Behavior of Erythrocytes in Various Solvent Systems IV

Water–Dimethylsulfoxide

By DONALD E. CADWALLADER and JOHN P. DRINKARD*

Hemolytic behavior of human erythrocytes in water–DMSO solutions was investigated. Complete hemolysis of erythrocytes took place in all DMSO solutions, with discoloration and precipitation occurring at DMSO concentrations greater than 35 per cent. The addition of 0.9 per cent NaCl or isotonic concentrations of other compounds ($CaCl_2$, dextrose, lactose, KBr, Na citrate, NaBr, NaI, and Na salicylate) prevented hemolysis in solutions containing 0.0 to 40 per cent DMSO. The addition of these compounds to solutions containing more than 40 per cent DMSO did not prevent hemolysis, discoloration, or precipitation of human red blood cells. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in various aqueous DMSO solutions. The DMSO concentration which afforded the greatest protection to erythrocytes against osmotic hemolysis was 26–27 per cent. In aqueous ternary solvent systems, DMSO did not affect the concentrations at which propylene glycol and polyethylene glycol 300 damage erythrocytes.

To prepare a safe, stable, and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous cosolvent. With this in mind, the hemolytic behavior of rabbit and human erythrocytes in aqueous solutions of glycerin, propylene glycol, and liquid polyethylene glycols has been investigated (1–3).

The solvent system selected for this investigation was water–dimethylsulfoxide (DMSO). In recent years, DMSO has been the subject of many research articles concerning its effect on biological systems and its unusual and numerous medicinal properties. In addition, a great deal of interest has been expressed in the excellent solvent properties of DMSO. Of the aprotic solvents, DMSO has one of the highest dielectric constants, 48.9 at 20°. Thus, DMSO is completely miscible in all proportions with water and most common organic solvents, including glycerin, acetone, and ethanol (4). DMSO has been found to be an unusually effective solvent for a variety of compounds, including proteins (5), glycogen (6), cellulose and related substances (7, 8), steroids (9), sugar and sugar esters (10), and alphaglucochloralose (11).

Results of preliminary pharmacological tests indicate that drugs dissolved in DMSO and administered systemically do not differ significantly in their lethality or cellular penetration (12). Willson *et al.* (13) carried out toxicity studies to determine if DMSO would be useful as a vehicle for the intravenous administration of water-insoluble antitumor agents. His and other reports (14, 16) indicate that DMSO has a very low toxicity; however, it appears that blood cells are hemolyzed in the presence of high concentrations of this vehicle.

The purpose of this investigation was to study the behavior of red blood cells in aqueous DMSO solutions. The hemolytic method was employed, and the experiments were designed so that standard hemolysis curves obtained for human blood in experiments using aqueous saline solutions could be compared to hemolysis curves obtained from experiments using sodium chloride–water–DMSO solutions. From these data it was possible to calculate *hemolytic* isotonic coefficients for sodium chloride in various water–DMSO solutions. Experiments were also carried out to observe what effect the addition of isotonic quantities of various compounds had in preventing hemolysis of erythrocytes in water–DMSO systems.

EXPERIMENTAL

Materials—Reagent grade dimethylsulfoxide supplied by the J. T. Baker Chemical Co. was used. All electrolytes and nonelectrolytes employed in this study were reagent grade.

Blood Samples—Human blood was used in all experiments and was obtained from several 20–25 year old male Caucasian donors just prior to each experiment. Approximately 10 ml. of blood was obtained from the forearm vein and placed in a 50-ml. round-bottom flask containing 10–15 glass beads. After gently rotating the flask for approximately 5 min., the defibrinated blood was decanted into a 50-ml. conical flask and aerated by gently swirling the flask for about 5 min.

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Preparation of Solutions—Dimethylsulfoxide solutions were calculated and prepared as volume–volume percentage solutions. Solutions containing glycerin, polyethylene glycol, and propylene glycol were prepared on a weight-volume basis. Concentrations of electrolytes and solid nonelectrolytes in solution were based on weight-in-volume calculations. Solutions containing isotonic concentrations of various compounds were prepared using data from an earlier paper [*viz.*, Table I (2)].

Quantitative Determination of Per Cent Hemolysis—The method used to determine the amount of hemolysis of erythrocytes in various solutions was described in earlier papers in this series (1, 2). Hemolysis experiments were carried out at 25 ± 1 and 37 ± 1°.

To determine the effect of time on hemolysis, several rate studies were carried out in which the blood-test solution mixtures were removed at 10-min. intervals, during a period of 60 min., centrifuged, and the amount of hemolysis determined colorimetrically. These rate experiments were carried out at 37 ± 1, 25 ± 1, and 20 ± 1° so that the effect of temperature on rate could be ascertained.

Calculation of i Values—An objective of this paper was to calculate *apparent i* values for sodium chloride when the salt was present in a water–DMSO system, *e.g.*, sodium chloride in 20% DMSO. When the concentrations of sodium chloride in water and water–DMSO solutions causing the same degree of hemolysis are known, the value of i (isotonic coefficient) for sodium chloride in aqueous DMSO solutions can be calculated according to a previously described equation (1) modified as follows:

$$\begin{pmatrix} i \text{ value for} \\ \text{NaCl in water} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl} \\ \text{in 100 ml. water} \end{pmatrix} = \begin{pmatrix} i \text{ value for} \\ \text{NaCl in DMSO soln.} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl in} \\ \text{100 ml. DMSO soln.} \end{pmatrix} \quad \text{(Eq. 1)}$$

The value of i for sodium chloride in water was taken as 1.86.

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–DMSO solutions were plotted on rectangular coordinate paper. From these curves the concentrations of sodium chloride in Gm./100 ml. of water and the other solvent, causing 25, 50, and 75% hemolysis were determined. These values were inserted into Eq. 1, and the values of i for sodium chloride in a particular water–DMSO solution, at concentrations giving 25, 50, and 75% hemolysis were determined. The various i values for sodium chloride in aqueous DMSO solutions are shown in Table I.

Preparation of Hemolysis Curves—Twenty experiments employing human blood were carried out. The average readings of these experiments were used to construct a standard hemolysis curve (left-hand side of Fig. 1.) Hemolysis curves of the various DMSO solutions shown in Fig. 1 were prepared in the same manner as described in previous papers (1, 3).

To determine DMSO concentrations that offered greatest protection to red blood cells, hemolysis experiments were carried out in various water–DMSO–sodium chloride solutions and the data portrayed on rectangular coordinate paper as described above. From these hemolysis curves the amounts of sodium chloride preventing hemolysis of 50% of the erythrocytes (or where 50% hemolysis occurred) in various DMSO solutions were determined and the results are shown in Fig. 2.

TABLE I—VALUES OF i FOR SODIUM CHLORIDE IN VARIOUS WATER–DMSO SOLUTIONS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

DMSO, % v/v	Hemolysis 25%	50%	75%	Av.
	Expt. at 25°			
5	1.87	1.88	1.87	1.87
10	1.94	1.97	1.97	1.97
15	1.99	1.98	1.99	1.99
20	2.16	2.15	2.13	2.15
30	2.13	2.11	2.11	2.12
35	2.06	2.06	2.05	2.06
40	1.96	2.08	2.07	2.04
	Expt. at 37°			
5	1.86	1.87	1.86	1.86
10	1.89	1.88	1.89	1.89
15	1.91	2.00	1.96	1.95
20	2.09	2.11	2.09	2.10
30	2.08	2.07	2.06	2.07
35	2.01	1.99	2.02	2.01
40	1.90	2.00	2.00	1.97

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

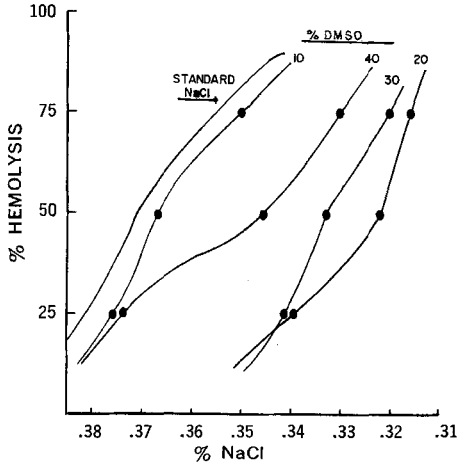

Fig. 1—Hemolysis of human erythrocytes after 45 min. at 37° in various DMSO–saline solutions.

RESULTS AND DISCUSSION

Complete hemolysis of human erythrocytes occurred in 0.0 to 100% DMSO solutions after 45 min. at 25 or 37°. Hemolysis in aqueous solutions containing 0.0 to 30% DMSO gave normal, clear red solutions; however, in solutions containing 35 to 45% DMSO, red-brown solutions were observed. Red-brown solutions containing a brown precipitate resulted when blood was placed in solutions containing 50% or more of DMSO. Precipitation became very heavy when the DMSO concentration reached 75 to 100%. These observations were

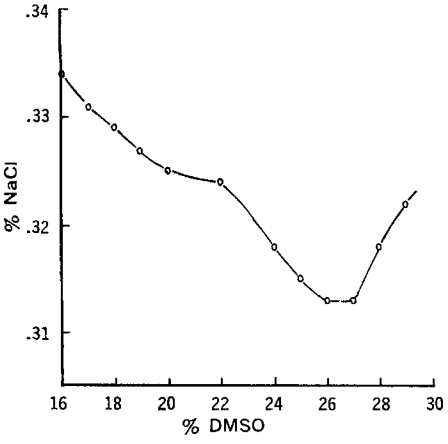

Fig. 2—Amount of sodium chloride preventing hemolysis of 50% of erythrocytes in various DMSO solutions at 37°.

similar to those reported by Ansel and Leake for rabbit blood (17).

Time studies showed that hemolysis and/or damage of red blood cells occurred immediately upon the addition of blood to aqueous DMSO solutions. The rate of hemolysis and cell damage was independent of experimental temperatures (20, 25, and 37°).

The addition of 0.9% sodium chloride prevented hemolysis of human erythrocytes in solutions containing 0.0 to 40% DMSO, however, in experiments where the concentration of DMSO was greater than 25%, trace hemolysis (less than 10%) occurred with several blood samples. Increased hemolysis was observed in solutions containing 40–45% DMSO, and brown-green solutions containing a dark brown precipitate resulted when blood was added to saline solutions containing between 46 and 49% DMSO. Discoloration and precipitation occurred in solutions containing higher concentrations of DMSO. This phenomenon was not prevented by the addition of 2 or 3% sodium chloride.

Aqueous DMSO solutions containing isotonic concentrations of various substances (1.15% calcium chloride, 9.39% dextrose, 8.16% lactose, 1.91% potassium bromide, 1.96% sodium citrate, 1.51% sodium bromide, 2.20% sodium iodide, and 2.45% sodium salicylate) gave results similar to those described above for DMSO–saline solutions. These compounds prevented hemolysis in 0.0 to 40% DMSO solutions but not in the more concentrated solutions.

The fragility of human erythrocytes in various water–DMSO solutions was modified by the addition of hypotonic quantities of sodium chloride. Typical sigmoid hemolysis curves (*viz.*, Fig. 1) resulted when blood was added to saline solutions containing 0.0 to 40% DMSO. These curves were constructed in the manner described under *Experimental* utilizing the data presented in Table I.

Hemolysis in 0.0 to 40% DMSO solutions is an osmotic phenomenon since addition of 0.9% sodium chloride to these solutions prevents hemolysis, and addition of hypotonic quantities of saline allows partial hemolysis to occur.

It was possible to calculate i values for sodium chloride in various water–DMSO solutions. The average i values for sodium chloride in these systems (Table I) were slightly greater than 1.86, the accepted value for 0.9% sodium chloride in water. This indicated that DMSO contributed very little to the tonicity of the extracellular aqueous solutions; however, it did not have a negative effect until, of course, the DMSO concentration became greater than 40%. This slight increase in i values can probably be attributed either to some effect of DMSO on the red cell membrane or to increased activity of sodium chloride in DMSO solutions. Sears *et al.* (18) determined the equivalent conductances in DMSO of a number of sodium and potassium salts including halides, but not sodium chloride, and found them to be completely dissociated in DMSO.

The i values at 37° were consistently slightly greater (approximately 1–3%) than the corresponding values at 25°. In previous studies (1, 2) with glycerin and propylene glycol systems, slightly greater i values were found at 37° than at 25°.

Although the range of hemolytic i values of sodium chloride in DMSO solutions was narrow, there was an optimum DMSO concentration which gave the most protection to erythrocytes against osmotic hemolysis. As can be seen in Fig. 2 the amount of sodium chloride needed to protect 50% of the erythrocytes against hemolysis decreased as the concentration of DMSO increased from 16 to 26–27%; the protective concentrations then increased with further increases in DMSO concentrations.

Kinetic studies were carried out in DMSO solutions (10, 20, and 30%) containing between 0.32 and 0.37% sodium chloride. Hypotonic concentrations of sodium chloride were used so that partial hemolysis of erythrocytes would occur in DMSO test solutions. Hemolysis did not appreciably increase from the first 10-min. reading to the 60-min. reading; final hemolysis readings were about 5% greater than initial readings. Essentially the same results were obtained for all experimental temperatures (20, 25, and 37°). These rate studies showed that complete or partial hemolysis in DMSO solutions occurs rapidly and is independent of time and temperature, indicating simple diffusion. The slight increases in hemolysis which did occur with time were probably due to some effect of DMSO on the red cell membrane.

Throughout this investigation it was observed that high concentrations of DMSO caused damage to red blood cells and that diluting the DMSO solutions with saline lowered the *in vitro* toxicity. This damage was not inhibited or prevented by the addition of sodium chloride or isotonic concentrations of various electrolytes and nonelectrolytes. DiStefano and Klahn (15) have shown that DMSO is a potent hemolytic agent when administered intravenously to cats. However, when DMSO was injected in a diluted form, it was far less hemolytic, and hemolysis was almost negligible when DMSO was diluted 8 times in isotonic saline. Willson *et al.* (13) reported that perivascular inflammation and local thrombosis resulted from repeated intravenous administration of undiluted DMSO into dogs, but that these reactions did not occur when the material was diluted adequately before use. In this current study the authors have found that the addition of 0.9% sodium chloride to solu-

tions containing more than 40% DMSO does not prevent damage to erythrocytes and/or the precipitation of blood components. It appears that aqueous solutions containing more than 40% DMSO would be unsafe as a vehicle for intravenous preparations.

To determine the effect of a ternary solvent system on erythrocytes, hemolysis experiments were run at 37° in solutions containing 20% DMSO, 0.9% sodium chloride, and various amounts of either propylene glycol or polyethylene glycol 300 (PEG 300). Solutions containing 0.0 to 30% propylene glycol did not hemolyze red blood cells, however, complete hemolysis with slight discoloration took place in those solutions containing more than 30% propylene glycol. Hemolysis did not take place in solutions containing 0.0 to 20% PEG 300; however, when blood was placed in solutions containing 25% or more PEG 300, the solutions became green-brown, and a brown precipitate formed. In these ternary solvent systems, the addition of DMSO did not alter the critical concentrations (in 0.9% saline) (2, 3) at which propylene glycol and PEG 300 have been reported to hemolyze red blood cells. The damaging effect of the glycols appeared to be solely dependent on their concentration in solution, and there was no additive effect contributed by the DMSO present.

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Fallacy in Concluding There are Zero-Order Kinetics from Blood Level and Urinary Excretion Data

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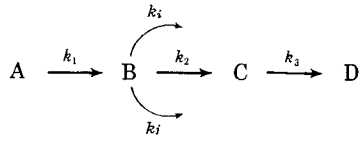
During the past several years there have been a number of reports in which the authors concluded their data proved zero-order formation of a metabolite or zero-order absorption of a drug in the animal or human body. For two of these reviewed in this paper—namely, the conjugation of benzoate with glycine in the rabbit following doses of 500 mg./Kg. of benzoic acid and the conversion of salicylate to salicyluarate in rats at doses above 20 mg./Kg.—the evidence is very convincing. However, the principal evidence in the remainder of the reports is the apparent linearity of a segment of a cumulative urinary excretion curve and/or the curvature of semilogarithmic plots of drug blood levels or of amounts of drug not excreted against time. Model studies and simulations presented here show that neither of the latter is sufficient evidence for concluding there is zero-order rate of formation of a metabolite or zero-order absorption of a drug. The simulations of pharmacokinetic data have far-reaching implications which go much beyond the zero-order–first-order problem. They show one cannot disregard first-order rate constants of relatively large magnitude in many cases; that graphical fitting of pharmacokinetic, and even chemical kinetic, data may often lead to serious misinterpretations; and that "Lineweaver-Burk" plots, which are artifacts, are produced by plotting the reciprocal of excretion rate of a metabolite against the reciprocal of the amount of metabolite remaining in the body.

To illustrate how one may erroneously conclude that there are parallel zero-order and first order kinetics when, in fact, all kinetics are first order, a model simulating the real situation is presented. Assume there is a catenary chain with branching of parallel paths at one point in the chain and that all rate constants are first-order rate constants. Then we may write the model as shown in Scheme I.



Scheme I

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