Behavior of Erythrocytes in Various Solvent Systems VI: Water–Tetramethylurea

DONALD E. CADWALLADER and JANIS R. PHILLIPS*

Abstract □ Hemolytic behavior of human erythrocytes in water–tetramethylurea (TMU) solutions was investigated. TMU freely permeates human red blood cells; however, hemolysis can be prevented by the inclusion of 0.9% sodium chloride in solutions containing up to 8% TMU. The addition of sodium chloride to solutions containing more than 8% TMU did not prevent hemolysis, discoloration, and precipitation of blood components. Divalent and trivalent anions gave greater protection against hemolysis than did the sodium chloride. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in aqueous solutions.

Keyphrases □ Erythrocytes, behavior—water–tetramethylurea system □ Hemolysis, erythrocytes—water–tetramethylurea system □ Tetramethylurea–sodium chloride system—erythrocyte hemolysis □ Isotonic coefficients—tetramethylurea–water systems

It is well known that to prepare a safe and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous solvent. For this reason, investigations have been made to study the hemolytic effects of aqueous solutions of glycerin, propylene glycol, and liquid polyethylene glycols on rabbit and human erythrocytes, and the hemolytic effects of aqueous dimethylsulfoxide and liquid amides on human erythrocytes (1–5).

This report is concerned with the investigation of water–tetramethylurea (TMU) systems. TMU is a dipolar aprotic solvent and possesses a number of desirable solvent properties. The solvent and reagent properties of TMU have been reviewed by Luttringhaus and Dirksen (6). The solvent has a mild and pleasant odor, a high degree of stability, low reactivity, a high boiling point (177.6°), and a water-white color. It also has the property of being completely miscible with water, alcohols, ether, benzene, chlorinated hydrocarbons, and many other polar and nonpolar solvents.

Due to the possible use of TMU as a drug solvent, Dixon *et al.* carried out toxicity studies on TMU (7). In their studies, TMU was also tested for central nervous system (CNS) pharmacological properties and the ability to increase survival time of tumor-bearing mice due to structural similarities between TMU and drugs capable of producing these effects. It was reported that the acute LD_{50} of TMU for mice was 2230 mg./kg. i.v. and 2920 mg./kg. given orally. Toxic doses were lower with the monkey, the drug being lethal at dosesas low as 750 mg./kg. i.v. TMU was found to possess weak anticonvulsant and tranquilizer activity, and it produced a significant increase in median survival time of mice bearing a plasma cell tumor.

It was the purpose of this investigation to observe the behavior of human erythrocytes in TMU solutions. In each experiment the hemolytic method was utilized. By comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium chloride–water–TMU solutions, it was possible to calculate the *hemolytic isotonic coefficients for sodium chloride in* various water–TMU solutions.

Table I—Values of *i* for Sodium Chloride in Various Water–TMU Solutions, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes[a]

Tetramethylurea, % v/v	Hemolysis 25%	Hemolysis 50%	Hemolysis 75%	Average
1	1.91	1.94	1.94	1.93
3	1.65	1.72	1.77	1.71
6	1.54	1.62	1.65	1.60
7	1.81	1.88	1.95	1.88

[a] Each value is an average of at least two blood samples.

EXPERIMENTAL

Materials—TMU (Aldrich Chemical Co.) had a specific gravity of 0.972 and was 99% pure. All electrolytes and nonelectrolytes employed in this study were reagent grade.

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of a 22-year-old male Caucasian donor. Fresh blood samples were used in all experiments. Approximately 10 ml. of blood was obtained from the donor and placed in a 50-ml. round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min.; then the blood was decanted into a 50-ml. conical flask and aerated by swirling the flask gently for about 5 min.

Preparation of Solutions—All of the TMU solutions were volume-in-volume percentage preparations. Sodium chloride solutions were prepared on a weight-in-volume basis. Data from a previous paper [*viz.*, Table I, (2)] were used in the preparation of isotonic solutions of calcium chloride, sodium citrate, sodium bromide, sodium sulfate, and sodium tartrate. All pH adjustments were made using Sorensen isotonic buffer systems. Distilled water was used to prepare all solutions.

Quantitative Determination of Percent Hemolysis—In each experiment, the hemolytic method was used to determine the degree of hemolysis of erythrocytes in the TMU solutions. This quantitative method is based on the fact that a hypotonic solution liberates oxyhemoglobin in direct proportion to the number of cells hemolyzed. Into each of two test tubes was transferred 5 ml. of standard sodium chloride solution (0.34%, 0.35% ... 0.45%, 0.46%) and 5 ml. of the mixed solvent system being tested. After the test tubes were brought to a constant temperature by placing in a water bath (37 ± 1°), 0.05 ml. of blood was pipeted into each tube. Each tube was then inverted several times to ensure thorough mixing and allowed to remain 45 min. at 37°. The absorbance of the supernatant liquid after centrifuging was measured using a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter. To find the percent hemolysis, these absorbance readings were divided by the absorbance readings for 0.05 ml. of blood in 5 ml. of distilled water (standard for 100% hemolysis) and multiplied by 100. A blank, made by placing 0.05 ml. of blood in 5 ml. of 0.9% sodium chloride solution, was used to cancel any light absorbance inherent to the blood sample. Since all solutions of TMU were slightly turbid, TMU solutions corresponding to the concentration range of test solutions were used as blanks to cancel any absorbance that might be caused as a result of this turbidity. Both the standard and the blanks were subjected to the same conditions of standing for 45

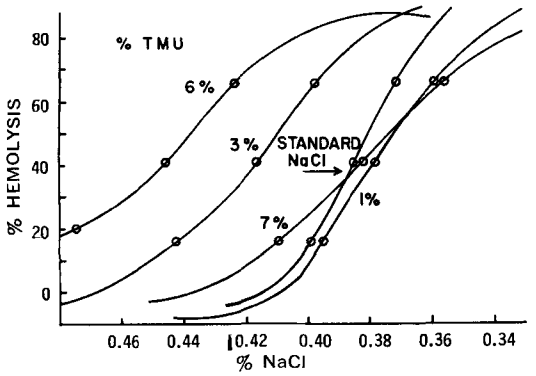

Figure 1—*Hemolysis of human erythrocytes after 45 min. at 37° in various TMU–saline solutions.*

min. at 37° followed by centrifuging. A pH meter (Corning model 7) was used for all pH measurements.

Calculations of *i* Values—Through use of the hemolytic method, concentrations of sodium chloride and the TMU solutions giving the same degree of hemolysis could be determined. Once these concentrations were ascertained, it was possible to calculate isotonic coefficients (*i* values) through use of the following equation:

$$\left(\begin{matrix}i \text{ value for NaCl} \\ \text{in water}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in} \\ \text{100 ml. of water}\end{matrix}\right) = \left(\begin{matrix}i \text{ value for NaCl} \\ \text{in TMU soln.}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in} \\ \text{100 ml. of TMU soln.}\end{matrix}\right) \quad \text{(Eq. 1)}$$

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.9% sodium chloride in water (8).

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–TMU solutions were plotted on rectangular coordinate graph paper. From these curves, it was possible to determine the concentrations of sodium chloride in g./100 ml. of water and the TMU solvent causing 25, 50, and 75% hemolysis. These values were inserted into Eq. 1, thereby giving the values of *i* for sodium chloride in a particular water–TMU solution at concentrations producing 25, 50, and 75% hemolysis. The various *i* values of TMU solutions are shown in Table I.

Preparation of Hemolysis Curves—Approximately 25 experiments employing human blood were carried out, A standard hemolysis curve (Fig. 1) was constructed from the average readings of these experiments. Hemolysis curves of the various TMU solutions were constructed using the *i* values previously calculated (Table I). Through rearrangement of Eq. 1 to give

$$\left(\begin{matrix}\text{g. of NaCl in 100} \\ \text{ml. of TMU soln.}\end{matrix}\right) = \frac{\left(\begin{matrix}1.86\ i \text{ value for} \\ \text{NaCl in water}\end{matrix}\right)\left(\begin{matrix}\text{g. of NaCl in 100 ml.} \\ \text{of water causing 25\%} \\ \text{hemolysis}\end{matrix}\right)}{\left(\begin{matrix}i \text{ value for NaCl in} \\ \text{TMU solution}\end{matrix}\right)} \quad \text{(Eq. 2)}$$

the grams of sodium chloride per 100 ml. in a TMU solution causing 25% hemolysis were calculated. Similar calculations were carried out at 50 and 75% hemolysis. By plotting these three points, the hemolysis curves for the various TMU solutions were constructed (Fig. 1). From these hemolysis curves (Fig. 1), the amounts of sodium chloride preventing hemolysis of 50% of the erythrocytes (or where 50% hemolysis occurred) in various TMU solutions were determined, and the results are shown in Fig. 2.

RESULTS

In this study it was found that complete hemolysis of human erythrocytes occurred in 0.0–100% TMU solutions after 45 min. at 37°. Upon addition of 0.9% sodium chloride, hemolysis of human erythrocytes was essentially prevented (less than 5%) in solutions containing 0.0–8% TMU. Hemolysis greatly increased in solutions containing 9–12% TMU even with 0.9% sodium chloride. These results are shown in Fig. 3.

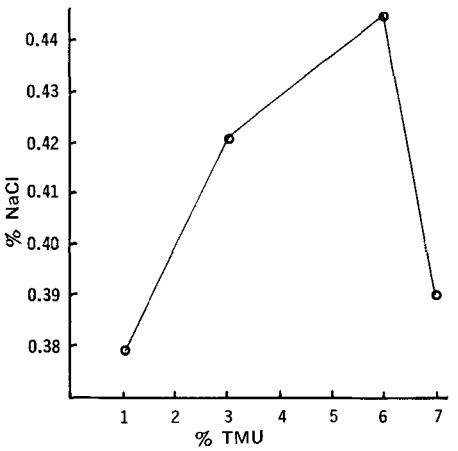

Figure 2—*Amount of sodium chloride preventing hemolysis of 50% of human erythrocytes in various TMU solutions at 37°.*

Reddish-brown solutions resulted when blood was added to 0.9% saline solutions containing 15–16% TMU. More pronounced discoloration and/or precipitation occurred in solutions containing higher concentrations of TMU. Solutions containing 50% TMU revealed a brownish-green color with no precipitation, while those at 75% showed a brown precipitate in addition to the brownish-green color.

It was possible to modify the fragility of human erythrocytes through the addition of hypotonic quantities of sodium chloride (0.34%, 0.35% . . . 0.45%, 0.46%) to various water–TMU solutions. When blood was added to saline solutions containing 0.0–7% TMU, typical sigmoidal hemolysis curves resulted (Fig. 1). These curves were constructed in the manner described in the *Experimental* section of this report utilizing the data presented in Table I.

Calculations of *i* values for sodium chloride in various water–TMU solutions were accomplished through use of Eq. 1 and are shown in Table I.

The pH readings for the TMU solutions were within a range of 5.1–7.0. Addition of isotonic phosphate buffer reduced hemolysis (less than 5%) in solutions containing as much as 10% TMU (Fig. 3).

Aqueous TMU solutions containing isotonic quantities of calcium chloride (1.15%) and sodium bromide (1.51%) gave results similar to those described for the TMU–saline solutions. Addition of sodium citrate (1.84%), sodium sulfate (1.27%), and sodium tartrate (1.61%) reduced hemolysis, producing trace hemolysis in solutions containing as much as 10.5% TMU. The hemolysis curves for TMU solutions containing these divalent and trivalent anion salts were similar to the mentioned curve for phosphate buffer (Fig. 3).

DISCUSSION

From the experimental data gathered in this study, it can be seen that TMU freely permeates human red blood cells and, therefore, offers no protection to these cells from hemolysis. Upon addition of

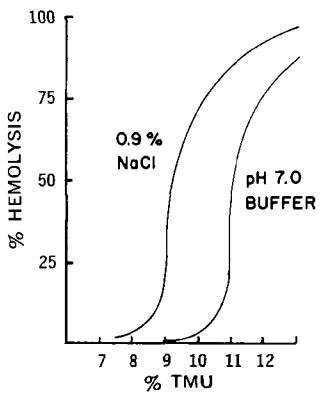

Figure 3—*Hemolysis of human erythrocytes after 45 min. at 37° in various TMU solutions containing 0.9% sodium chloride or pH 7.0 isotonic Sorensen buffer.*

0.9% sodium chloride, complete hemolysis can be prevented in the TMU solutions containing up to 8% TMU. Since addition of 0.9% sodium chloride to these TMU solutions prevents hemolysis and since addition of hypotonic quantities of sodium chloride to these solutions (and TMU solutions of lower concentration) prevents complete hemolysis, the lysis of red cells in solutions containing 7% or less TMU can be attributed to osmotic hemolysis.

There is a critical concentration for TMU at which addition of 0.9% sodium chloride will not prevent hemolysis and/or precipitation of blood components. The transition from nonhemolytic concentrations to destructive concentrations is rather abrupt, as shown by the hemolysis curve in Fig. 3.

Further destruction of red cells occurs as TMU concentrations are increased above the critical concentration. This is apparent as the color changes from reddish-brown to greenish-brown with a brown precipitate.

The van't Hoff factor or isotonic coefficient can be expressed as the ratio of any colligative property of a real solution to that of an ideal solution of a nonelectrolyte (9). The isotonic coefficients (*i* values) for aqueous solutions containing 3 and 6% TMU were found to be less than 1.86. This is evidence that these concentrations offer no protection to human erythrocytes against osmotic hemolysis. However, *i* values calculated for 1 and 7% TMU were slightly higher than 1.86, indicating that these strengths contribute slightly to the tonicity of the extracellular aqueous solutions. This is shown graphically when the concentration of TMU is plotted against the amount of sodium chloride preventing hemolysis of 50% of the erythrocytes (Fig. 2). No *i* values were calculated for 8% TMU solutions since this concentration gave very erratic results and appears to be too critical an area for determination of *i* values.

Upon first observations, pH of the TMU solutions appeared to have some effect upon the solution's tendency to damage human red blood cells, since addition of pH 7.0 Sorensen phosphate buffer considerably reduced hemolysis in solutions containing up to 12% TMU (Fig. 3). However, it did not seem that the reduction of hemolysis was due to the pH effect since the pH range (5.1–7.0) of plain aqueous TMU solutions was near neutral, especially in the higher concentrations. Through further experiments using isotonic quantities of other salts, it was determined that divalent and trivalent anions gave greater protection against hemolysis than did the 0.9% sodium chloride. Therefore, it was surmised that the effect of hemolysis reduction with the phosphate buffer system was due to its phosphate anion properties, not its pH-limiting properties.

In a series of papers (10–15) concerning the hemolysis of human erythrocytes in relation to lattice structure of water, Good established the idea that the effect of a solute upon the structural properties of the water lattice is basic to the mechanism of hemolysis. He found that large simple anions produce simple steric distortion and electrostatic screening which disturb the lattice locally and free water molecules which will tend to associate themselves with a malonamide stabilized zone. Thus the fluidity of the extracellular phase is reduced and hemolysis is slower. Therefore, the large anion will inhibit hemolysis by contributing free water molecules to the malonamide-stabilized lattice. Divalent and trivalent anions used in these studies could possibly act in a similar way whereby they inhibit hemolysis by contributing free water to a TMU-stabilized lattice.

REFERENCES

(1) D. E. Cadwallader, *J. Pharm. Sci.*, **52,** 1175(1963).

(2) D. E. Cadwallader, B. W. Wickliffe, and B. L. Smith, *ibid.* **53,** 927(1964).

(3) B. L. Smith and D. E. Cadwallader, *ibid.*, **56,** 351(1967).

(4) D. E. Cadwallader and J. P. Drinkard, *ibid.*, **56,** 5(1967).

(5) D. E. Cadwallader and J. R. Phillips, *ibid.*, **58,** 1220(1969).

(6) A. Luttringhaus and W. H. Dirksen, *Angew. Chem., Int. Ed. Eng.*, **3,** 260(1964).

(7) R. L. Dixon, R. H. Adamson, M. Ben, and D. P. Rall, *Arch. Int. Pharmacodyn. Ther.*, **160,** 333(1966).

(8) T. S. Grosicki and W. J. Husa, *J. Amer. Pharm. Ass., Sci. Ed.*, **43,** 632(1954).

(9) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 192.

(10) W. Good, *Biochim. Biophys. Acta*, **44,** 130(1960).

(11) *Ibid.*, **48,** 229(1961).

(12) *Ibid.*, **50,** 485(1961).

(13) *Ibid.*, **52,** 545(1961).

(14) *Ibid.*, **53,** 549(1961).

(15) *Ibid.*, **57,** 104(1962).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 17, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication February 3, 1970.

* Undergraduate Research Participant, NSF/URP Grant GY-4290.