9}
Methyl Thiourea	14,800	8.9×10^{14}	9,500	3.1×10^{10}
Ethyl Thiourea	9,400	3.2×10^{10}	7,200	4.4×10^{8}
1.3 Diethyl Thiourea	11,500	2.8×10^{12}	6,600	1.4×10^{8}
Ethylene Glycol	9,000	1.5×10^{10}	6,300	9.5×10^{7}
Propylene Glycol	10,000	1.1×10^{11}	5,600	2.1×10^{7}
Trimethylene Glycol	11,200	9.3×10^{11}	8,000	2.1×10^{9}
Glycerol	7,500	1.3×10^{8}	5,700	7.1×10^{6}
	0.10 gm./100 ml.		0.05 gm./100 ml.	
Phlorhidzin	6,400	5.1×10^{7}	5,300	8.7×10^{6}

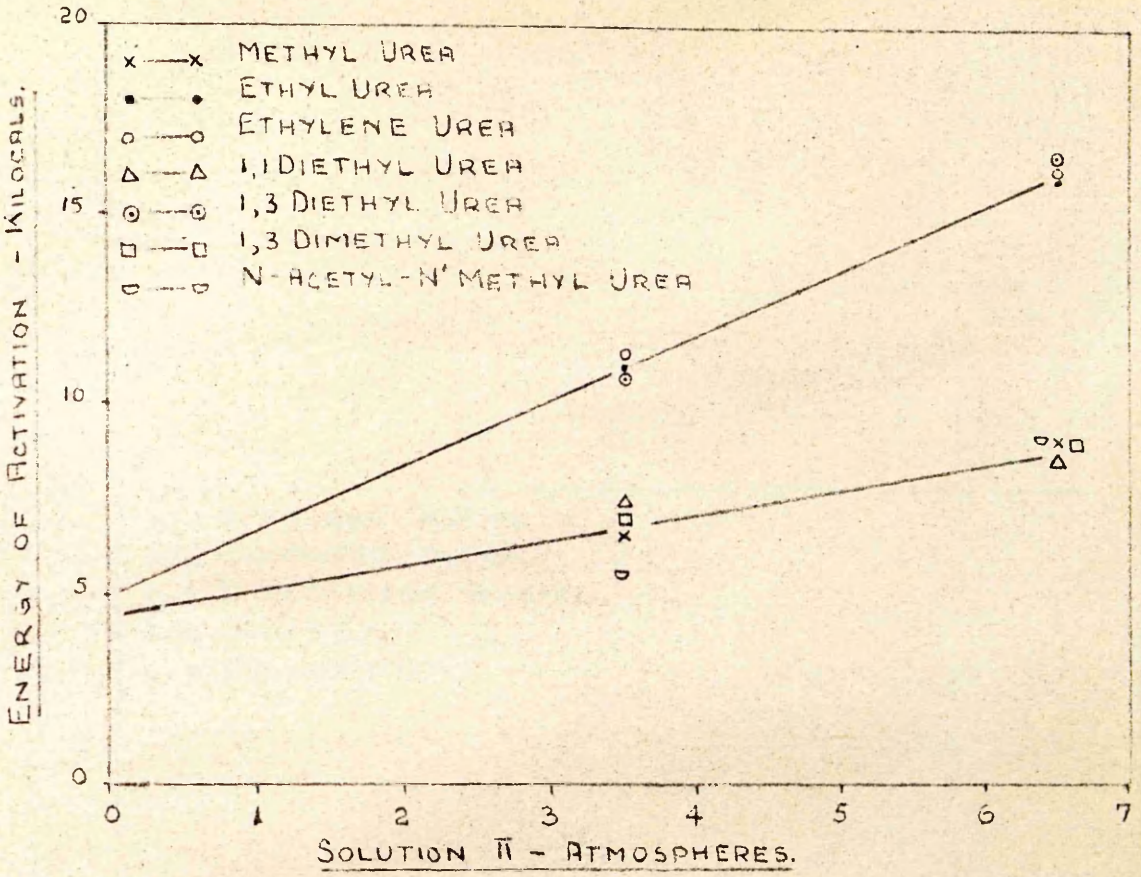

METHYL UREA
ETHYL UREA
ETHYLENE UREA
1,1 DIETHYL UREA
1,3 DIETHYL UREA
1,3 DIMETHYL UREA
N-ACETYL-N' METHYL UREA
ENERGY OF ACTIVATION - KILOCALS.
SOLUTION π - ATMOSPHERES.
0
5
10
15
20
1
2
3
4
6
7

FIG. 35.

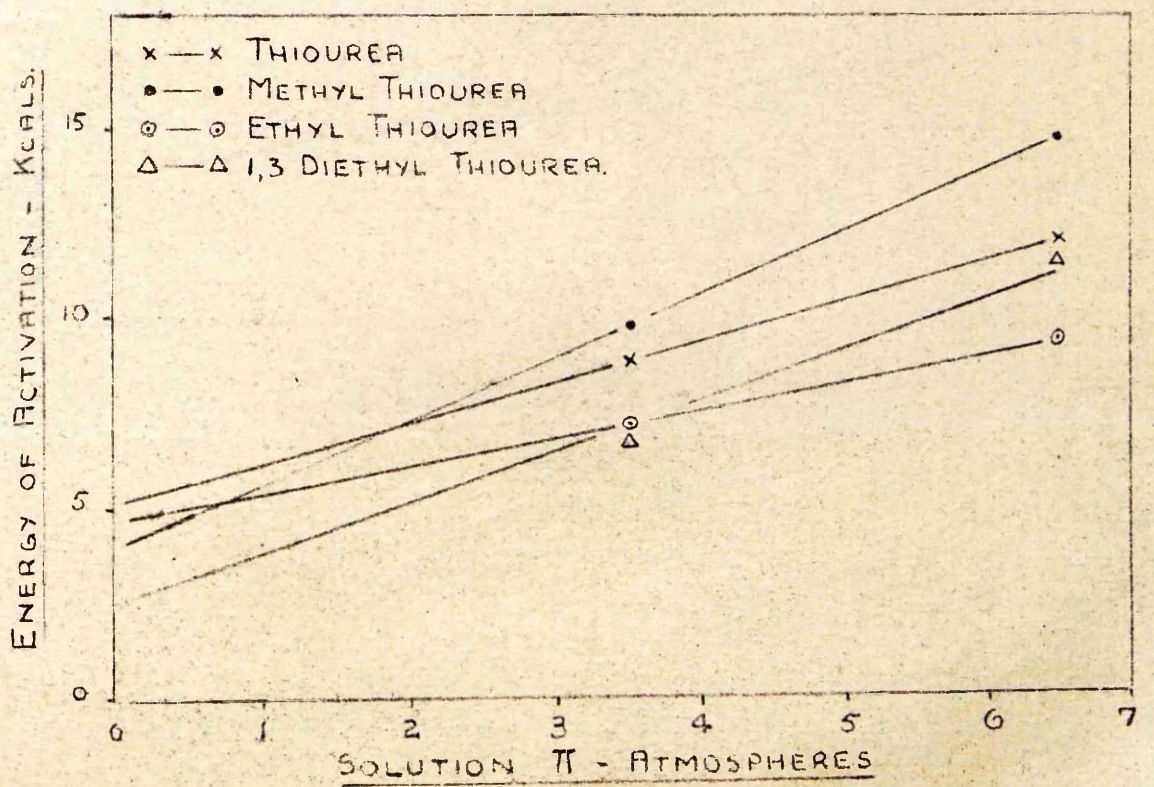

THIOUREA
METHYL THIOUREA
ETHYL THIOUREA
1,3 DIETHYL THIOUREA.
ENERGY OF ACTIVATION - KCALS.
SOLUTION π - ATMOSPHERES
0
5
10
15
1
2
3
4
6
7

FIG. 36.

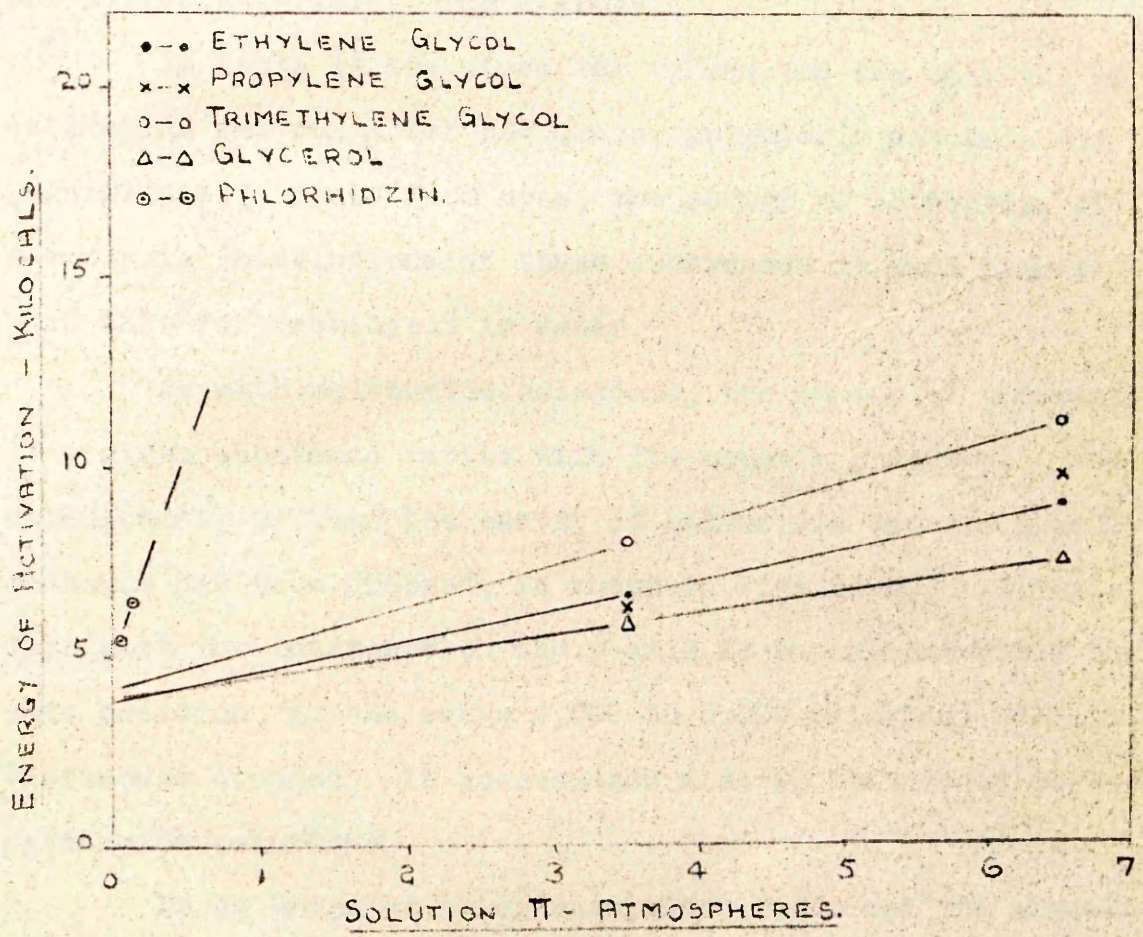

•—• ETHYLENE GLYCOL
×—× PROPYLENE GLYCOL
o—o TRIMETHYLENE GLYCOL
Δ—Δ GLYCEROL
⊙—⊙ PHLORHIDZIN.
ENERGY OF ACTIVATION - KILOCALS.
20
15
10
5
0
0
1
2
3
4
5
6
7
SOLUTION π - ATMOSPHERES.

FIG. 37.

Table 13 shows that formamide, nicotinamide and methyl alcohol have E-values greater than that found for water, while the E-values of the urethanes are less. For the remaining substances, at this osmotic pressure, E does not differ materially from E-water.

In Table 14 are given the values for the energies of activation and for A for the ureas, polyhydric alcohols and phlorhidzin. Apart from urea, the energy of activation of haemolysis in solutions of these substances is much higher than that for haemolysis in water.

As with malonamide solutions, the energy of activation of a given substance varies with its osmotic pressure. The relationship between the energy of activation and the osmotic pressure has been graphed, as shown in Figs.35-37. It is seen that the intercept on the y-axis is in approximately the same position, in the range 4,000 to 6,000 calories, for all substances studied; it corresponds also to that found for malonamide solutions.

It is proposed to discuss these facts and the significance of the A term in the following section.

Section 4.

Discussion.

4.1. The Validity of the Results.

In these, as in most experiments with biological material, an element of more or less serious abnormality is present. Although the cells are not in any way manipulated - other than by the mixing required to ensure oxygenation - they cannot remain "normal" in an environment grossly different from plasma. This unavoidable difficulty is to some extent mitigated by the use of control systems, differing from the test systems only by the particular factor under review. It is believed, however, that the methods used in this work give a better approximation to the normal behaviour of erythrocytes than would be obtained if the blood were stored in the cold prior to experiment, or if the cells were washed free of plasma and added to the systems in the form of saline suspensions.

With normal blood the reproducibility of the malonamide curves is satisfactory, considering the inherent variability of biological material. Out of 17 experiments to determine the rate of haemolysis in malonamide solution of 3.5 atmospheres the mean value is 13.1 per cent per minute. The standard deviation is 1.1 per cent per minute and the coefficient of variation is 8.5 per cent. Although the numbers of experiments for malonamide haemolysis in presence of inhibitors are too few to warrant statistical analysis,

the reproducibility appears to be no less good. The alkaline haematin method enables the degree of haemolysis to be measured with an error of less than 1 per cent, but the time factor with respect to the centrifugation of specimens is open to considerable variation. From the point of view of this factor alone, the reproducibility is quite good.

4.2. The Behaviour of Erythrocytes Suspended in Hypotonic Solutions of Malonamide at Constant Temperature.

It has been shown that delayed haemolysis occurs in hypotonic solutions of malonamide and that the duration of the zero haemolysis lag phase decreases and the rate of haemolysis - measured by the average slope of the curve - increases with decreasing tonicity of the medium. Further, delayed haemolysis having commenced at low tonicity, it can be stopped abruptly by the addition of solid malonamide to the system. The degree of haemolysis remains constant for about 15 minutes, thereafter increasing steadily until complete haemolysis is reached. The permeation of malonamide is intermediate between that of glucose, which at equal tonicity and temperature permeates only slowly (Hendry (1951)), and urea which, under the same conditions, permeates with great rapidity (Klinghoffer (1935)).

In the system of lowest tonicity of malonamide (concentration = 1.49 gm./100 ml., π = 3.5 atmospheres and tonicity

= 0.5), the cells are considerably swollen; they have increased their volume by a factor of 1.75 and are very near the point of purely osmotic haemolysis. They are, at this point, in osmotic equilibrium with the external medium. A small amount of malonamide entering the cell will have very little effect on the extra-cellular tonicity - since water goes into the cell with it - but the amount of water which goes into the cell, to maintain osmotic equilibrium, produces that extra swelling which is just sufficient to cause the cell to swell beyond its critical volume. Haemolysis begins and, in time, all the cells undergo lysis.

In the system of intermediate tonicity of malonamide (concentration = 2.13 gm./100 ml., π = 5.0 atmospheres and tonicity = 0.7), the cells swell initially to about 1.2 times their original volume under the influence of osmosis. The critical volume has to be reached by the entry of malonamide plus water, the latter being required in larger volume. The start of haemolysis will therefore take longer, and when haemolysis does eventually begin, its progress will be slower.

If the extra-cellular concentration of malonamide is still further increased the rate of swelling and the rate of haemolysis are further decreased.

When malonamide, or any other solute which does not penetrate the cell or penetrates only very slowly, is added

during the haemolytic phase, the extra-cellular tonicity (but not the intra-cellular tonicity) is suddenly increased. Water is withdrawn from the remaining unhaemolysed cells and haemolysis is arrested. It restarts only when sufficient malonamide - and water - have penetrated the cell and restored it to the critical volume.

While explaining the phenomenon of delayed haemolysis up to a point, the argument does not explain why malonamide should penetrate more slowly from a more concentrated solution. A possible explanation is as follows.

Since malonamide enters the cell relatively slowly it would appear to function as a "colloid" under the conditions of these experiments. In the ordinary sense of the word malonamide could never be classed as a colloid, but it "appears to function as a colloid" in the sense that it exerts its osmotic pressure (or the bulk of its osmotic pressure) only on the extra-cellular side of the membrane. If it acts even to a limited extent in this capacity as a "colloid", it will then exert, to that limited extent, a "colloidal osmotic pressure" and this "colloidal osmotic pressure" will tend to retain water on the extra-cellular side of the membrane, i.e., preventing swelling to some extent and postponing haemolysis.

There is nothing essentially novel about this idea,

and although malonamide is always regarded as a simple crystalloid, there is no sharp dividing line between "colloid" and "crystalloid". The essential feature is that malonamide penetrates the cell relatively slowly and therefore exerts an osmotic pressure across the cell membrane.

Similar arguments apply in other cases, such as erythrocytes suspended in solutions of the alkali halides. The penetration or escape of sodium etc. across the cell membrane is very slow - negligibly slow compared with malonamide. In such cases the salt exerts its osmotic pressure in the extra-cellular fluid. Delayed haemolysis, such as has been shown to occur in hypotonic solutions of malonamide, does not occur in sodium chloride of the same tonicity. Yet if cells are suspended in a sodium chloride solution sufficiently hypotonic to produce a partial degree of haemolysis, this degree of haemolysis increases very slowly over a period of 5 hours (Hendry (1947)).

Urea, on the other hand, enters the cell so rapidly that in a matter of a few seconds it is equally distributed between intra- and extra-cellular fluid. Under these circumstances there is no external "colloidal osmotic pressure". There is, however, an internal "colloidal osmotic pressure" due to haemoglobin; this draws water into the cell which rapidly swells and haemolyses. That the free permeability

of urea prevents this substance from exerting an extracellular osmotic pressure is shown in the following tables. Table 15 shows that no cell swelling takes place when the urea concentration of whole blood is raised far beyond its normal value. Table 16 shows that the osmotic fragility of erythrocytes in sodium chloride solution is independent of urea added to the system.

Table 15.

Percent Cell Volume in Presence of Excess Urea.

Concn. Excess Urea (mg./100 ml. blood)	P.C.V. Haematocrit (%)
+200	47.8
+400	48.4
+600	47.7
+1500	46.8

Table 16.

Erythrocyte Fragility in Sodium Chloride Solution in Presence of Urea.

Concn. NaCl gm./100 ml.	percent haemolysis		
Solution Medium	Water	0.2M Urea	0.4M Urea
0.400	12	14	14
0.350	71	68	69
0.300	94	93	96

From these considerations it follows that the effect of the addition of a second solute to a malonamide haemolysing system will depend on one factor only, that is, the rate at which the second solute penetrates the cell. At one extreme are the solutes which do not penetrate the cell. Such solutes are sodium chloride, mannitol, sucrose, etc. At the other extreme are the solutes which penetrate immediately; urea, ethylene glycol and glycerol, for instance. Solutes of the first group, since they are retained in the extra-cellular phase, will exert a "colloidal osmotic pressure" in that phase, thereby slowing down the rate of entry of water and thus slowing the haemolytic process. Solutes of the second group, since they are equally distributed across the membrane in a very short time, exert no such osmotic pressure difference. The system behaves as it would in their absence. Between these two extremes there exist solutes, the rates of penetration of which cover an intermediate range. Mannose, galactose and glucose are members of this group. The first two penetrate at much the same rate as malonamide and their delaying effect is much the same as that of an iso-molar concentration of malonamide. Glucose penetrates more slowly than malonamide. The bulk of its concentration exerts an extra-cellular "colloidal osmotic pressure" which slows down the haemolytic process considerably.

The phenomenon of osmosis has been known, but not understood, for some 200 years. Since osmosis was exhibited consistently by colloidal systems and involved an apparent pressure, the term "colloidal osmotic pressure" came into use. The term has remained in biological thought, since living systems are essentially colloidal in nature. In point of fact there is no fundamental difference in the osmotic effects of "colloid" or "crystalloid". The only requirements are that the membrane between the phases is freely permeable to water and impermeable to solute or suspensoid.

4.3. The Action of Electrolytes on Malonamide-induced Haemolysis.

Osmotic pressure is classified with the colligative properties of solutions, its magnitude depending on the number of particles in solution and not on their nature. For this reason it might be expected that osmotically equivalent concentrations of non-penetrating solutes would have the same effect on malonamide-induced haemolysis. This is not so even for substances as closely related as the alkali metal halides.

With the alkali metal salts a change of cation or a change of anion, at constant temperature and osmotic pressure, has a marked effect on the extent of inhibition observed

(Figs.13, 23, 26, 27). A change of cation among the alkaline earth chlorides at the same temperature and osmotic pressure also has a considerable effect on the observed inhibition (Fig.17).

In these systems the osmotic pressure of the haemolysing solution is constant at 5.0 atmospheres. The osmotic contribution of malonamide is 3.5 atmospheres in each case, and that of electrolyte, alkali metal salt or alkaline earth chloride, is 1.5 atmospheres. That haemolysis does occur shows that malonamide and water continue to penetrate the cell. The reduced rate of haemolysis - or observed inhibition - must therefore be due either to a reduced rate of penetration of malonamide, or to reduced rate of flow of water. The alkali metal halides especially are closely related in their physical and chemical properties and there is no reason to believe that they react in any way with malonamide. While cations might alter the permeability of the membrane, it is extremely unlikely that monovalent anions would do this, since they attain diffusion equilibrium with great rapidity. If these ions do not alter membrane permeability or react with malonamide, the large differences observed, with the different salts at constant osmotic pressure, must be due to their effect on the flow of water into the cell. It is not unreasonable to suppose that ions "hold"

water in differing amounts, since in solution they are hydrated to different extents.

The literature abounds with highly discrepant data concerning the number of water molecules in an ionic hydration sheath. Bockris (1949) pointed out that different methods measured different degrees of hydration and suggested the terms primary, secondary and total hydration. Primary hydration is defined as the number of molecules of water associated with an ion in its electrolytic transport or Brownian movement. Such solvent molecules have completely lost their separate degrees of translational freedom. Secondary hydration covers all other attracted molecules not included in the primary sheath, and total hydration is the sum of these two numbers. Primary hydration is constant for a given ion, while secondary, and therefore total hydration vary under different conditions. The values given by Conway and Bockris (1954) are shown below.

Table 17.

Mean Primary Hydration Numbers.

Ion	Hydration Number
Li^+	5 ± 1
Na^+	5 ± 1
K^+	4 ± 2
Rb^+	3 ± 1
F^-	4 ± 1
Cl^-	1 ± 1
Br^-	1 ± 1
I^-	1 ± 1

On theoretical grounds Bockris (1954) considers that Cs^+ would have a primary hydration number zero. The effect of primary hydration is therefore inadequate to account for the differences observed.

The heat of hydration gives a measure of the total interaction between ion and solvent (Kortum and Bockris, 1951, p.134). The heat of hydration is therefore a measure of the extent to which an ion retains its primary and secondary hydration sheaths. Since the values of individual ionic heats of hydration depend on the assumptions made when the experimental values of heats of hydration of salts are divided, the theoretical values of Eley and Evans (1938) have been used. Conway and Bockris (1954) consider these to be in fair agreement with the experimental values and further, Eley and Evans give a large number of values, all of which have been obtained in the same way. The relevant values are tabulated on the next page.

Table 18.

Theoretical Values for Heats of Hydration.

Ion	Heat of Hydration (k cals./gm. ion)
F^-	-91
Cl^-	-59
Br^-	-52
I^-	-45
Li^+	-133
Na^+	-115
K^+	-90
Rb^+	-81
Cs^+	-73
Mg^{++}	-501
Ca^{++}	-428
Sr^{++}	-381
Ba^{++}	-347

The changes in inhibition caused by changes of ion, at constant osmotic pressure of the salt, are given in Table 19, overleaf. The degree of inhibition is expressed as the difference in the times required to reach 50 per cent haemolysis by test and control.

Table 19.

Degree of Ionic Inhibition at Constant Osmotic Pressure.

Osmotic Pressure = 1.5 atmospheres

(Chlorides)		(Sodium and Potassium Salts)		
Ion	Degree of Inhibition		Ion	Degree of Inhibition
Li^+	17	(Na^+)	F^-	1
Na^+	10		Cl^-	9
K^+	1		Br^-	22
Rb^+	2		I^-	36
Cs^+	3	(K^+)	F^-	3
Mg^{++}	50		Cl^-	6
Sr^{++}	43		Br^-	8
Ba^{++}	37		I	10

Cations increase the cohesion between the molecules of the erythrocyte membrane (Danielli (1937)). Since this action depends mainly on the electrostatic charge carried by the ion, the bivalent cations should be more effective than equivalent concentrations of monovalent cations. That this is the case has been shown in Table 19.

If the observed inhibition were due solely to the action of the ions on the membrane then, since the charge

on Mg^{++} is twice as great as that on Li^{+}, and since their ionic radii are very similar (Pauling, 1945, p.346), magnesium should be twice as effective as an inhibitor. In point of fact magnesium ion is about three times as effective an inhibitor compared with lithium ion. Further, Ca^{++} has about the same ionic radius as Na^{+}, and twice the ionic charge, yet calcium chloride produces an infinite degree of inhibition compared with sodium chloride, in osmotically equivalent concentration. Thus, although the cations do affect membrane stability, this action is not adequate to account for the different degrees of inhibition at constant osmotic pressure.

With respect to the alkaline earth cations, only calcium combines with the membrane in such a way that permeability is grossly altered. The remaining members of this series, although capable of forming very insoluble salts with cell surface phosphate groups, do not appear to enter into chemical combination to anything like the same extent as calcium.

Although a mechanism has been put forward by Danielli and Davson (1934), explaining the action of Ca^{++} on the basis of insoluble salt formation with surface anions, it is difficult to understand why the other members of this group should not act in the same way. It is suggested by the observations

that the action of Ca^{++} is highly specific and that the interpretations presented in the literature are inadequate.

Figure 38 shows the relationship between the inhibition caused by cations and their heats of hydration. In each case the haemolysing solution contained malonamide, osmotically equivalent to 3.5 atmospheres, and alkali metal or alkaline earth chloride, osmotically equivalent to 1.5 atmospheres.

With sodium, lithium, barium, strontium and magnesium ions, the higher the heat of hydration, the greater the degree of inhibition. Also, the higher the heat of hydration, the more extensive the total hydration. Since the erythrocyte membrane is impermeable to cations - as far as the duration of these experiments is concerned - the different inhibitory effects, at constant osmotic pressure, appear to be due to differing abilities to retain water in the extra-cellular phase. Magnesium is therefore the most effective, and sodium the least effective, with respect to the inhibition of malonamide-induced haemolysis, at constant osmotic pressure.

The cations, K^{+}, Rb^{+} and Cs^{+}, which have low heats of hydration, give a low degree of inhibition: with these ions the degree of inhibition increases with decreasing heat of hydration, and therefore their effects are not accounted

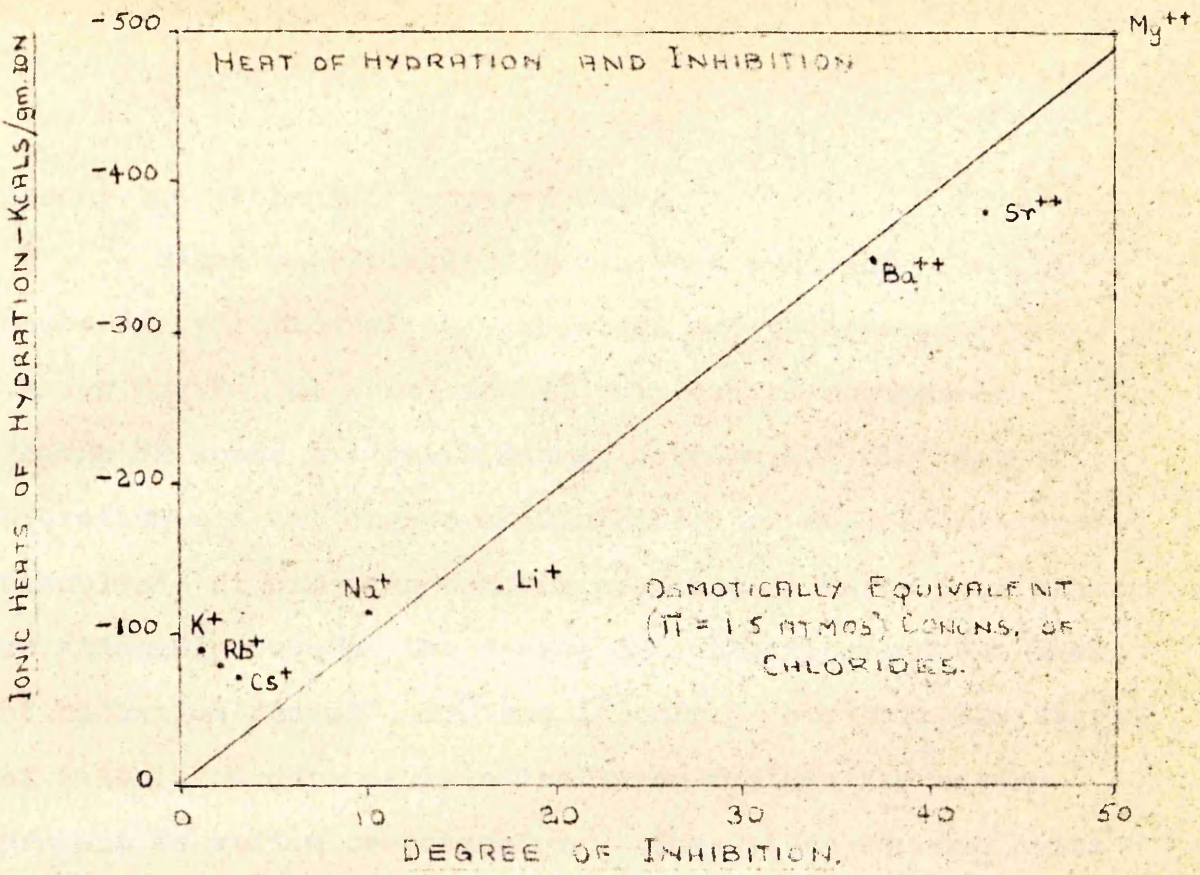

HEAT OF HYDRATION AND INHIBITION
IONIC HEATS OF HYDRATION - KCALS/gm. ION
DEGREE OF INHIBITION.
-500
-400
-300
-200
-100
0
0
10
20
30
40
50
Mg++
Sr++
Ba++
Li+
Na+
K+
Rb+
Cs+
OSMOTICALLY EQUIVALENT (Π = 1·5 ATMOS) CONCNS. OF CHLORIDES.

FIG. 38.

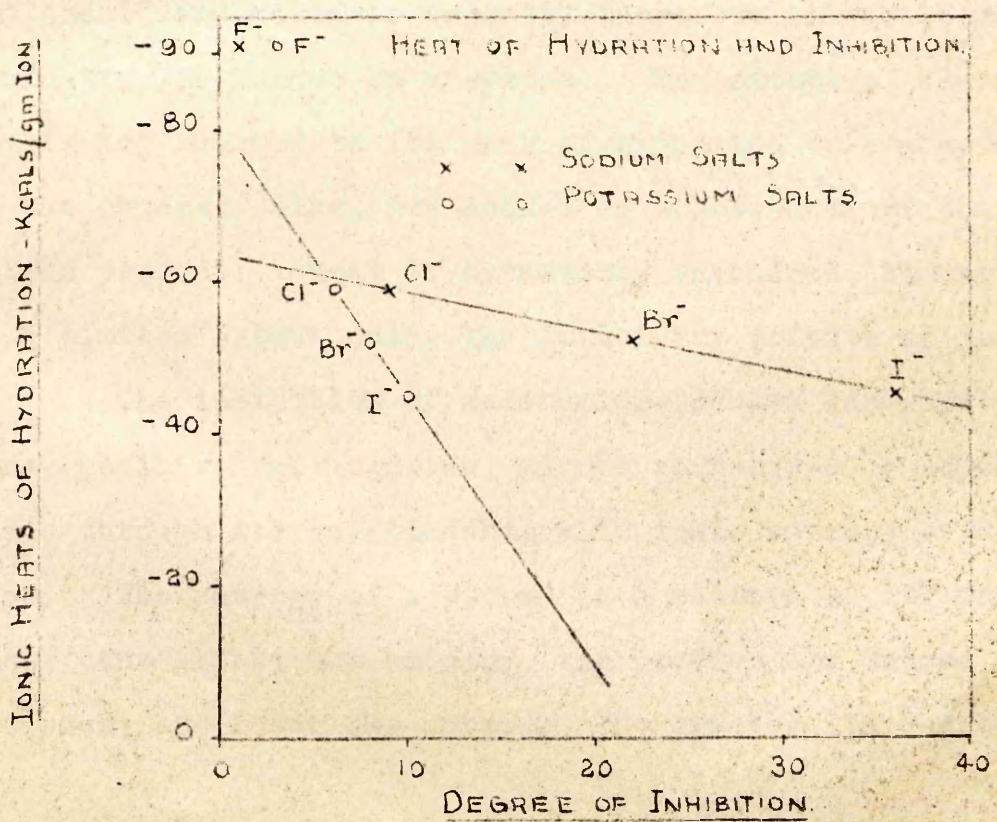

HEAT OF HYDRATION AND INHIBITION.
IONIC HEATS OF HYDRATION - KCALS/gm ION
DEGREE OF INHIBITION.
F⁻ of F⁻
-90
-80
-60
-40
-20
0
0
10
20
30
40
Sodium Salts
Potassium Salts
Cl⁻
Br⁻
I⁻

FIG. 39.

for by the mechanism proposed above.

Since no reliable figures were available for the heats of hydration of the oxyanions and thiocyanate, the values for F^-, Cl^-, Br^- and I^- only can be considered. Figure 39 shows the relationship between anionic heat of hydration and the degree of inhibition of malonamide-induced haemolysis at constant osmotic pressure. There is a linear relationship between the degree of inhibition and the heat of hydration for Cl^-, Br^- and I^- only. Further, the degree of inhibition depends principally on whether the cation present is sodium or potassium. The values for the heats of hydration of K^+ and Na^+ are about twice those for Cl^-, Br^- and I^- and would be expected therefore to have the dominating influence on a system. The effect of fluoride ion is not related to its heat of hydration in the same way as the others: also, its action is independent of the cation present. Heat of hydration, therefore, summarises, to a limited extent only, the inhibitory effects of ions.

The inhibition of malonamide-induced haemolysis by ions, positive and negative, may be compared on a common basis through its relationship with ionic entropy of hydration. The entropy of a system is a measure of its randomness; the higher the entropy, the greater the degree of disorder; the lower the entropy, the greater the degree of

order.

The entropies of hydration are all negative and since the ions are themselves unchanged when they enter into solution, the negative values reflect the lowering of the entropy of water - that is, increasing the degree of order - in the vicinity of the ion (Ulich (1934)). The entropy of hydration is therefore a measure of the extent to which water molecules in the vicinity of the ion lose their translational degrees of freedom. Theoretical values for entropies of hydration - which are in good agreement with experimental values - are given in Table 20 (Conway and Bockris (1954)). There is of course a qualitative relationship between entropy of hydration and heat of hydration, but the former is calculated independently by statistical-mechanical methods.

Table 20.

Theoretical Values for the Entropies of Hydration of Ions.

Ion	$\Delta S_{s,i}$
Li^+	-34.7
Na^+	-28.3
K^+	-21.8
Rb^+	-19.9
Mg^{++}	-72.4
Ba^{++}	-50.9
F^-	-35.2
Cl^-	-24.2
Br^-	-15.4
I^-	- 8.8

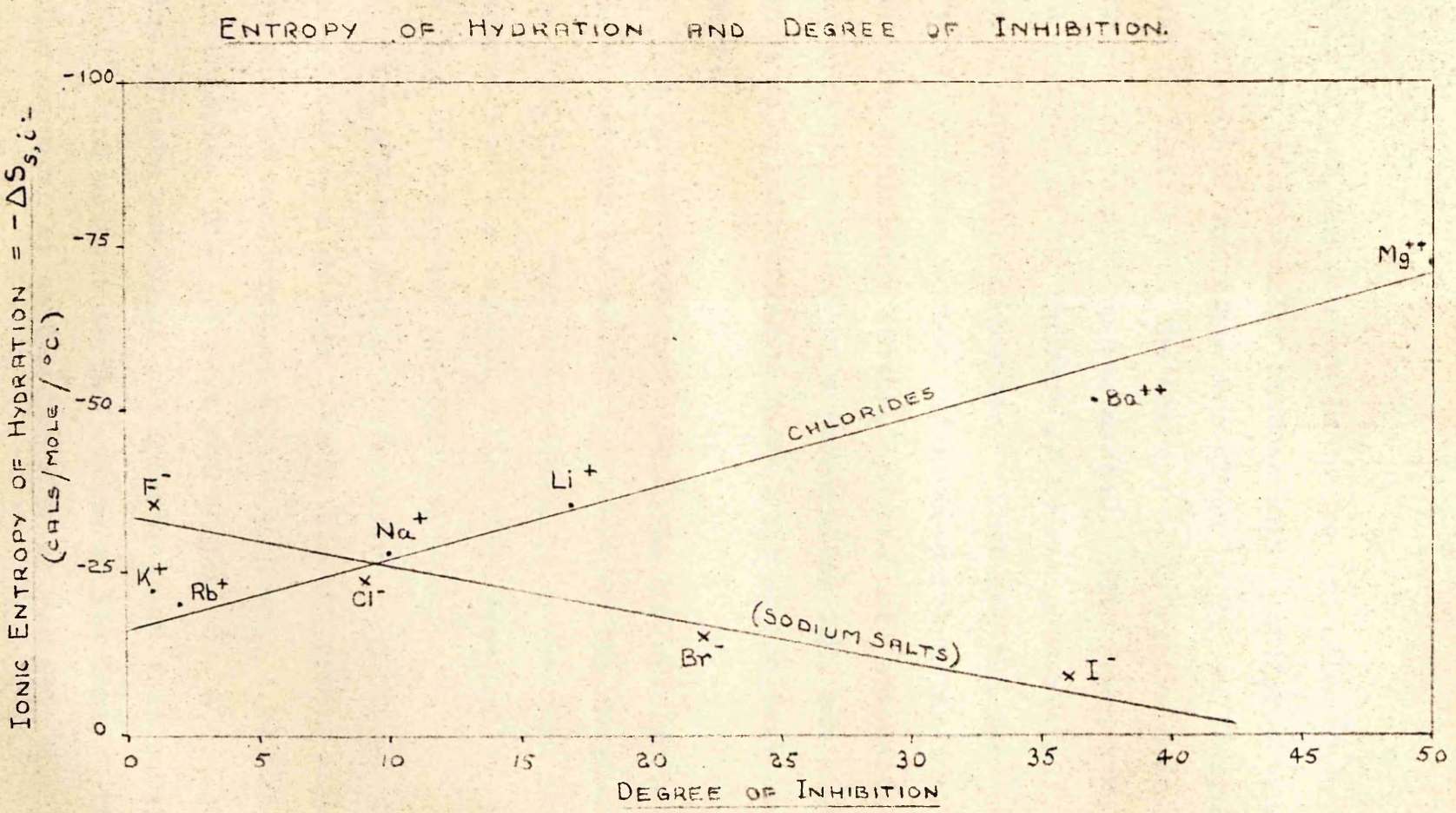

ENTROPY OF HYDRATION AND DEGREE OF INHIBITION.
IONIC ENTROPY OF HYDRATION = $-\Delta S_{s,i}$
(CALS/MOLE / °C.)
-100
-75
-50
-25
0
0
5
10
15
20
25
30
35
40
45
50
DEGREE OF INHIBITION
CHLORIDES
(SODIUM SALTS)
Mg^{++}
Ba^{++}
Li^+
Na^+
K^+
Rb^+
F^-
Cl^-
Br^-
I^-

FIG. 40.

Reliable values are not available for the other ions.

The relationships between degree of inhibition and ionic entropy of hydration are given in Fig.40. With respect to cations, as the entropy of hydration decreases, the degree of inhibition increases. With respect to the halide anions, as the entropy of hydration decreases, the degree of inhibition decreases. That is, the more extensive the "water ordering action" of a non-penetrating ion, the greater its inhibitory effect, and the more extensive the "water ordering action" of a penetrating ion, the less it inhibits malonamide-induced haemolysis at constant osmotic pressure.

It is very gratifying that the entropy/inhibition lines for cations and anions intersect at the common point, sodium chloride. This intersection is a valuable check on the accuracy of the results, which were obtained at different times, and on different specimens of blood.

The effects of the electrolytes considered above are therefore due to a balance between the inhibiting action of cation and the accelerating action of anion. In the system, sodium chloride $\pi = 1.5$ plus malonamide $\pi = 3.5$, the opposing ion effects are equal and opposite, and this substance is "neutral" with respect to the action of its ions. The inhibition produced by sodium chloride is probably wholly osmotic in nature.

This explanation accounts for the fact that the haemolysis curves are the same in systems of 5.0 atmospheres, whether this osmotic pressure is due to malonamide alone, or to malonamide 3.5 atmospheres + sodium chloride 1.5 atmospheres, as shown in Fig.4. Further, the energies of activation for these two systems are also the same, within the limits of experimental error, viz., 13,000 calories for malonamide $\pi = 5.0$ and 13,500 calories for malonamide $\pi = 3.5$ plus sodium chloride $\pi = 1.5$.

The concept that the orderly arrangement of water molecules is a factor in malonamide-induced haemolysis, necessitates a discussion on the modern views of the structural properties of water. The action of ions will be further considered in the light of their effects on water structure.

4.4. The Structure of Water.

Bernal and Fowler (1933) were first to postulate that an orderly arrangement of molecules persisted in liquid water. They assumed that the water molecule carried a negative charge at the oxygen atom, balanced by two positive charges on the hydrogen atom, and owing to the shape of the molecule and the charge distribution, electrostatic forces promoted the formation of a tetrahedral 4-coordinated structure. Though later workers have shown that this charge distribution

is incorrect, the general picture of tetrahedral coordination remains the same.

It is now understood that water association occurs through hydrogen bonding. Forslind (1952) discusses the electronic configurations which give rise to directional hydrogen bonds between water molecules, and the part these bonds play in the formation of the water lattice. This author has shown that structurally water differs only slightly from ice and that the quasi-crystalline structure persists even at boiling point (Forslind (1954)).

In the ice lattice the molecules perform thermal vibrations around their positions of equilibrium. With the application of heat the amplitudes of the vibrations increase, eventually breaking the weaker bonds. Some molecules therefore break free from the lattice and pass into the interstitial lattice spaces, leaving behind vacant points in the structure. These structural defects, or Frenkel defects, are defined as one vacant lattice point and one interstitial non-associated molecule. Each component is free to diffuse in the lattice. When holes reach the boundary of the molecular lattice they are annihilated, causing a reduction in volume, and leaving behind the lone interstitial molecules to increase the bulk density. By increasing the bulk density the interstitial molecules reduce the amplitudes of the

thermal vibrations of the lattice molecules. The formation of interstitial molecules goes on until the increasing density attenuates the thermal vibrations of the lattice molecules sufficiently to prevent the further formation of Frenkel defects. At this point the lattice is in thermal equilibrium.

In this paper Forslind (1954) has shown that even at 100°C some 25 per cent only of the molecules in the system are present in the interstitial lattice spaces. For that temperature range within which biological systems generally function, the number of interstitial molecules varies between 16 and 22 per cent of the total number of water molecules in the system. That is, with regard to temperature only, water is about 80 per cent polymerised in the biological range.

The action of ions on the structure of water has been dealt with by Forslind (1954) from the point of view of their steric effects on the lattice. In addition to the action of an ionic field in attracting water molecules - as measured by the heat of hydration - the steric effect of the ion on the water lattice must be considered. Steric distortion of the lattice only takes place when the mean radius of the ion exceeds the radius of the interstitial space. For this geometrical reason simple ions of radius larger than the potassium ion induce lattice distortion. This is in accord

with the observations of Stewart (1939), who found, by X-ray diffraction methods, that the addition of ions to water had the same effect on the diffraction pattern as raising the temperature of the water.

The effect of sodium chloride on water structure is accounted for by Forslind as follows. .The large chloride ions distort the lattice sterically and augment the number of Frenkel defects. An increase in the number of lone interstitial molecules is therefore required to restore thermal equilibrium. The sodium ions, of smaller radius than the interstitial spaces, cannot prevent the movement of freed molecules into the interstitial spaces and the density therefore increases. The effect of increased density dominates over the loss in lattice rigidity and results in an increased activation energy for viscous flow.

The potassium ion, which is large enough to have the same attenuating effect on the lattice vibrations as an interstitial molecule requires, for a given lattice distortion, a smaller density increase to restore thermal equilibrium. Hence, in a solution of potassium chloride, the density effect on viscosity is smaller in relation to the effect of the decrease in lattice rigidity, and the result is a decrease in solution viscosity.

All simple negative ions, by virtue of their size,

disturb the stability of the water lattice, but the resulting effect of a dissolved electrolyte on the rheological properties of water is determined by the size and shape of the cation. The effects of the polyatomic anions are generally similar, with the exception of certain oxyanions. The perchlorate ion, for example, closely resembles the water tetrahedron with respect to shape and geometrical dimensions. The oxygen atoms may form hydrogen bonds with adjacent water molecules, and the ion can substitute for a water tetrahedron without greatly disturbing the water lattice.

An essential feature of the Forslind water model is that the tetrahedral coordination is a result of the directional properties of the hydrogen bond. Forslind (1952) points out that when non-associated water vapour molecules are adsorbed on a polar substrate, without the formation of directed hydrogen bonds, the orientation of these molecules is unfavourable with respect to bond formation with the second layer. He cites as evidence of this the work of Freymann and Freymann (1951) who showed that water adsorbed on silica or alumina gel behaves as a non-associated substance, as expected from the series H_2S, H_2Se, etc. When the adsorption is specific, i.e., when directed hydrogen bonds are formed between suitably placed active sites of the substrate, the association process is facilitated. Forslind

(1954) has shown that these conditions are satisfied by certain lyophillic clays. He states that the adsorption of water on the surface of a clay crystal appears to be a very specific process, depending on directed valence forces from active surface groups, suitably spaced to cooperate with the organising forces of the water lattice. The coupling between the water and the clay crystal imparts an increased rigidity to the water lattice, effective over distances greater than 300 Å units.

4.5. The Inhibitory Action of Electrolytes in Relation to the Structure of Water.

Since the steric effects of ions on the water lattice depend on their radii, the values for ionic radii quoted by Pauling (1945, p.346) are given below.

Table 21.

Radii of Ions.

Ion	Crystal Radius
Li^{+}	0.60
Na^{+}	0.95
K^{+}	1.33
Rb^{+}	1.48
Cs^{+}	1.69
Mg^{++}	0.65
Ca^{++}	0.99
Sr^{++}	1.13
Ba^{++}	1.35
F^{-}	1.36
Cl^{-}	1.81
Br^{-}	1.95
I^{-}	2.16

The cations, Li^+, Na^+, Mg^{++} and Sr^{++}, have smaller radii than the potassium ion and therefore do not distort the water lattice. Their different inhibitory effects are due to the different extents to which they stabilise the water lattice, by virtue of their differing hydration energies. Ba^{++} distorts the lattice due to its size, but this action is greatly outweighed by the stabilising effect of its high energy of hydration.

The cations, K^+, Rb^+ and Cs^+, distort the water lattice considerably and do not have the compensating factor of a high energy of hydration. The different inhibitory effects of the sodium and potassium halides may be explained by the different effects of the cations on the water lattice. K^+ has a lower energy of hydration than Na^+ and will, therefore, not strengthen the water lattice to the same extent as Na^+. K^+ also contributes sterically to lattice distortion, while Na^+ does not. The inhibiting effects of potassium salts are therefore much less than the inhibiting effects of corresponding sodium salts.

It is not clear why Rb^+ and Cs^+ should cause increasing inhibition with increasing lattice distortion. The presence of malonamide in the system may be responsible for this. Malonamide, as will be suggested later, may be able to act as a nucleus for water lattice formation. If it does

so, then the greater the structure breaking action of the ions, the greater the relative importance of the malonamide. Since the strengthening effect of an ion on the water lattice is due to electrostatic forces, and that of malonamide is probably due to hydrogen bond formation, it may reasonably be supposed that, over a given lattice volume, the malonamide nucleated structure will be the stronger. The cations K^+, Rb^+ and Cs^+, only contribute sterically to lattice distortion and their increasing inhibition with increasing ion size may be due to the greater relative importance of the action of malonamide. Where the cation has no structure breaking action, the more rigid lattice is the result of the combined effects of ion and malonamide.

By virtue of their size, the halide anions all disturb the water lattice. Further, if the electrostatic field of a cation strengthens the lattice, the electrostatic field of an anion should weaken it. The rigidity of the water lattice might be expected to decrease with increasing anionic energy of hydration. The inhibition due to anions will therefore decrease with increasing energy of hydration. This has been shown to be the case for the halide anions.

It has been pointed out in the preceding section that the perchlorate ion may replace a water tetrahedron in the lattice without greatly disturbing the structure. This possibly occurs also, but to a lesser extent, with the

chlorate ion. The arrangement of the atoms in this molecule is pyramidal, with the chloride atom at the apex and the three oxygen atoms forming the base (Kujumzelis (1938)). Thus, in the chlorate ion, the oxygen atoms have much the same arrangement as the three oxygen atoms at the base of the perchlorate tetrahedron. This ion may also form hydrogen bonds with the water lattice and not greatly disturb the structure and its slight acceleration of malonamide-induced haemolysis is probably related to this factor.

The iodate ion, although also pyramidal, will, owing to its large size, contribute more extensively to lattice breakdown, and inhibition, probably by the same mechanism as mentioned above, is observed.

The nitrate ion is planar with respect to the arrangement of its atoms (Kujumzelis (1938)) and therefore, although hydrogen bonding may occur, the steric lattice distortion is considerable and, once again, inhibition is observed.

The thiocyanate ion is unlikely to form hydrogen bonds with water molecules. It is also very large, and, consequently, its structure breaking action is very great. The inhibition observed is also considerable. The inhibition caused by potassium thiocyanate is so much greater than that shown by the remaining potassium salts that there must be some considerable doubt as to whether a compensating action

by malonamide could account for it. Both ions contribute sterically to weaken, if not destroy, the water lattice and yet there is considerable inhibition. It may be that the steric effects of the ions are very localised and the lattice strengthening effect of malonamide relatively extensive. Although this explanation for the inhibitory effect of large ions remains open to doubt, that given for the action of small ions - relative to the water lattice - would appear to be reasonably satisfactory.

4.6. The Inhibitory Action of Non-electrolytes in Relation to the Structure of Water.

If, as mentioned in the section dealing with water structure, the steric and directional conditions are fulfilled by the active sites of a lattice boundary, hydrogen bonds are formed between the lattice and the boundary. This bonding causes an increase in lattice stability, which extends over a considerable distance. It would appear not unreasonable to suppose that if the same conditions were met by an internal lattice boundary - a solute molecule - hydrogen bonding would also take place.

By a geometrical analysis of the relationship between the active sites of the deoxyribonucleic acid molecule and the water lattice, Jacobsen (1953) has shown that hydrogen

bond formation is very probable. Further, the anomalous physical properties of the deoxyribonucleic acid molecule in aqueous solution are adequately explained by assuming that such bonding does in fact take place.

There would appear to be no reason why such bonding should not occur between the water lattice and smaller solute molecules, provided that the steric and directional conditions are met.

It is suggested that such bonding does occur and provides a reasonable way of accounting for the different inhibitory effects of osmotically equivalent concentrations of the carbohydrates and polyhydric alcohols observed in these experiments.

The rates of haemolysis - as measured by the slopes of the haemolysis curves - are here used for comparing the inhibitory effects of these substances. This is just a matter of convenience, since substances which have a high degree of inhibition have also a slow rate of haemolysis. Degree of inhibition and rate of haemolysis are merely different ways of measuring the same factor; this point will be discussed in the following section.

In the experiments depicted in Figs.31 and 32 the osmotic pressures of the solutions are constant, 3.5 atmospheres malonamide plus 0.5 atmosphere carbohydrate or poly-

hydric alcohol. There is no reason to suppose that the inhibitors react with malonamide or with the cell membrane, and as far as the duration of the experiments is concerned, the membrane is impermeable to these substances. Despite these facts there are considerable variations in the rate of haemolysis. These variations are shown in Table 22.

Table 22.

The Inhibition of Malonamide-induced Haemolysis by Carbohydrates and Polyhydric Alcohols.

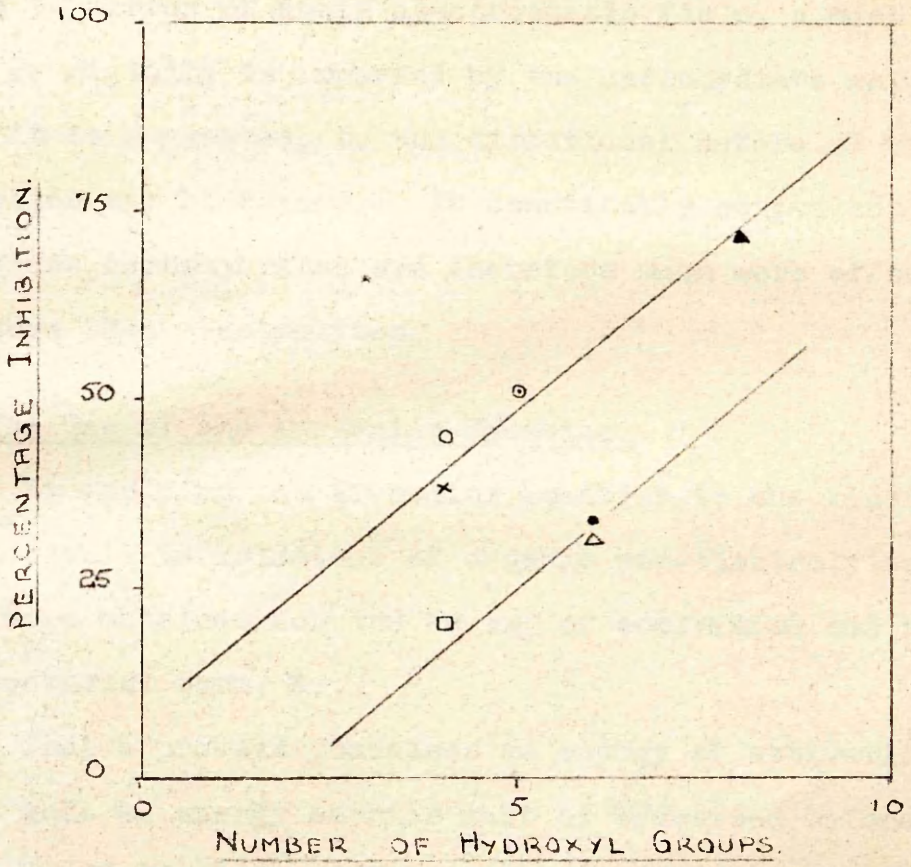
Inhibitor	Rate of Haemolysis (% Haemolys/mm.)	Percent Inhibition
D-Arabinose	8.2	38
α-Methyl-D-glucoside	7.1	45
D-Fructose	6.4	51
Sucrose	3.7	72
DL-Erythritol	10.5	20
Dulcitol	9.1	31
Mannitol	8.7	34
No inhibitor	13.1	-

These rates of haemolysis are, with the exception of erythritol, outside the lower limit of variation for 3.5 atmospheres malonamide, namely, 10.9% haemolysis per minute.

The table indicates - very approximately - that there is increasing inhibition with increasing number of hydroxyl groups. It may be mentioned that the inhibition caused by maltose and lactose, though not quoted in the table or shown in Fig.31, were practically the same as sucrose. The inhibitory effects of these substances are shown, in relation to their number of hydroxyl groups, by Fig.41. It is appreciated that this is an extremely crude method of representation, but it does illustrate a point, namely, that carbohydrates are relatively more effective inhibitors than the polyhydric alcohols. It is suggested that this is due to the different molecular structures of these substances in solution. The carbohydrates form 5- and 6-member rings, while the polyhydric alcohols are linear molecules. Since, in the carbohydrate molecule, the hydroxyl groups are directed tetrahedrally from the corners of a ring structure, these groups are more favourably orientated with respect to bond formation with the water lattice than are the corresponding groups of a polyhydric alcohol.

Without a detailed crystallographic study of the geometrical relationships between the active sites of the solute molecule and the water lattice, no definite statement can be made regarding the formation of bonds, but their occurrence is strongly favoured by the experimental evidence.

THE INHIBITION OF MALONAMIDE HAEMOLYSIS IN RELATION TO THE NUMBER OF HYDROXYL GROUPS ON NON-PENETRATING CARBOHYDRATE AND POLYHYDRIC ALCOHOL MOLECULES.



× D-ARABINOSE
○ α-METHYL-D-GLUCOSE
⊙ D-FRUCTOSE
▲ SUCROSE (MALTOSE, LACTOSE)
□ DL-ERYTHRITOL
• MANNITOL
△ DULCITOL

FIG. 41.

The inhibition caused by these substances is therefore fundamentally the same as that caused by small cations. With both groups water tends to remain in the extra-cellular phase. Although the cations do impart rigidity to the lattice by virtue of their electrostatic field, a much greater degree of rigidity is imparted by the carbohydrate molecule owing, it is suggested, to the directional nature of the bonds which may be formed. In osmotically equivalent concentrations carbohydrates are therefore much more effective inhibitors than electrolytes.

4.7. The Use of the Arrhenius Equation.

By applying the Arrhenius equation to the kinetic data for haemolysis in solutions of organic non-electrolytes, values were obtained for the energy of activation and the non-exponential term, A.

That a process possesses an energy of activation implies that an energy barrier must be traversed before the process can take place. This energy barrier may be the result of molecular inertia, as in chemical reactions in the gas phase: or it may be due to molecular attraction, as in viscous flow. The energy of activation is the amount of energy a molecule requires to traverse the energy barrier. In the first example, this is achieved by increasing the kinetic energy of the molecules: in the second, by overcoming

the intermolecular attraction, in each case by supplying energy in the form of heat.

(a) Malonamide-induced Haemolysis.

With respect to malonamide-induced haemolysis, it might be thought that the diffusion of malonamide into the cell would be the governing factor, and that the observed energy of activation of haemolysis was, in fact, the energy of activation of the diffusion process. If this interpretation were correct, then the graph of E against concentration (or osmotic pressure) should pass through the origin. It has been shown that this graph has an intercept on the y-axis, approximately equal to 5,000 calories. This figure, which has been verified experimentally for zero concentration, is identified with the energy of activation for the viscous flow, or self diffusion of water.

For the reasons mentioned in Section 4.2, the haemolysis curve may be regarded as a graphical representation of the course of cell swelling. The slow penetration of malonamide results in slow swelling of the cell, due to the influx of excess water required to maintain osmotic equilibrium at any instant. During the lag phase the swelling is approaching the critical volume, and during the lytic phase the swelling is carried beyond the critical volume. Both phases therefore measure the same process, but the main

lytic phase is the more sensitive of the two to volume change, since a very slight increase in the critical volume of the cells induces haemolysis. Changes in the degree of haemolysis - which includes the lag phase - or in the slope of the graph imply therefore changes in the rate of swelling of the cell. The rate of swelling, although itself caused by the penetration of water, depends on the rate of penetration of malonamide and we return to the hypothesis - already rejected - that the observed activation energy is that for the diffusion of malonamide.

With respect to the main lytic phase, since its slope is the sensitive measure of volume change, a change in the critical volume would cause a change in the slope of the graph. A larger critical volume would result in a decrease in the gradient of the graph, since more malonamide - and water - are required to diffuse into the cell to cause the swelling necessary for haemolysis. A smaller critical volume will result in an increase in the gradient of the graph since a smaller quantity of malonamide and water will cause haemolysis.

It has been shown that such changes of critical volume do, in fact, occur when there is a change in the cell suspending medium. Ponder and Robinson (1934) have shown that the cells of the same animal haemolyse at a smaller

critical volume in hypotonic saline than they do in hypotonic plasma. Ponder (1947) has also shown that erythrocytes have a large critical volume in presence of sub-haemolytic concentrations of resorcinol. It is pointed out that hypotonic plasma is more viscous than hypotonic saline, i.e., the water lattice is more rigid. Also, resorcinol may well confer some rigidity on the water lattice owing to hydrogen bonding with the lattice and its two hydroxyl groups.

It would appear therefore, that the rigidity of the extra-cellular water lattice modifies the critical volume of the cell. The larger critical volume occurring in presence of a more rigid water lattice may be due to a greater degree of purely mechanical support for the membrane - although this is perhaps rather much to expect from the water lattice - or it may have a more fundamental explanation. In whatever way the increase in critical volume with increasing lattice rigidity may be explained, the point remains that the main lytic phase of the graph may be regarded as a measure of that rigidity.

The energy of activation of haemolysis therefore appears to be due to the activation energy of defect formation in the water lattice. This is offered as a reasonable explanation of the intercept, 5,000 calories.

In considering now the Arrhenius equation, $V = Ae^{-E/RT}$, it is seen that when $e^{-E/RT}$ is equal to unity, $V = A$. Now $e^{-E/RT}$ approaches unity when $^{-E}/_{RT}$ approaches zero. Since E and R are constants, the quantity approaches zero only when T approaches infinity. That is, A may be considered to represent the rate of the process at infinite temperature. Clearly, infinite temperature can have no meaning for any system, biological, physical or chemical, but this artifice is commonly used for the purpose of interpretation.

In chemical reactions between atoms in the gas phase A is considered to represent the frequency of collisions of reacting molecules at infinite temperature. With viscous flow Andrade (1934) considers that A expresses the viscosity at infinite temperature, assuming the substance to remain a liquid. That is, A is the viscosity of the liquid when all attractive forces between the molecules have been overcome.

For the purpose of this work therefore, A is considered to be a measure of the attractive forces between the molecules of the liquid in the system. With respect to aqueous solutions, A will reflect not only the number of bonds formed between the solute molecule and the water lattice, but also the strengths of these bonds. Thus A is a constant only when the number of bonds and their strengths are con-

stant; that is, A is also a measure of lattice rigidity, but on the microscopic scale. If A depended wholly on the numbers of molecules then it would have a constant value for all solutions. That it differs among isomolar solutions - as shown by different rates of haemolysis - indicates that all molecules do not stabilise the lattice to the same extent.

With respect to malonamide-induced haemolysis, there appears to be some considerable stabilisation of the water lattice. Structurally malonamide would appear to provide four active sites for lattice bonding. These sites, two in each amide group, are tetrahedrally directed and may undergo axial rotation about the central C-C chain. Without detailed examination of the crystallographic relationships between the active sites on molecule and lattice, bonding can only be suggested, but the conditions do appear to be very favourable for this to occur. The experimental results, such as delayed haemolysis and slow rate of haemolysis in the lytic phase, also the linear extrapolation to E = 5,000 calories, are reasonably explained by such an interpretation.

(b) Rapidly Penetrating Substances.

The same type of mechanism is postulated to explain the haemolytic properties of these substances. As in the

other cases it remains a suggestion pending crystallographic analysis.

Regarding the action of lipid solvents on the cell membrane, the results show that the energy term is the same, whether the cells undergo haemolysis in diethyl ether osmotically equivalent to 6.5 atmospheres, or whether haemolysis occurs in water. This would seem to confirm that the limiting factor in these experiments is the viscous flow of water.

Urethanes are the only substances so far investigated which give an activation energy value definitely less than that for pure water. It is therefore suggested that urethanes break up the water lattice and have no compensating bond formation to diminish this effect.

Although the E values for the amides and alcohols are fairly close to that of pure water, there are slight differences which are thought to be real. The point is that the experimental methods in this section are relatively crude and are open to error. The observed differences could be accounted for on the basis of water lattice distortion.

With respect to the amide group, its dimensions are such that it should fit into, and bond with, the water lattice. The substituent group on the carbon atom will have a considerable effect on the resulting degree of lattice

rigidity. Lattice rigidity depends on two main factors, namely, the strength of the bonds formed and the structure breaking action of non-bonding groups.

Thus, a change in the electromeric distribution of charge within the molecule, due to change in substituent group, will strengthen or weaken the bonds between the water lattice and the molecule. Increasing the size of the non-bonding substituent group will reduce lattice rigidity through steric distortion and lattice breakdown. Further, a substituent group which can be accommodated in the interstitial space will cause little steric distortion. The resulting effect on water lattice rigidity is a combination of these - and probably other - factors.

To return to the aliphatic amides, reference to the energies of activation shows that these values vary with the substituent group. This is true also of the alcohols. From the data it appears that formamide and methyl alcohol owe their slightly higher energy term to their strengthening the water lattice. The presence of a methyl group on the same carbon atom as the bonding unit reduces the value for energy of activation; this may be explained on the basis that the structure breaking effect predominates over the bond strengthening action of the electromeric effect. When the methyl group is moved to the next carbon atom the electromeric

effect predominates - and perhaps the structure breaking action is also less - the result is a slight increase in the energy term. These effects are more obvious when the relevant values of A are considered.

With respect to urea and the substituted ureas, the action is considered to be similar. A methyl group is less effective for lattice stabilisation than an ethyl group. A second methyl or ethyl group appears to make little difference when present on the second nitrogen atom. In this case the size of a substituent group (the second amino group of the urea molecule) is being increased; so also is the electromeric inductive effect and the observed value of E may be due to a balance of these. In the case of 1.1-diethyl urea the structure breaking effect is the dominating factor.

Although thioureas have the same electromeric distribution of charge as ureas, i.e., dipolar thioamide structure, hydrogen bonds do not generally form with the sulphur atom. It can only be said that the effect of substituent groups with regard to structure breaking and bond formation may well differ from that of the ureas.

The rapidly penetrating polyhydric alcohols show well the effect of molecular structure on the lattice. The dihydric glycols are similar in their effects, the molecule

having two bonding groups separated by one carbon atom being the most effective for lattice stabilisation. Glycerol, on the other hand, although it has three active sites, does not stabilise the lattice so much. It is therefore assumed to have a dominating structure breaking effect.

The effect of phlorhidzin is most reasonably explained by postulating an extensive lattice stabilising action. It does not seem possible that a phosphorylation process could be interfered with - or an inhibitory reaction take place with the cell membrane - in the short time required for complete haemolysis. Since the solution is made up some time before the blood is added, the lattice stabilisation would be an accomplished fact prior to the experiment.

It cannot be said at this point, whether the non-penetration of substances like sucrose is due solely to their interaction with the water lattice, or to other factors such as size. Since the large haemoglobin molecule leaves the cell, apparently without damage to the membrane, it may well be that the apertures in the cell surface are much larger than hitherto presumed, especially when the cell is spherical. In this circumstance the rigidity of the water lattice, and the extent to which solutes "fit" that lattice, would determine whether or not the solutes would penetrate. The confused situation regarding membrane structure, derived

in part, as mentioned in the introduction, from the relationships between the distribution of substances between oils and water, and their related haemolytic activity, may have arisen through lack of knowledge of water structure. The distribution of a substance between a non-polar solvent - such as an oil - and water could be reasonably regarded as a measure of its ability to form hydrogen bonds with the water lattice. The greater the bond forming property of a substance, the greater its concentration in the aqueous phase.

It has been said by Pauling (1945, p.285) that, "as the methods of structural chemistry are further applied to physiological problems it will be found that the significance of the hydrogen bond for physiology is greater than that of any other structural feature".

It is claimed that the results and interpretations presented here fully justify the above statement.

Summary.

1. An investigation has been made of the haemolysis of human erythrocytes in hypotonic solutions of malonamide and other organic substances.

2. It has been found that the Arrhenius equation adequately describes the effect of temperature on all the haemolytic systems investigated.

3. Electrolytes do not inhibit malonamide haemolysis to the same degree, when present in osmotically equivalent concentrations.

4. Non-penetrating non-electrolytes do not inhibit malonamide haemolysis to the same degree, when present in osmotically equivalent concentrations.

5. Interpretation of the terms of the Arrhenius equation with respect to the haemolytic process, the effects of electrolytes and the effects of non-electrolytes, have led to the conclusion that the lattice structure of water is of prime importance in haemolysis.

6. The inhibition due to ions depends on their size and the magnitude of the electrostatic effect which they exert on the water lattice.

7. The inhibition due to non-penetrating non electrolytes depends on their great strengthening action on the water lattice.

8. Of the organic substances investigated, those that penetrate slowly are likely to strengthen the water lattice, while those that penetrate rapidly are unlikely to strengthen the water lattice.

Bibliography.

Andrade, E.N. da C., (1934)
Phil.Mag., 17.7.S., 698.

Bartlett, G.R., and Marlow, A.A., (1951)
Bull.Scripps Metabolic Clinic., 2, 9.

Bernal, J.D., and Fowler, R.H., (1933)
J.Chem.Phys., 1, 515.

Bockris, J.O'M., (1949)
Quart.Revs.Chem.Soc., 3, 173.

Bockris, J.O'M., (1954)
Personal communication.

Bourdillon, J., and Lavietes, P.H., (1936)
J.Clin.Invest., 15, 301.

Brooks, S.C., (1935)
J.Cell and Comp.Physiol., 7, 163.

Clegg, J.W., and King, E.J., (1942)
Brit.Med.Jour., 2, 329.

Collander, R., (1926)
Soc.Sc.Fenn.Comment., 2, 6.

Collander, R., and Barlund, H., (1933)
Acta Bot.Fenn., 11, 1.

Collins, D.A., and Scott, F.H., (1932)
Am.J.Physiol., 101, 21.

Conway, B.E., and Bockris, J.O'M., (1954)
In Press.

Danielli, J.F., (1937)
Proc.Roy.Soc. B., 122, 155.

Danielli, J.F., and Davson, H., (1934)
J.Cell. and Comp.Physiol., 5, 495.

Danowski, T.S., (1941)
J.Biol.Chem., 139, 693.

Davson, H., (1941)
J.Cell. and Comp.Physiol., 18, 173.

Davson, H., and Danielli, J.F., (1952)
The Permeability of Natural Membranes, Cambridge.

Dirken, M.N.J., and Mook, H.W., (1931)
J.Physiol., 73, 356.

Eley, D.D., and Evans, M.G., (1938)
Trans.Far.Soc., 34, 1093.

Forslind, E., (1952)
Proc.Swed.Cement and Concrete Res.Inst., No.16.

Forslind, E., (1954)
Proc. 2nd. Internat.Congress Rheol.

Freymann, M., and Freymann, R., (1951)
Compt.Rend.Acad.Sci., 252, 1096.

Fricke, H., (1935)
J.Gen.Physiol., 18, 103.

Fricke, H., and Curtis, H.J., (1935)
J.Gen.Physiol., 18, 821.

Furchgott, R.F., (1940)
J.Exptl.Biol., 17, 30.

Furchgott, R.F., and Ponder, E., (1940)
J.Exptl.Biol., 17, 117.

Furchgott, R.F., and Ponder, E., (1941)
J.Gen.Physiol., 24, 447.

Glasstone, S., (1947)
Text Book of Physical Chemistry, London.

Gourley, D.R.H., (1951)
Am.J.Physiol., 164, 213.

Govaerts, J., and Lambrechts, A., (1946)
Nature, 157, 301.

Grijns, G., (1896)
Pflüger's Arch., 63, 86.

Guest, G.M., and Siler, V.E., (1934)
J.Lab and Clin.Med., 19, 757.

Haden, R.L., (1934)
Am.J.Med.Sci., 188, 441.

Hahn, L., and Hevesy, G., (1942)
Acta Physiol.Scand., 3, 193.

Hald, Pauline M., Heinsen, A.J., and Peters, J.P., (1948)
Am.J.Physiol., 152, 77.

Ham, T.H., Shu Chu Shen, Fleming, Eleanor M., and Castle, W.B., (1948)
Blood, 3, 373.

Handbook of Chemistry and Physics, (1949)
Cleveland.

Harris, E.J., (1941)
J.Biol.Chem., 141, 579.

Hedin, S., (1897)
Pflüger's Arch., 68, 229.

Hedin, S., (1898)
Pflüger's Arch., 70, 525.

Heller, V.G., and Paul, H., (1934)
J.Lab. and Clin.Med., 19, 777.

Hendry, E.B., (1947)
Edin.Med.J., 54, 476.

Hendry, E.B., (1948a)
Edin.Med.J., 55, 142.

Hendry, E.B. (1948b)
Edin.Med.J., 55, 427.

Hendry, E.B., (1949)
Edin.Med.J., 56, 320.

Hendry, E.B., (1952)
J.Gen.Physiol., 35, 605.

Hendry, E.B., (1954)
Unpublished Data.

Hevesy, G., and Jacobsen, C.F., (1941)
Acta Physiol.Scand., 1, 11.

Hinshelwood, C.N., (1940)
The Kinetics of Chemical Change, Oxford.

Höber, R., (1936)
Physiol.Revs., 16, 53.

Höber, R., (1945)
The Physical Chemistry of Cells and Tissues, London.

Höber, R., and Orskov, S.L., (1933)
Pflüger's Arch., 231, 599.

Hunter, F.R., Stringer, L.D., and Weiss, H.D., (1940)
Science, 91, 579.

International Critical Tables (1929)
New York.

Jacobs, M.H., and Parpart, A.K., (1931)
Biol.Bull., 60, 95.

Jacobs, M.H., Glassman, H.N., and Parpart, A.K., (1935)
J.Cell. and Comp.Physiol., 7, 197.

Jacobs, M.H., Glassman, H.N., and Parpart, A.K., (1936)
J.Cell. and Comp.Physiol., 8, 403.

Jacobsen, B., (1953)
Nature, 172, 666.

Jarisch, A., (1921)
Pflüger's Arch., 192, 255.

Kjumzelis, T.G., (1938)
Z.Physik., 109, 586.

Klinghoffer, K.A., (1935)
Am.J.Physiol., 111, 231.

Knisely, M.H., (1936)
Anat.Rec., 65, 131.

Kozawa, S., (1914)
Biochem.Z., 60, 231.

Landolt Börnstein Roth Tabellen (1912)
Berlin.

Latta, H., (1952)
Blood, 7, 508.

Maizels, M., and Harris, E.J., (1952)
J.Physiol., 118, 40.

Masing, E., (1914)
Pflüger's Arch., 156, 401.

Michaelis, L., and Fujita, A., (1926)
Biochem.Z., 170, 18.

Moelwyn-Hughes, E.A., (1933)
The Kinetics of Reactions in Solution, Oxford.

Monaghan, Betty R., and White, H.L., (1936)
J.Phys.Chem., 40, 1063.

Mond, R., and Gertz, H., (1929)
Pflüger's Arch., 221, 623.

Mueller, C.B., and Hastings, A.B., (1951)
J.Biol.Chem., 189, 869.

Mukai, G., (1921)
J.Physiol., 55, 356.

Mullins, L.J., Fenn, W.O., Noonan, T.R., and Haege, L., (1942)
Am.J.Physiol., 135, 93.

Orr, W.J.C., and Butler, J.A.V., (1935)
J.Chem.Soc., 1273.

Orskov, S.L., (1946)
Acta Physiol.Scand., 12, 192.

Overton, E., (1902)
Pflüger's Arch., 92, 115.

Parpart, A.K., (1931)
Biol.Bull., 61, 500.

Parpart, A.K., (1935)
J.Cell. and Comp.Physiol., 7, 153.

Parpart, A.K., (1940)
Cold Spr.Harb.Symp.Quant.Biol., 8, 25.

Parpart, A.K., and Ballentine, R., (1952)
Modern Trends in Physiology and Biochemistry, New York.

Pauling, L., (1945)
The Nature of the Chemical Bond, 2nd Edn., New York.

Ponder, E., (1933)
Quart.J.Exptl.Physiol., 23, 305.

Ponder, E., (1935)
J.Physiol., 85, 439.

Ponder, E., (1947)
J.Exp.Biol., 23, 350.

Ponder, E., (1948)
Haemolysis and Related Phenomena, New York.

Ponder, E., and Marsland, D., (1936)
J.Gen.Physiol., 19, 35.

Ponder, E., and Robinson, E.J., (1934)
J.Physiol., 83, 34.

Ruhland, W., and Hoffman, C., (1925)
Planta, 1, 1.

Saslow, G., (1929)
Quart.J.Exptl.Physiol., 19, 329.

Saslow, G., (1932)
J.Physiol., 74, 262.

Sidgwick, N.V., (1950)
The Chemical Elements and their Compounds, Oxford.

Smith, P.K., Winkler, A.W., and Schwartz, B.M., (1939)
J.Biol.Chem., 129, 661.

Smith, P.K., Eisenman, Anna J., and Winkler, A.W., (1941)
J.Biol.Chem., 141, 555.

Stewart, G.W., (1939)
J.Chem.Phys., 7, 869.

Teitel-Bernard, A., (1932)
Arch.Roumain.Path., 5, 389.

Teorell, T., (1952)
J.Gen.Physiol., 35, 669.

Tulin, M., Danowski, T.S., Hald, Pauline M., and Peters, J.P., (1947)
Am.J.Physiol., 149, 678.

Ulich, H., (1934)
Z.Physikal.Chem., 168, 141.

Wakeman, A.M., Eisenman, Anna J., and Peters, J.P., (1927)
J.Biol.Chem., 73, 567.

Waugh, D.F., and Schmidt, F.O., (1940)
Cold Spr.Harb.Symp.Quant.Biol., 8, 233.

Whitby, L.E.H., and Hynes, M., (1935)
J.Path.Bact., 40, 219.

Wilbrandt, W., (1937)
Trans.Faraday Soc., 33, 956.

Wilbrandt, W., (1938)
Ergebn.Physiol., 40, 204.

Wolf, R., (1939)
Compt.rend.de la Soc.de Biol., 131, 1082.

Young, L.E., (1947)
New York State J.Med., 47, 1875.