and 3,5-dinitrobenzoyl chloride reagent. The titration was carried out potentiometrically beyond the second break (titration of the benzoate ester) using a glass-saturated calomel electrode system and one of several different bases as titrant. Following this, solid sodium or ammonium acetate was added to determine whether the potential of the solution could be reversed partially and made to stabilize at the beginning of the second break. At this point, color development due to the ester is near a maximum, while color due to the 3,5-dinitrobenzoic acid is negligible. In this manner solvent systems involving pyridine, acetonitrile, dimethylformamide, acetone and chloroform alone, and some selected mixtures, were tested to arrive at the solvent mixture used in the procedure. Tetrabutylammonium hydroxide (1 *M* in methanol) and KOH (1 *M* in water) were tested as bases before turning to concentrated ammonia.

It is obvious that any of the reactive compound types would constitute a positive interference in the analysis of any other. Similarly, reactive acyl functions (anhydrides or halides) will tend to show negative interference depending on the efficiency with which they compete with the 3,5-D reagent for the available hydroxyl groups. The fact that both hydrochloric and 3,5-dinitrobenzoic acids are formed during the esterification reaction indicates that these types of compounds would not constitute interferences if present in small amounts. Water will react preferentially with 3,5-D, but will not interfere seriously unless present in sufficient quantity to consume a large proportion of the available reagent. Additional compounds which have been tested and shown not to interfere seriously in the determination of hydroxyl groups are summarized in the data of Table II. Note also in Table I that the multifunctional steroids imply a lack of interference by unsaturated moieties and by simple and conjugated ketones.

SUMMARY

A method has been presented and described for the colorimetric determination of organic alcohol, amine, and thiol groups. The method is free of interference from most common solvents and other functional groups. The procedure is rapid and the resulting products are adequately stable to provide ease of measurement.

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Behavior of Erythrocytes in Various Solvent Systems III

Water–Polyethylene Glycols

By B. LESTINA SMITH* and DONALD E. CADWALLADER

Hemolytic behavior of human and rabbit erythrocytes in aqueous solutions of polyethylene glycol (PEG) 200, 300, 400, and 600 was investigated. Complete hemolysis occurred in all PEG 200 and 300 solutions, with discoloration occurring in $\geq$ 25 per cent PEG 200 and $\geq$ 15 per cent PEG 300 solutions. Sodium chloride was effective in preventing hemolysis in $\leq$ 25 per cent PEG 200 or $\leq$ 40 per cent PEG 300 solutions. When possible, *i* values were calculated for sodium chloride in the various water–PEG 200 and 300 solutions. PEG 400 and 600 protected blood cells from damage in > 10 per cent to < 40 per cent solutions, and *i* values were calculated for these PEG's. Solutions containing $\geq$ 40 per cent PEG 400 or 600 (with and without NaCl) were damaging to red cells. The ability of liquid PEG's to penetrate rabbit and human erythrocytes appeared to be 200 > 300 > 400 > 600.

PREVIOUS PAPERS in this series have reported the behavior of erythrocytes in various water–glycerin and water–propylene glycol systems (1, 2). Among other nonaqueous solvents that might be used in the preparation of parenterals would be the liquid polyethylene glycols.

Polyethylene glycols (PEG's) are products possessing a very low order of toxicity. Spiegel and Noseworthy (3) have reviewed the physical properties, toxicities, and parenteral applications of these liquids. Skin penetration studies on

Received September 19, 1966, from the School of Pharmacy, University of Georgia, Athens, GA 30601
Accepted for publication December 15, 1966.
Abstracted in part from a thesis submitted by B. Lestina Smith to the Graduate School, University of Georgia, Athens, in partial fulfillment of Master of Science degree requirements.
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Previous paper: Cadwallader, D. E., Wickliffe, B. W., and Smith, B. L., *J. Pharm. Sci.*, **53**, 927(1964).

many of these products show that they do not penetrate the skin in harmful amounts (4). Meyer and Sturmer (5) reported low oral and subcutaneous toxicities of PEG 200 and 600 in mice. Swanson and co-workers (6) found that sodium amobarbital in 60% PEG 200 and sodium secobarbital in 50% PEG 200 had approximately the same potency and toxicity as aqueous solutions of these barbiturates. Lee and Anderson (7) determined the toxicity of vancomycin in 50% PEG 200 and of PEG 200 alone. Their results indicated that PEG 200 produced no apparent toxic effects when given to dogs at 1.0 ml./Kg./day for 80 days intramuscularly, or 0.5, 1.0, 2.5, and 5.0 ml./Kg. as single intravenous doses.

The purpose of this investigation was to conduct experiments to study the behavior of red blood cells in aqueous polyethylene glycol solutions. Experiments were designed to determine the effect that aqueous solutions of PEG's 200, 300, 400, and 600 have in preventing hemolysis alone, and in the presence of sodium chloride.

EXPERIMENTAL

Materials—Polyethylene glycols 200, 300, 400, and 600, supplied by Union Carbide Chemical Corp., were used without further purification. The sodium chloride used was reagent grade.

Collection of Blood—Approximately 10 ml. of blood was obtained from rabbits by heart puncture. An 18-gauge, 2-in. needle attached to a 10-ml. syringe was used to make entrance into the heart. The blood was placed in a 50-ml. round-bottom flask which contained 10–15 glass beads. After gently rotating the flask for approximately 5 min., the defibrinated blood was decanted into a 50-ml. conical flask. The blood was aerated by gently swirling the flask for about 5 min.

The human blood used was obtained from the forearm veins of several 20–25-year-old healthy male Caucasians. The blood was treated in the same manner as the rabbit blood. Fresh blood samples were used in all experiments.

Preparation of Solutions—All of the polyethylene glycol and sodium chloride solutions were weight-in-volume percentage preparations.

Quantitative Determination of Per Cent Hemolysis—The method used to determine the degree of hemolysis in this investigation was dependent upon the fact that the amount of oxyhemoglobin liberated from the red corpuscles in hypotonic solutions is a direct function of the number of cells hemolyzed. A quantitative determination of partial hemolysis in any mixed solvent system was made by centrifuging solutions containing unhemolyzed cells and determining the oxyhemoglobin in the supernatant solutions by means of a photoelectric colorimeter.

The general method consisted of transferring 5 ml. of standard sodium chloride solutions (0.34, 0.36, . . ., 0.44, 0.46%) into each of two test tubes. Identical amounts of the mixed solvent systems were also transferred into each of two test tubes. Then 0.05 ml. of blood was added to each test tube and the tubes inverted several times to obtain complete mixing. After 45 min. at 37°, the blood mixtures were centrifuged. Because of the viscosity of the PEG solutions, especially in high concentrations, it was necessary to use centrifuge speeds of 2000–3000 r.p.m. to bring about complete settling of intact cells. After centrifuging, the light absorbance of the supernatant liquid was measured using a Klett-Summerson photoelectric colorimeter equipped with a No. 54 filter. 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 to obtain per cent hemolysis occurring in each test solution. A blank, used to cancel any light absorbance inherent to the blood sample, was prepared by placing 0.05 ml. of blood in 0.9% sodium chloride solution, allowing to stand for 45 min., and centrifuging in a like manner.

Water–PEG solutions absorbed a small amount of light, and this absorbance increased with an increase in PEG content. This absorbance was determined for the various concentrations of PEG's used in the experiments, and these blank readings were subtracted from the colorimeter readings obtained at the end of the hemolysis experiments.

A battery-operated model M Beckman pH meter was used in all pH measurements.

Calculation of i Values—The i values (isotonic coefficients) for polyethylene glycols were calculated according to the equation of Grosicki and Husa (8) which was modified as follows.

$$\frac{\left(\begin{matrix}i\text{ value}\\ \text{for NaCl}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of NaCl}\\ \text{in 100 ml.}\\ \text{soln.}\end{matrix}\right)}{(\text{Gm.-mol. wt. of NaCl})} = \frac{\left(\begin{matrix}i\text{ value for}\\ \text{PEG}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of PEG}\\ \text{in 100 ml.}\\ \text{of soln.}\end{matrix}\right)}{(\text{Gm.-mol. wt. of PEG})} \quad \text{(Eq. 1)}$$

Concentrations of sodium chloride and polyethylene glycol causing the same degree of hemolysis (*e.g.*, 25, 50, and 75%) were used in the above equation to calculate i values for the polyethylene glycols.

Experiments were carried out to obtain data for calculating *apparent* i values for sodium chloride when the salt was present in a water–polyethylene glycol system in which the polyethylene glycol itself exhibited no protection to red blood cells, *e.g.*, sodium chloride in 10% PEG 200. In these calculations, it was necessary to assume that polyethylene glycol did not contribute to the osmotic behavior of the solutions and that sodium chloride was solely responsible for the tonicity of the solutions. Since the calculations were concerned with i values of only sodium chloride in different solvents, the molecular weights in Eq. 1 are identical and the equation becomes

$$\left(\begin{matrix}i\text{ value for}\\ \text{NaCl in water}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of NaCl}\\ \text{in 100 ml. of}\\ \text{water}\end{matrix}\right) = \left(\begin{matrix}i\text{ value for}\\ \text{NaCl in PEG}\\ \text{soln.}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of NaCl}\\ \text{in 100 ml.}\\ \text{PEG soln.}\end{matrix}\right) \quad \text{(Eq. 2)}$$

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

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–PEG solutions were plotted on rectangular coordinate paper. From these curves the concentrations of sodium chloride in Gm./100 ml. of water and PEG, causing 25, 50, and 75% hemolysis, were determined. These values were inserted into Eq. 2, and the values of i for sodium chloride in a particular water–PEG solution at concentrations giving 25, 50, and 75% hemolysis were determined.

Preparation of Hemolysis Curves—Experiments employing human blood were carried out to determine *apparent* i values for sodium chloride in various PEG 200 and 300 solutions. The average readings of these experiments were used to construct a standard hemolysis curve. In constructing the hemolysis curves of the various PEG solutions the grams of sodium chloride per 100 ml. of solution causing 25, 50, and 75% hemolysis were calculated with reference to the standard hemolysis curve. By utilizing Eq. 2, the grams of sodium chloride per 100 ml. in a polyethylene glycol solution causing 25% hemolysis was calculated as

$$X = \frac{A \times B}{C} \qquad \text{(Eq. 3)}$$

where X = Gm. of sodium chloride in 100 ml. of polyethylene glycol solution causing 25% hemolysis, A = 1.86 as the i value for sodium chloride in water, B = Gm. of sodium chloride in 100 ml. of water causing 25% hemolysis (obtained from standard hemolysis curves), and C = previously calculated i value for sodium chloride in appropriate polyethylene glycol solution. Similar calculations were carried out to obtain i values at 50 and 75% hemolysis. With these three i values at 25, 50, and 75% hemolysis, the hemolysis curves for the various polyethylene glycol solutions were constructed.

RESULTS

Polyethylene Glycol 200 and 300—The hemolysis of rabbit and human erythrocytes after 45 min. at 37° in various water–PEG 200 and 300 solutions are shown in Figs. 1 and 2. All of the PEG 200 and 300 solutions void of sodium chloride caused complete hemolysis of rabbit and human erythrocytes; however, hemolysis of red blood cells in aqueous solutions containing more than 15–25% PEG 200 or 300 resulted in brown-green solutions instead of normal red solutions. The concentrations at which this discoloration occurred are shown in Table I.

The inclusion of 0.9% sodium chloride in aqueous solutions containing 0.0 to 25% PEG 200 or 300 afforded complete protection (no hemolysis) to rabbit and human erythrocytes. However, the inclusion of sodium chloride in solutions containing 25% and more of PEG 200 or 40% and more of PEG 300 did not prevent damage of blood cells. At these critical concentrations (see Table I) the red blood cells were destroyed resulting in brown-green solutions. The addition of 2, 3, or 5% sodium chloride to 25, 30, 40, and 50% PEG 200 and 300 solutions prevented hemolysis and discoloration of blood.

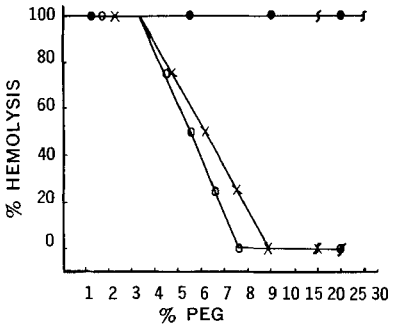

Fig. 1—Hemolysis of rabbit erythrocytes after 45 min. at 37° in various polyethylene glycol–water solutions. Key: ●, PEG 200 and 300; ×, PEG 400; ○, PEG 600; S, discoloration occurred.

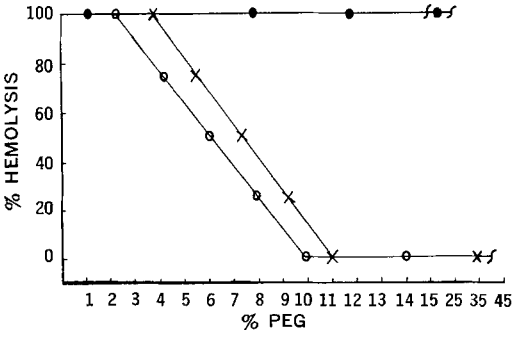

Fig. 2—Hemolysis of human erythrocytes after 45 min. at 37° in various polyethylene glycol–water solutions. Key: ●, PEG 200 and 300; ×, PEG 400; ○, PEG 600; S, discoloration occurred.

TABLE I—CONCENTRATIONS (Gm./100 ml.) OF PEG IN AQUEOUS SOLUTIONS AT WHICH DISCOLORATION OF BLOOD OCCURRED[a]

	No NaCl		0.9% NaCl	
PEG	Rabbit Blood	Human Blood	Rabbit Blood	Human Blood
200[b]	25	25	30	40
300[b]	15	15	25	25
400[c]	15	40	40	40
600[c]	20	40	40	40

[a] Each value represents an average of at least four blood samples. [b] Discoloration in PEG 200 and 300 solutions resulted in brown-green solutions. [c] Discoloration in PEG 400 and 600 solutions resulted in brown-black precipitates. The solutions were not colored.

The fragility of human erythrocytes in various water–PEG 200 and 300 solutions was modified or corrected by the addition of sodium chloride. It was possible to calculate i values for sodium chloride in various water–PEG 200 and 300 solutions. The average i values for sodium chloride in 5, 10, and 20% PEG 200 and 300 solutions are shown in Table II. The i values for sodium chloride in 10 and 20% PEG 200 solutions were less than 1.86 (the accepted value for 0.9% sodium chloride in water). The i values were greater than 1.86 for PEG 300 solutions.

The pH readings for PEG 200 and 300 solutions

TABLE II—VALUES OF *i* FOR NaCl IN VARIOUS WATER–PEG SOLUTIONS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES AT 37°[a]

% w/v	Hemolysis, % 25	50	75	Av.
PEG 200				
5[b]	1.8	2.1	2.2	2.0
10	1.7	1.7	1.9	1.8
20	1.6	1.6	1.7	1.6
PEG 300				
5	4.0	5.2	6.6	5.3
10	2.6	4.4	6.9	4.6
20	2.8	3.1	3.4	3.1

[a] Unless otherwise indicated each *i* value represents an average of at least two blood samples. [b] Average of four blood samples.

before and after the addition of blood were within a range of 3.5 to 4.5.

Hemolysis curves showing the amount of laking that occurred when human blood was added to various water–PEG 200 and PEG 300–sodium chloride solutions are shown in Figs. 3 and 4. These curves were constructed in the manner described under *Experimental* utilizing the data presented in Table II. Unusual data were obtained for experiments using 5% PEG 200 solutions containing various amounts of sodium chloride and the results are shown in Fig. 5. Instead of the continual increase in hemolysis with decreasing sodium chloride concentration, hemolysis in 5% PEG 200 solutions decreased in those solutions containing 0.38 to 0.35% sodium chloride and then increased with further decrease in sodium chloride concentrations.

Polyethylene Glycol 400 and 600—The hemolysis of rabbit and human erythrocytes after 45 min. at 37° in various water–PEG 400 and 600 solutions is shown in Figs. 1 and 2. Hemolysis was prevented in various aqueous PEG 400 and 600 solutions up to critical concentrations where damage of red blood cells occurred resulting in brown-black precipitates (see Table I).

It was possible to calculate *i* values for PEG 400 and PEG 600. Average *i* values are shown in Table III.

The addition of 0.9% sodium chloride to solutions containing less than 40% PEG 400 or 600

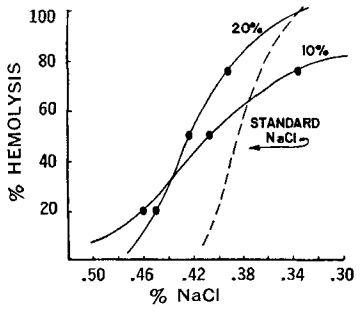

Fig. 3—Hemolysis of human erythrocytes after 45 min. at 37° in various polyethylene glycol 200–saline solutions.

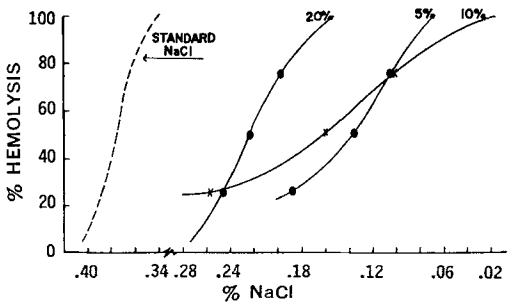

Fig. 4—Hemolysis of human erythrocytes after 45 min. at 37° in various polyethylene glycol 300–saline solutions.

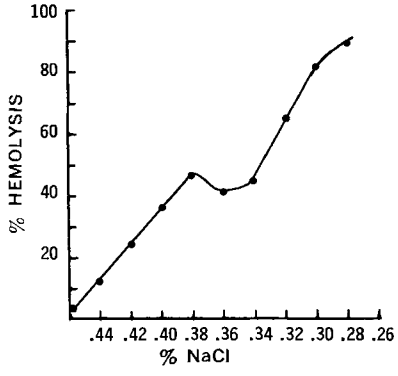

Fig. 5—Hemolysis of human erythrocytes after 45 min. at 37° in 5% polyethylene glycol 200–saline solutions.

TABLE III—VALUES OF *i* FOR PEG 400 AND 600 AT CONCENTRATIONS (Gm./100 ml.) CAUSING 25, 50, and 75% HEMOLYSIS OF RABBIT AND HUMAN ERYTHROCYTES AT 37°[a]

	Hemolysis, % 25	50	75	Av.
	Rabbit Blood			
PEG 400	0.7	0.8	1.0	0.8
PEG 600	1.2	1.4	1.6	1.4
	Human Blood			
PEG 400	0.5	0.6	0.8	0.6
PEG 600	0.9	1.2	1.6	1.2

[a] Each *i* value represents an average of two to seven blood samples.

afforded complete protection (no hemolysis) to rabbit and human erythrocytes; however, at critical concentrations (see Table I) damage of red blood cells occurred as in PEG 400 and 600 solutions void of sodium chloride. This damage was not prevented by the addition of 2, 3, or 5% sodium chloride to 40, 50, and 60% solutions of PEG 400 and 600.

The pH readings of all PEG 400 and 600 test solutions before and after the addition of blood was within a range of 3.5 to 4.5.

DISCUSSION

The concentrations of PEG 200, 300, 400, and 600 in water that are iso-osmotic with 0.9% sodium chloride, according to calculations using the osmotic factor equation (9),

$$\text{osmotic factor} = \frac{\left(\begin{matrix}\text{No. of particles}\\ \text{from 1 molecule}\\ \text{of solute}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of solute}\\ \text{in 100 ml. of}\\ \text{soln.}\end{matrix}\right)}{\text{(Gm.-mol. wt. of solute)}} \quad \text{(Eq. 4)}$$

are 5.8, 8.7, 11.6, and 17.4%, respectively. Regardless of the polyethylene glycol content in aqueous solution, PEG 200 and 300 solutions failed to prevent hemolysis of rabbit and human erythrocytes. These experimental data point out that when water–PEG 200 or water–PEG 300 are used as vehicles for intravenous solutions, the finished product should not be assumed hypertonic with respect to blood, even when there is a high concentration of polyethylene glycol present. In experimental studies, tissue reactions have been observed following parenteral doses of undiluted polyethylene glycols which are severe enough to warrant very thorough study of safety before any parenteral applications are made (10). Hemolysis has resulted from high concentrations injected into the blood stream. Viscosity and lack of diffusion have resulted in discomfort or pain after subcutaneous injections of undiluted material and ischemic necrosis has also been seen after intramuscular injections. In a study of the polyethylene glycols as vehicles for intramuscular and subcutaneous injections by Carpenter and Shaffer (11), tissue reactions at the site of subcutaneous and intramuscular injections of undiluted PEG 300 in dosages 2.5 to 10 times that anticipated for human use caused blanching of the skin and scab formation in 48 hr. In a study by McCabe *et al.* (12), it was found that daily administration of 240 mg. of nitrofurantoin in PEG 300 to 30 patients caused severe metabolic acidosis and nephropathy in seven patients resulting in two deaths. These damaging effects were attributed to polyethylene glycol rather than nitrofurantoin.

Sodium chloride is effective in preventing hemolysis of human erythrocytes in aqueous polyethylene glycol solutions as long as the polyethylene glycol concentrations do not exceed 25 to 40%. At these higher polyethylene glycol concentrations, erythrocytes are not protected from discoloration by the addition of 0.9% sodium chloride. Concentrations of polyethylene glycols causing discoloration of human and rabbit erythrocytes are summarized in Table I. Higher concentrations of sodium chloride prevented discoloration in PEG 200 and 300 solutions but not in PEG 400 and 600 solutions. This damage does not appear to be hemolytic in character, but seems to be a chemical type of destruction. It appears that pH was not a factor since the pH of all concentrations of polyethylene glycol solutions remained within a range of 3.5 to 4.5 before and after the addition of blood.

The van't Hoff factor (i value or isotonic coefficient) is defined as the ratio of the colligative effect produced by a concentration (molal) of electrolyte divided by the effect observed for the same concentration of nonelectrolyte (13). In studying the effect of low concentrations of various substances on erythrocytes, previous workers (1, 2, 8) used molar concentrations in place of molal concentrations in their calculations. The use of molar instead of molal concentration would introduce only a small error at electrolyte concentrations of 0.1 M or less (8). However, in the present study, comparatively high concentrations of liquid polyethylene glycols (4–9%) were involved in calculation of i values. This meant that enough volume of the test solution was occupied by the polyethylene glycol to produce a substantial difference between molar and molal concentrations. Molal concentrations of the test solutions used in this study were calculated by direct proportion (no shrinkage was noticed when polyethylene glycols and water were mixed) and i values calculated using molal concentrations. They were found to be 12 to 15% lower than the i values calculated on a molar basis. However, it was decided to calculate *hemolytic* i values using molar concentrations since pharmaceutical calculations in the area of isotonic solutions are based on this concentration expression.

The i values for sodium chloride in most aqueous PEG 200 solutions were less than 1.86. These low i values indicate that PEG 200 offers no protection to red blood cells against osmotic hemolysis. In fact, there probably is some deleterious effect since more sodium chloride is needed to protect the red blood cells against hemolysis in aqueous PEG 200 solutions than in water. The higher values of i for sodium chloride in aqueous PEG 300 solutions indicate that PEG 300 contributes to the tonicity of aqueous solutions. The fact that i values could be calculated for PEG 400 and 600 shows that these polyethylene glycols have the ability to protect erythrocytes against osmotic hemolysis. Since i values for PEG 400 were less than i values for PEG 600, it can be assumed that PEG 600 gives greater protection to red blood cells than PEG 400. Therefore, the order in which the liquid polyethylene glycols protect rabbit and human erythrocytes against hemolysis is $200 < 300 < 400 < 600$. The ability of the liquid polyethylene glycols to contribute to the tonicity of aqueous solutions is dependent on the molecular weight. A possible explanation for this behavior might be that the lower molecular weight polyethylene glycols are able to penetrate the red blood cell membrane and therefore have little or no effective concentration in the extracellular solutions. It appears that increasing the molecular weight decreases the membrane penetrating properties of liquid polyethylene glycols.

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