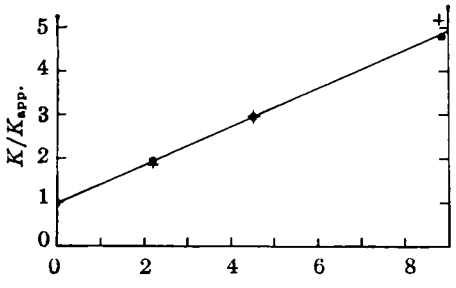

Fig. 6.—Effect of beta-cyclodextrin on the rate of benzocaine hydrolysis in 0.04 N $Ba(OH)_2$ at 30°. Key: +, experimental; ●, calculated.

relationship between the ratio of $K_s/K_{app.}$ and the concentration of free beta-cyclodextrin. This linear relationship is shown in Fig. 6, and further substantiates this 1:1 stoichiometry and the benzocaine hydrolysis in system containing the beta-cyclodextrin is dependant on the uncomplexed benzocaine in solution.

Studies involving inclusion formation and molecular complexation are of importance in the area of drug stabilization and solubilization. Since inclusion formation plays a role in the chemistry of living cells, studies of this type are also important in that data obtained may provide information with respect to the mechanism of drug absorption, transport, and metabolism in biological systems.

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Behavior of Erythrocytes in Various Solvent Systems II

Effect of Temperature and Various Substances on Water–Glycerin and Water–Propylene Glycol Solutions

By D. E. CADWALLADER, B. W. WICKLIFFE, and B. L. SMITH

Hemolytic behavior of rabbit and human erythrocytes in water–glycerin and water–propylene glycol solutions was studied at 25 and 37°, and the effect of various added substances to these systems was investigated. Human *i* values obtained for sodium chloride in aqueous glycerin or propylene glycol solutions at 37° were slightly greater than the corresponding *i* values at 25°. Increase in temperature from 25 to 37° decreased the concentrations of propylene glycol in 0.9 per cent saline solution needed to cause hemolysis of erythrocytes. Mono-monovalent salts, sugars and sugar alcohols, magnesium chloride, and sulfate and sodium succinate afforded essentially the same degree of protection as sodium chloride against hemolysis by propylene glycol. Isotonic concentrations of sodium or potassium sulfate, potassium sodium or disodium tartrate, or trisodium citrate afforded greater protection to erythrocytes than 0.9 per cent sodium chloride. The order in which the anions of the above salts appeared to protect human erythrocytes against propylene glycol hemolysis is citrate > tartrate > gluconate > sulfate; for rabbit erythrocytes the order is sulfate, tartrate > citrate. Much lower concentrations of propylene glycol were required to hemolyze erythrocytes in solutions containing isotonic concentrations of calcium chloride.

THE PREVIOUS PAPER in this series (1) discussed the behavior of erythrocytes in water–glycerin and water–propylene glycol systems in experiments carried out at 25°. Hemolytic *i* values were obtained for sodium chloride in the presence of various concentrations of glycerin and propylene glycol. It was observed that complete hemolysis took place in most glycerin solutions, but the addition of suitable amounts of sodium chloride prevented hemolysis of rabbit and human erythrocytes. Complete hemolysis occurred in all propylene glycol solutions, and in solutions containing 45–50% or more of propylene glycol the addition of iso- and hypertonic quantities of sodium chloride failed to prevent complete hemolysis of rabbit and human red blood cells.

The presently reported experiments are in three areas. (*a*) The behavior of rabbit and human red blood cells was compared at 37 and 25° in aqueous polyhydric alcohol systems,

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TABLE I.—ISOTONIC VALUES OF VARIOUS SUBSTANCES

Compound	Isotonic Values — Rabbit Blood: *i* Value[a]	Rabbit Blood: % Compn. (anhydr.) equiv. to 0.9% NaCl	Human Blood: *i* Value[a]	Human Blood: % Compn. (anhydr.) equiv. to 0.9% NaCl
Calcium chloride	2.85	1.11	2.76	1.15
Dextrose	...	...	0.55[b]	9.39
Glycine	...	...	1.21	1.78
Lactose	1.24	7.91	1.20	8.16
Magnesium chloride	2.97	0.92	2.90	0.94
Magnesium sulfate	...	...	1.99	1.73
Mannitol	...	...	1.37(3)	3.83
Potassium bromide	1.81	1.88	1.79	1.91
Potassium gluconate	...	...	2.28(4)	2.94
Potassium sodium tartrate	...	...	3.30	1.82
Potassium sulfate	...	...	3.06	1.63
Sodium benzoate	...	...	1.85	2.24
Sodium chloride	1.86	0.90	1.86	0.90
Sodium citrate	4.18	1.77	4.02	1.84
Sodium iodide	1.92	2.21	1.93	2.20
Sodium nitrate	1.83	1.33	1.86	1.30
Sodium tartrate	3.36	1.61	3.37	1.60
Sodium succinate	...	...	3.27	1.35
Sodium sulfate	3.20	1.26	3.19	1.27
Sorbitol	1.28(3)	4.07	1.36(3)	3.83
Sucrose	...	...	1.37	7.16

[a] All *i* values were calculated from hemolysis data obtained at 37° and represent an average of at least two blood samples. Except for dextrose, these *i* values proved to be the same as those previously reported at 25°. [b] Hemolytic *i* value at 25°, 1.17 (2).

especially water–propylene glycol. (*b*) Because of the inability of 0.9% sodium chloride to prevent complete hemolysis in 45–50% propylene glycol solutions, experiments were conducted to determine the critical hemolytic concentrations of propylene glycol in 0.9% saline for rabbit and human erythrocytes. (*c*) Experiments were also carried out to observe what effect the addition of isotonic quantities of various compounds had in preventing hemolysis of erythrocytes in glycerin and propylene glycol systems.

EXPERIMENTAL

Materials.—All chemicals were U.S.P., N.F., or reagent grade.

Collection of Blood.—Rabbit and human blood samples were collected and defibrinated in the manner described by Husa and co-workers (2–4). Human blood was obtained from several female and male donors.

Solutions.—All solutions were weight-in-volume preparations. Isotonic concentrations of the various compounds used in this study are shown in Table I. The hemolytic *i* value of each compound was used to calculate the concentration in water of anhydrous compound equivalent to 0.9% sodium chloride. Previously reported *i* values of most compounds in Table I had been calculated from data obtained at 25°. Since hemolysis experiments at 37° were planned, *i* values were determined for this temperature. It was found that *i* values were the same at 37 and 25° for all compounds in Table I except dextrose. Human *i* values are reported for the first time for lactose and sucrose.

Quantitative Determination of Hemolysis.—The method used to determine the degree of hemolysis of erythrocytes in various solutions was described in the first paper of this series (1).

Hemolysis experiments were carried out at 25 ± 1° and 37 ± 1°. In obtaining data for the calculation of *i* values at 37°, the procedure was essentially the same as that referred to except that four tubes of each concentration were used: two each were kept at 37° for 45 minutes, while two were maintained at 25° for the same period of time.

In determining the critical concentrations of propylene glycol in the presence of 0.9% sodium chloride that caused hemolysis of rabbit and human erythrocytes, blood was added to aqueous solutions of 0.9% sodium chloride containing various amounts of propylene glycol. The blood mixtures were allowed to stand 45 minutes—two tubes at 37° and two tubes at 25°. The results are shown in Fig. 1. Similar experiments in which isotonic equivalents of various substances were added to propylene glycol solutions were carried out at 37°. The results are depicted in Figs. 2 and 3.

RESULTS

Water–Glycerin Solution.—Complete hemolysis of rabbit and human erythrocytes occurred in 0.0

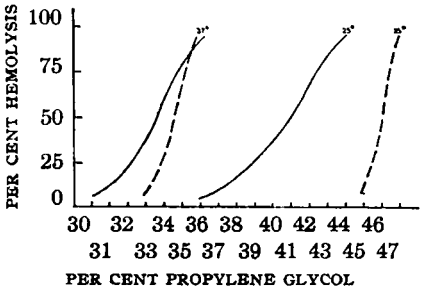

Fig. 1.—Hemolysis of erythrocytes after 45 minutes in propylene glycol solutions containing 0.9% sodium chloride. Key: – – – –, human blood; ———, rabbit blood.

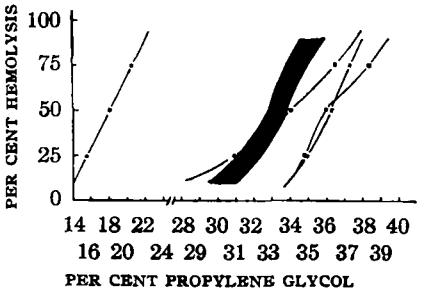

Fig. 2.—Hemolysis of rabbit erythrocytes at 37° in propylene glycol solutions containing isotonic concentrations of various substances. [Isotonic concentrations of various substances refer to isotonic concentrations of these substances in water (Table I).] Key: shaded area includes lactose, $MgCl_2$, KBr, NaCl, NaI, $NaNO_3$, and sorbitol; ●, $CaCl_2$; O, Na_3citrate; ×, Na_2SO_4; □, Na_2tartrate.

to 100% glycerin solutions after 45 minutes at 37°.

Human i values for sodium chloride calculated from data obtained at 37° were slightly greater than the corresponding 25° i values, including the i values previously reported at 25° (1); the increases were not more than 5%.

Compounds in Table I, when added in isotonic concentrations to aqueous solutions containing 0.0 to 60% glycerin (solubility permitting), prevented hemolysis of rabbit and/or human erythrocytes at 37 and 25°.

Water-Propylene Glycol Solutions.—Rabbit and human erythrocytes were completely hemolyzed in 0.0 to 100% propylene glycol after 45 minutes at 37°.

Human i values obtained for sodium chloride in 5, 10, 20, and 30% propylene glycol solutions at 37° were slightly greater (2–5%) than the corresponding i values at 25°, including i values previously reported at 25° (1).

Inclusion of 0.9% sodium chloride in 40% propylene glycol solution did not protect rabbit and human erythrocytes from complete hemolysis at 37°; nor did addition of up to 10% sodium chloride prevent complete hemolysis.

The exact concentrations of propylene glycol in 0.9% sodium chloride that caused hemolysis of rabbit and human red blood cells after 45 minutes at 25 and 37° are shown in Fig. 1. The increase in temperature from 25 to 37° greatly decreased the concentrations of propylene glycol in 0.9% saline solution needed to cause hemolysis of rabbit and human erythrocytes. The concentration of propylene glycol needed to produce 50% hemolysis of rabbit red blood cells decreased from 41% (at 25°) to 33.7% (at 37°). For 50% hemolysis of human blood the concentration dropped from 46% (at 25°) to 34.6% (at 37°), a decrease of more than 11%. At 25° rabbit red corpuscles were hemolyzed by much lower concentrations of propylene glycol than were human red blood cells. However, these differences were markedly decreased at 37°.

Hemolysis experiments were also carried out in which isotonic equivalents of various compounds were added to propylene glycol solutions at 37°. Hemolysis curves plotted from the data obtained are shown in Figs. 2 and 3. The mono-monovalent salts, sugars and sugar alcohols, magnesium chloride and sulfate, glycine, and sodium succinate had sigmoid hemolysis curves for rabbit and/or human blood that fell within the boundaries of the wide, shaded curves in Figs. 2 and 3. Thus, these compounds afforded essentially the same degree of protection as sodium chloride against hemolysis by aqueous propylene glycol solutions. Sodium benzoate gave slightly less protection to human erythrocytes than the above-mentioned compounds.

Isotonic concentrations of sodium or potassium sulfate, potassium sodium or disodium tartrate, or trisodium citrate afforded greater protection to rabbit and/or human red blood cells than 0.9% sodium chloride; in their presence higher concentrations of propylene glycol were necessary to initiate and bring about complete hemolysis of erythrocytes. The order in which the anions of the above salts appeared to protect human erythrocytes against propylene glycol hemolysis was citrate > tartrate > gluconate > sulfate. The curves were not as well defined for rabbit blood; however, the order of protection against hemolysis appeared to be sulfate, tartrate > citrate.

When blood was added to propylene glycol solutions containing isotonic concentrations of calcium chloride, hemolysis of erythrocytes took place in much lower concentrations of propylene glycol than solutions containing isotonic amounts of other compounds. Eight to ten per cent less propylene glycol was required to hemolyze human erythrocytes in aqueous solutions containing 1.15% calcium chloride than in solutions containing isotonic concentrations of other chloride salts. The decrease in propylene glycol concentrations for hemolysis of rabbit erythrocytes in 1.11% calcium chloride solutions was approximately 15%.

DISCUSSION

Current experiments showed that at 37° complete hemolysis of rabbit erythrocytes occurred in all aqueous glycerin solutions, whereas in previous work at 25° (1) hemolysis was incomplete in 50 to 70% glycerin solutions. This is consistent with the observations that for most permeants containing hydroxyl groups the usual hemolytic response to an increase in temperature is increased hemolysis.

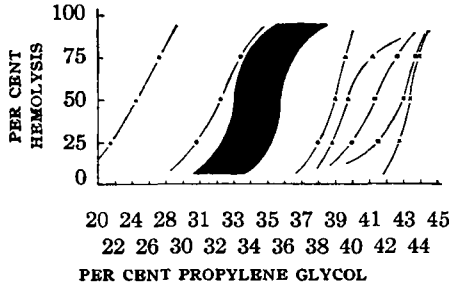

Fig. 3.—Hemolysis of human erythrocytes at 37° in propylene glycol solutions containing isotonic concentrations of various substances. [Isotonic concentrations of various substances refer to isotonic concentrations of these substances in water (Table I).] Key: shaded area includes dextrose, glycine, lactose, $MgCl_2$, $MgSO_4$, mannitol, KBr, NaCl, NaI, $NaNO_3$, Na_2succinate, sorbitol, and sucrose; ●, $CaCl_2$; O, Na benzoate; ▲, K_2SO_4 and Na_2SO_4; △, K gluconate; ■, KNa tartrate; □, Na_2tartrate; ×, Na_3citrate.

Eisenberg (5) found that hemolysis caused by water, ethanol, and glycerin was hastened and increased by a rise in temperature.

It appeared that all the hemolytic phenomena encountered in glycerin solutions were of an osmotic character since hemolysis was prevented by inclusion of isotonic concentrations of sodium chloride or of other salts and nonelectrolytes.

In a series of papers (6–11) concerning the hemolysis of human erythrocytes in relation to the lattice structure of water, Good established the view that the effect of a solute upon the structural properties of the water lattice is basic to the mechanism of hemolysis. A nonpenetrating, structure-promoting substance stabilizes the extracellular water lattice, and by thus reducing the fluidity of the extracellular phase, it slows the rate of movement of water into the cells. Hemolysis is therefore inhibited to an extent dependent upon the stabilizing power of the solute. Conversely, a nonpenetrating, structure-breaking substance reduces the stability of the extracellular lattice, thereby facilitating the flow of water into the cell. Its inhibition is therefore less than that of a structure former. Good found that glycerin, propylene glycol, and ethylene glycol did not greatly influence the stability of a malonamide–water lattice, and that hemolysis was essentially unaffected in the presence of small concentrations of these substances. He also reported that isosmotic concentrations of glycerin and glycols took longer to induce hemolysis than water alone, the longest time for complete hemolysis being reported for glycerin.

In the present study and in the first paper of this series (1) the hemolytic i values of sodium chloride in various glycerin and propylene glycol solutions at 37 and 25° were found to be slightly higher than 1.86, the i value for sodium chloride in water. This indicated that neither glycerin nor propylene glycol contributed much to the tonicity of the extracellular aqueous solutions, or employing the semantics of the above discussion, they did not promote stability of the extracellular water lattice to any significant extent. The slightly higher i values for sodium chloride in glycerin solutions indicated only a slight stabilizing effect of glycerin on the water lattice.

An interesting observation in the present study was an abrupt change in behavior of erythrocytes in concentrated propylene glycol solutions containing 0.9% sodium chloride. At a propylene glycol concentration of about 30%, a sudden initiation of hemolysis occurred, and over the next 2 to 3% range complete hemolysis took place. The exact concentrations where hemolysis occurred varied and were dependent on temperature and the species origin of erythrocytes.

This sudden initiation of hemolysis occurred unexpectedly as the addition of 0.9% sodium chloride protects against hemolysis over 5 to 30% concentrations of propylene glycol in aqueous solutions. Furthermore the hemolytic i value of sodium chloride increases slightly as the propylene glycol concentration increases from 5 to 30% (1) so that theoretically, even less sodium chloride should have been needed to prevent hemolysis at the higher propylene glycol concentrations.

The hemolysis of red blood cells in aqueous solutions containing less than critical hemolyzing concentrations of propylene glycol was apparently of an osmotic character since it was inhibited by 0.9% or less sodium chloride. That hemolysis was not prevented above critical propylene glycol concentrations would indicate some other hemolytic mechanism at these levels.

The behavior of rabbit and human erythrocytes in aqueous propylene glycol solutions containing isotonic concentrations of substances other than sodium chloride (Table I) was similar to that of erythrocytes in glycol solutions containing 0.9% sodium chloride. For solutions containing isotonic concentrations of most compounds, the propylene glycol concentrations causing 50% hemolysis of rabbit erythrocytes were between 32.8 to 33.8%, and for human erythrocytes the range was 33 to 35.8%.

If the same hemolyzing concentration of propylene glycol had been observed with all added compounds, it probably would have been proper to assume that propylene glycol was cytotoxic and therefore caused a complete breakdown of the cell membrane at this particular strength. However, differences in hemolyzing concentrations of propylene glycol were observed upon the addition of other substances, particularly upon the addition of salts having di or trivalent anions. Of the polyvalent organic salts employed in this study, all except sodium succinate provided increased protection of erythrocytes to hemolysis in propylene glycol solutions. From these observations it would appear that some other phenomenon was responsible for the above-mentioned behavior rather than destruction of the membrane.

Stein (12) has shown that the theory of simple diffusion fails at higher concentrations of ethylene glycol, diethylene glycol, 1,2-dihydroxypropane, and 1,3-dihydroxypropane. Experimental results were accounted for by the assumption that interactions occur at these high concentrations between glycol molecules in the main bulk of the permeant solution. These interactions lead to the formation of hydrogen-bonded dimers and oligomers which penetrate the membrane at a different rate from that of the monomer.

The increased inhibition of propylene glycol hemolysis by salts having di and trivalent anions might be explained by classical behavior of erythrocytes to these polyvalent anions. Although permeable to anions (7), the red blood cell is generally thought of being essentially impermeable to cations. However, many cations are able to diffuse slowly and in small proportions into the interior of the cell (13). If a sodium ion can traverse the membrane, then a chloride ion could follow, or a potassium ion could come out of the cell in order to maintain electrical neutrality. If a sodium and chloride ion entered the cell, then there would be an influx of water. The sodium salts of succinic, tartaric, fumaric, and citric acids are known to be fairly indifferent to various cells and tissues (14). In the case of sodium citrate, the anion cannot diffuse through the membrane, so that there can be no immediate change in concentration. This phenomenon could account for sodium citrate and other similar salts providing increased protection against propylene glycol hemolysis.

Isotonic concentrations of calcium chloride considerably reduced the propylene glycol concentrations necessary to induce and complete hemolysis of rabbit and human erythrocytes. This behavior was

probably due to some effect of calcium ion on the red blood cell which made the cell more permeable to extracellular permeants. Calcium does not cross the red cell membrane under normal conditions (15). However, Davson and Danielli (16) have shown that calcium ion in concentrations greater than 0.01 *M* accelerates the penetration of extracellular potassium into the cell and increases loss of intracellular sodium from the cat erythrocyte. They also point out that unusual reponses of cell membranes take place in the presence of alkaline earth ions. Ruyssen and Croes (17) found that alkaline earths appear to thicken the wall of the red blood cell by forming insoluble salts with phosphatide acids. Dentzer (18) reported that calcium ion increased saponin hemolysis of human and cattle erythrocytes. These literature reports emphasize the fact that the calcium ion is capable of producing unusual responses in hemolysis studies.

The hemolysis behavior of the other divalent cation, magnesium, was similar to that of the majority of compounds employed in this investigation.

Jacobs, *et al.* (19), found that the rate of osmotic hemolysis of ox erythrocytes in solutions of penetrating nonelectrolytes (glycerin and ethylene glycol) were considerably increased by the addition of low concentrations of certain electrolytes. Salts with bivalent cations were more effective than those with univalent cations, while salts with bi- or trivalent anions usually had a retarding effect. In most instances, the effect of these ions on the rate of hemolysis are analogous to our present findings for the protective effect of similar ions against hemolysis of rabbit and human erythrocytes in concentrated propylene glycol solutions; calcium afforded the least protection while polyvalent anions gave the most protection.

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Inclusion Compounds of α-Lipoic Acid Methyl Ester with Urea and Thiourea

By HIROYUKI MIMA and MASAO NISHIKAWA

Recently several works have been reported on stabilization of various pharmaceuticals by formation of urea, thiourea, or deoxycholic acid adducts. Methyl α-lipoate, a relatively unstable hepatotonic, was found to form adducts both with urea and thiourea. X-ray diffraction patterns and infrared spectra showed that these adducts were typical inclusion compounds. Methyl α-lipoate in the urea adduct was relatively stable under exposure to sunlight or to ultraviolet light, whereas it was not so stabilized in the thiourea adduct. It was also found that in these adducts methyl α-lipoate became its free radical under such irradiation and that this radical remained stable in these channels for several hours to one day even after the irradiation was stopped.

Inclusion compounds are described as crystalline compounds which consist of two or more distinct components and in which one of the components fits into cavities provided by the other. The component which forms the cavity is designated as the host molecule, and the component which is included in the cavity as the guest molecule.

Urea and thiourea form such adducts or inclusion compounds with various other organic molecules such as hydrocarbons, acids, and esters. These interesting properties, found by Bengen (1), Angla (2), and Schlenk (3) in the 1940's, have been the subject of many investigations. X-ray studies have revealed the general details of the structure of these adducts. Ordinary urea has a tetragonal crystal structure but when crystallized from methanol containing normal paraffins, normal fatty acids, or other straight-chain molecules, it adopts a hexagonal crystal

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