Our findings with berberine on the gastrointestinal, cardiovascular, and respiratory systems are in agreement with those of other workers (6).

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Behavior of Erythrocytes in Various Solvent Systems I

Water-Glycerin and Water-Propylene Glycol

By DONALD E. CADWALLADER

The hemolytic behavior of rabbit and human erythrocytes in water–glycerin and water–propylene glycol solutions was investigated. Complete hemolysis of erythrocytes took place in all propylene glycol solutions and most glycerin solutions. Aqueous solutions containing 50, 60, and 70 per cent glycerin prevented complete hemolysis of rabbit erythrocytes but not human erythrocytes. The addition of sodium chloride to various glycerin solutions prevented hemolysis. The addition of sodium chloride to propylene glycol solutions prevented hemolysis of rabbit erythrocytes in 5–30 per cent solutions and of human erythrocytes in 5–40 per cent solutions. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in various water-glycerin and water–propylene glycol solutions. Unusual behavior was displayed by erythrocytes in 40–50 per cent propylene glycol solutions. The addition of sodium chloride to solutions containing 50 per cent or more of propylene glycol did not prevent complete laking of red blood cells.

SINCE THE DEVELOPMENT of the hemolytic method by Husa and co-workers (1, 2), many investigations have been carried out (3–12) to study the behavior of erythrocytes to various compounds. In the aforementioned investigations, water was used as the solvent for all of the substances studied. Water, however, is not the only solvent used for intravenous preparations. To prepare a safe, stable, and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous co-solvent. Two nonaqueous solvents that are used in the formulation of parenteral preparations are glycerin and propylene glycol.

Husa and Adams (1) showed that glycerin and propylene glycol did not prevent hemolysis at concentrations which were calculated to be isotonic according to physicochemical data. They also reported that 0.3 to 0.5% sodium chloride would prevent hemolysis when added to hypo-osmotic concentrations of the polyhydric alcohols in water. Hammarlund and Pedersen-Bjergaard (13) demonstrated that complete hemolysis of blood takes place in iso-osmotic concentrations of glycerin and propylene glycol.

Hemolytic studies with glycerin and propylene glycol solutions were carried out by Zanowiak and Husa (8). They found that complete hemolysis took place in each 10% polyhydric alcohol–water solution, even though this concentration was well above the iso-osmotic concentration of each substance. They also reported that the addition of 0.2% sodium chloride did not prevent hemolysis of blood in the 10% glycerin or propylene glycol solutions. The presence of 0.6% sodium chloride, however, in these 10% solutions did prevent hemolysis. The purpose of this investigation was to conduct experiments to study further the behavior of red blood cells in aqueous glycerin and propylene glycol solutions. The hemolytic method was employed, and the experiments were designed so that standard hemolysis curves of human and rabbit blood obtained from experiments using sodium chloride–water solutions could be compared to hemolysis curves obtained from experiments using sodium chloride–water–polyhydric alcohol solutions. From these

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data it was possible to calculate *hemolytic* isotonic coefficients for sodium chloride in various water–polyhydric alcohol solutions (*e.g.*, *i* values for NaCl in 5, 10, 20% propylene glycol).

EXPERIMENTAL

Materials.—Propylene glycol U.S.P. and reagent grade glycerin and sodium chloride were used.

Preparation of Solutions.—All of the polyhydric alcohol solutions were weight-in-volume percentage preparations. The sodium chloride solutions were prepared in the same manner described by other workers (1–10).

Collection of Blood.—The blood was collected and defibrinated in the same manner reported by Husa and co-workers (1–10). The human blood used in these experiments was obtained from the forearm veins of a 31-year-old white male.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis of erythrocytes in the various solutions involved in this investigation was essentially that described by Grosicki and Husa (2). The laking of blood in distilled water was used as the standard for 100% hemolysis. Because of the viscosity of the polyhydric alcohol 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.

Water-glycerin and water–propylene glycol solutions absorbed a small amount of light, and this absorbance increased with an increase in polyhydric alcohol content. This absorbance was determined for the various concentrations of polyhydric alcohols used in experiments, and these blank readings were subtracted from the Klett-Summerson colorimeter readings obtained at the end of hemolysis experiments.

After several experiments, complete hemolysis of blood in polyhydric alcohol solutions gave absorbance readings indicating greater than 100% hemolysis. These higher readings appeared to be because of the darkening of the color of the oxyhemoglobin solutions. Experiments were carried out to determine to what extent this darkening took place in various glycerin and propylene glycol solutions. A solution representing 100% hemolysis was prepared by adding 1 ml. of blood to 100 ml. of distilled water. The resulting oxyhemoglobin solution was added in like amounts to each of two colorimeter tubes, one containing a measured amount of distilled water and the other containing a like amount of water–polyhydric alcohol solution. The colorimeter readings of these solutions were compared. After correcting for the absorbance of the polyhydric alcohol, per cent hemolysis readings were 2–3% higher for the water–polyhydric alcohol–blood solutions than water–blood solutions. This increase in hemolysis readings was attributed to a darkening of the red color by the polyhydric alcohols. The necessary corrections were made by subtracting this excess colorimetric reading, expressed as per cent, from the per cent hemolysis readings obtained for various glycerin and propylene glycol solutions.

Calculation of *i* Values.—When the concentrations of sodium chloride and any other compound causing the same degree of hemolysis are known, the value of *i* (isotonic coefficient) for the other compound can be calculated according to the osmotic equation used by Grosicki and Husa (2)

$$\frac{\left(\begin{matrix}i\text{ value}\\ \text{for NaCl}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of NaCl}\\ \text{in 100 ml. of}\\ \text{solution}\end{matrix}\right)}{(\text{Gm.–molecular wt. of NaCl})} = \frac{\left(\begin{matrix}i\text{ value}\\ \text{for other}\\ \text{compound}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of other}\\ \text{compound in 100}\\ \text{ml. of solution}\end{matrix}\right)}{\left(\begin{matrix}\text{Gm.—molecular wt. of other}\\ \text{compound}\end{matrix}\right)} \quad \text{(Eq. 1)}$$

The objective of this paper, however, was to calculate *apparent* *i* values for sodium chloride when the salt was present in a water–polyhydric alcohol system, *e.g.*, sodium chloride in 10% glycerin. In these calculations, it was necessary to assume that glycerin and propylene glycol did not contribute to the osmotic behavior of the solutions and that the sodium chloride was solely responsible for the

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

Glycerin, % w/v	Hemolysis, % 25	50	75	Av.
Rabbit Blood				
5	1.94	2.00	2.08	2.00
10	2.02	2.13	2.20	2.17
20	2.24	2.52	3.04	2.60
30	2.30	2.62	3.00	2.64
40	2.53	3.02	3.44	3.00
50	2.70	3.20	3.80	3.23
60	4.21	7.12	22.7	...
70	2.7	3.8	25.0	...
Human Blood				
5	1.86	1.90	1.91	1.89
10	1.88	1.92	1.95	1.92
20	1.90	1.95	1.99	1.95
30	1.96	2.02	2.07	2.02
40	2.03	2.09	2.18	2.10
50	2.08	2.16	2.25	2.16
60	2.20	2.58	3.13	2.64
70	2.28	2.87	3.81	2.99

[a] All *i* values represent an average of two blood samples.

TABLE II.—VALUES OF *i* FOR SODIUM CHLORIDE IN VARIOUS WATER-PROPYLENE GLYCOL SOLUTIONS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT AND HUMAN ERYTHROCYTES[a]

Propylene Glycol, % w/v	Hemolysis, % 25	50	75	Av.
Rabbit Blood				
5	1.88	1.89	1.88	1.88
10	1.91	1.90	1.90	1.90
20	1.96	1.98	1.97	1.97
30	1.95	2.02	2.04	2.00
Human Blood				
5	1.92	1.97	2.00	1.96
10	1.91	1.94	1.95	1.93
20	1.98	1.98	1.97	1.98
30	2.05	2.09	2.05	2.06
40	1.94	1.98	1.97	1.96

[a] All *i* values represent an average of two blood samples.

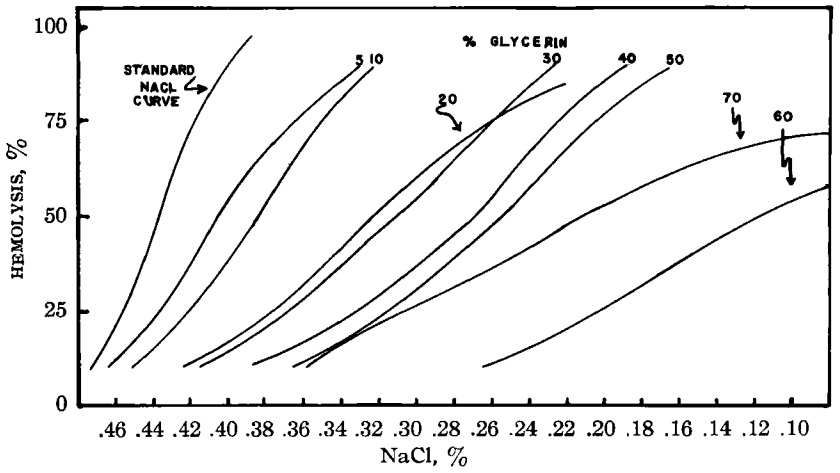


Fig. 1.—Per cent hemolysis of rabbit erythrocytes after 45 minutes at 25° in various glycerin – saline solutions.

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

$$\begin{pmatrix} i \text{ value for} \\ \text{NaCl in water} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl} \\ \text{in 100 ml.} \\ \text{water} \end{pmatrix} = \begin{pmatrix} i \text{ value for} \\ \text{NaCl in other} \\ \text{solution} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl} \\ \text{in 100 ml.} \\ \text{other solution} \end{pmatrix} \quad \text{(Eq. 2)}$$

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 (2).

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–polyhydric alcohol 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. 2, and the values of i for sodium chloride in a particular water–polyhydric alcohol solution, at concentrations giving 25, 50, and 75% hemolysis were determined. The various i values for sodium chloride in aqueous glycerin and propylene glycol solutions are shown in Tables I and II.

Preparation of Hemolysis Curves.—Fourteen experiments employing rabbit blood were carried out. The average readings of these experiments were used to construct a standard hemolysis curve (left hand side of Figs. 1 and 3). In constructing the hemolysis curves of the various polyhydric alcohol solutions, the grams of NaCl 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 Gm. of NaCl per 100 ml. in polyhydric alcohol solution causing 25% hemolysis was calculated as

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

where X = Gm. of NaCl in 100 ml. polyhydric alcohol solution causing 25% hemolysis, A = 1.86 as the i value for NaCl in water, B = Gm. of NaCl in 100 ml. of water causing 25% hemolysis (obtained from standard hemolysis curves in Figs. 1 and 3), and C = i value for NaCl in appropriate polyhydric alcohol solution (obtained from Tables I and II). 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 glycerin and propylene glycol solutions were plotted. In this manner, Figs. 1 and 3 are a compilation of the experiments run with rabbit blood, and all the curves have in common a standard hemolysis curve.

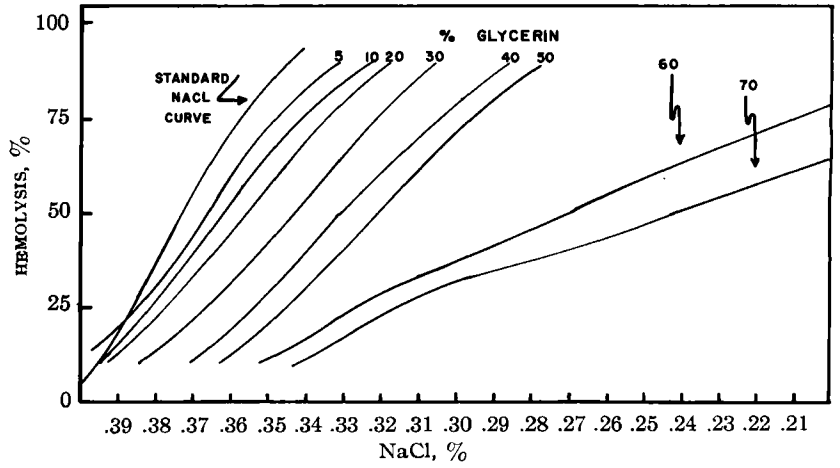


Fig. 2.—Per cent hemolysis of human erythrocytes after 45 minutes at 25° in various glycerin – saline solutions.

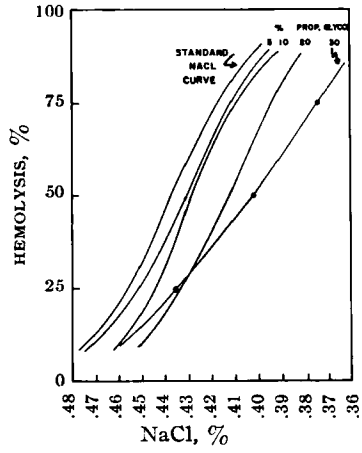


Fig. 3.—Per cent hemolysis of rabbit erythrocytes after 45 minutes at 25° in various propylene glycol–saline solutions.

Twelve similar 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 Figs. 2 and 4). The hemolysis curves for human blood in various polyhydric alcohol solutions were constructed in the manner previously described for the rabbit blood hemolysis curves. Figures 2 and 4 are a compilation of the experiments run with human blood.

RESULTS

Water-Glycerin Solutions.—The hemolysis of rabbit erythrocytes after 45 minutes at 25° in various water-glycerin solutions is shown in Fig. 5. All of the glycerin solutions void of sodium chloride caused complete hemolysis of rabbit erythrocytes except the 50, 60, and 70% solutions in which a small decrease in hemolysis was noticed.

Complete hemolysis of human erythrocytes took place in aqueous solutions containing 0.0 to 100% glycerin.

The fragility of rabbit and human erythrocytes in various water-glycerin solutions was modified or corrected by the addition of sodium chloride. It was possible to calculate *i* values for sodium chloride in various water-glycerin solutions. The average *i* values for sodium chloride in various glycerin solutions are given in Table I. In all cases, the *i* values for sodium chloride in water-glycerin solutions were greater than 1.86, the accepted value for 0.9% sodium chloride in water. This increase in *i* values can probably be attributed to some effect of glycerin on erythrocytes rather than increased activity of sodium chloride in the binary solvent. The higher *i* values for sodium chloride for rabbit blood than for human blood indicate that glycerin renders rabbit erythrocytes more resistant toward osmotic hemolysis. The sodium chloride *i* values increased with an increase in glycerin concentration which meant that less sodium chloride needed to be added to a more concentrated glycerin solution in order to make the solution isotonic to blood. The only exception to this was the higher *i* values for sodium chloride in 60% glycerin than in 70% glycerin solution when rabbit blood was employed. This can be accounted for by the fact that the least amount of hemolysis in water-glycerin solutions *sans* sodium chloride took place in 60% glycerin solution as shown in Fig. 5.

Values of *i* for sodium chloride in water-glycerin solutions containing more than 70% glycerin were not calculated because of inadequate data. Rabbit or human blood was placed in 80 and 90% glycerin solutions containing 0.2 to 0.4% sodium chloride. The opacity of these mixtures indicated that sodium chloride prevented complete hemolysis of rabbit and human erythrocytes in 80–90% glycerin solutions. The high viscosity of these solutions made it difficult to spin down the unhemolyzed cells which

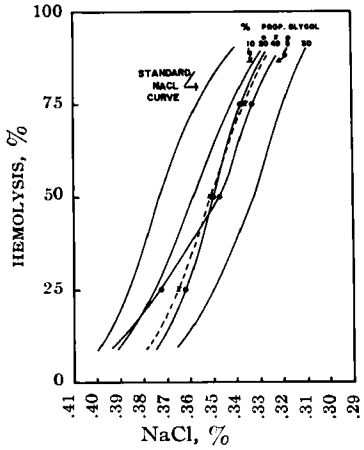
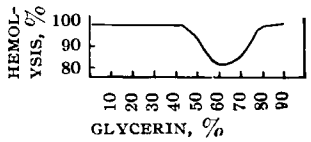


Fig. 4.—Per cent hemolysis of human erythrocytes after 45 minutes at 25° in various propylene glycol–saline solutions.

Fig. 5.—Per cent hemolysis of rabbit erythrocytes after 45 minutes at 25° in various glycerin solutions.



resulted in inaccurate colorimeter readings. After prolonged high speed centrifugation, these solutions retained a slight opaqueness.

Hemolysis curves showing the amount of laking that occurred when rabbit and human blood were added to various water–glycerin–sodium chloride solutions are shown in Figs. 1 and 2. These curves were constructed in the manner described in the *Experimental* section of this report utilizing the data presented in Table I. The addition of 0.48 to 0.28% sodium chloride to various water-glycerin solutions prevented osmotic hemolysis of rabbit erythrocytes. Hemolysis of human erythrocytes was prevented in water-glycerin solutions by the addition of 0.42 to 0.35% sodium chloride.

The average *i* values for human blood (Table I) were used to calculate the amount of sodium chloride that should be added to various water-glycerin solutions to make them isotonically equivalent to 0.9% sodium chloride solution. It was found that between 0.89 and 0.80% sodium chloride should be added to solutions containing 5–40% glycerin. The 50, 60, and 70% glycerin solutions would be made equivalent to isotonic saline solution by the addition

of 0.75, 0.63, and 0.55% sodium chloride, respectively. These data are shown in Fig. 6.

Water–Propylene Glycol Solutions.—All aqueous propylene glycol solutions ranging in concentration from 0.0 to 100% caused complete hemolysis of rabbit and human erythrocytes.

The addition of sodium chloride to water-propylene glycol solutions prevented hemolysis of rabbit erythrocytes in those solutions containing up to 30% propylene glycol. The *i* values for sodium chloride in these solutions were determined; the average values are given in Table II. The *i* values were slightly greater than 1.86. The hemolysis curves plotted from the data obtained after the addition of rabbit blood to various water–propylene glycol–sodium chloride solutions are shown in Fig. 3. Hemolysis of rabbit erythrocytes was prevented by the addition of 0.49 to 0.46% sodium chloride to 5–30% propylene glycol solutions. Peculiar hemolytic behavior of rabbit erythrocytes was noticed in 40% propylene glycol solutions. Complete protection of rabbit erythrocytes could not be achieved by the addition of sodium chloride up to concentrations of 6.0%. As the sodium chloride content increased from 0.4 to 1.0%, there was a decrease in hemolysis to a minimum of 15%; higher concentrations of sodium chloride caused an increase in hemolysis. This unusual behavior of rabbit erythrocytes in 40% propylene glycol is shown in Fig. 7. The complete hemolysis of rabbit erythrocytes in aqueous solutions containing 45% and more propylene glycol could not be prevented by the addition of up to 10.0% sodium chloride.

Hemolysis of human erythrocytes was prevented by the addition of 0.41 to 0.37% sodium chloride to 5–40% propylene glycol solutions. The *i* values for sodium chloride in these solutions were determined; the average values are given in Table II. The hemolysis curves prepared from the data obtained after the addition of human blood to various water–propylene glycol–sodium chloride solutions are illustrated in Fig. 4. Interesting behavior of human erythrocytes occurred in 45% propylene glycol solutions. There was a decrease in hemolysis in solutions containing 0.40 to 0.75% sodium chloride. Higher concentrations of sodium chloride resulted in increases in hemolysis. Complete hemolysis of human erythrocytes took place in 45% propylene glycol solutions containing 3.0% and more sodium chloride. This behavior is depicted in Fig. 8. The addition of various amounts of sodium chloride (up to 10.0%) did not prevent complete hemolysis of human erythrocytes in aqueous solutions containing 50% or more propylene glycol.

The average *i* values for human blood (Table II) were used to calculate the amount of sodium chloride that should be added to various water–propylene glycol solutions to make these solutions isotonically equivalent to 0.9% sodium chloride solution. Between 0.87 to 0.80% sodium chloride should be added to aqueous solutions containing 5–40% propylene glycol.

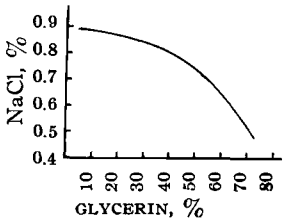


Fig. 6.—Amount of sodium chloride required to render various glycerin solutions isotonic with human blood.

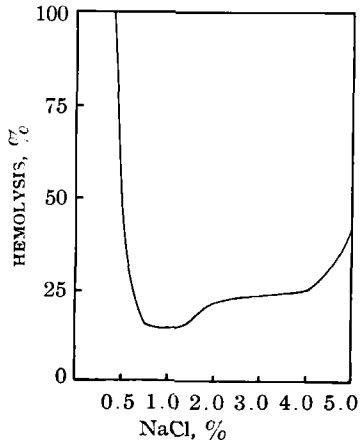


Fig. 7.—Per cent hemolysis of rabbit erythrocytes after 45 minutes at 25° in various saline solutions containing 40% propylene glycol.

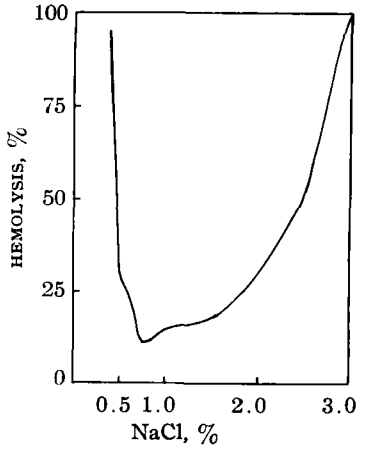


Fig. 8.—Per cent hemolysis of human erythrocytes after 45 minutes at 25° in various saline solutions containing 45% propylene glycol.

DISCUSSION

The concentrations of glycerin and propylene glycol in water that are iso-osmotic to 0.15 *M* sodium chloride and human blood, according to osmotic calculations, are 2.6 and 2.0%, respectively. The polyhydric alcohol content of aqueous solutions studied in this paper exceeded those concentrations having the same osmotic pressure as rabbit and human blood. Previous workers (1, 13) have shown that aqueous glycerin and propylene glycol solutions having concentrations that are hypo- and iso-osmotic to 0.15 *M* sodium chloride cause complete hemolysis of rabbit and human erythrocytes. Regardless of the polyhydric alcohol content, water-glycerin and water–propylene glycol solutions failed to prevent hemolysis of rabbit and human erythrocytes. These experimental data point out that when water-glycerin or water–propylene glycol are used as vehicles for intravenous solutions, the finished

product should not be assumed hypertonic with respect to blood. All aqueous glycerin and propylene glycol solutions studied in this investigation were hypotonic with respect to rabbit and human erythrocyte membranes.

Sodium chloride is effective in preventing hemolysis of rabbit and human erythrocytes in glycerin and propylene glycol solutions when the alcohols were in hypo-osmotic concentrations (1). Zanowiak and Husa (8) reported that 0.6% sodium chloride present in 10% glycerin and propylene glycol solutions prevented hemolysis of rabbit and human erythrocytes. The present investigation shows the presence of sodium chloride will prevent hemolysis of rabbit and human erythrocytes in aqueous solutions containing 5–90% glycerin. Sodium chloride prevented hemolysis of rabbit and human erythrocytes in aqueous propylene glycol solutions as long as the glycol concentration did not exceed 40–45%. Water–propylene glycol solutions containing more than 40–45% propylene glycol could not be made isotonic to rabbit and human erythrocyte membranes by the addition of sodium chloride. These data demonstrate that propylene glycol has greater hemolytic activity than glycerin. Jacobs, *et al.* (14), reported that each successive hydroxyl group added to the propane molecule decreased the rate of penetration of the alcohol into ox and rabbit erythrocytes.

Brittain and D'Arcy (15) have reported on the *in vivo* hematological effects in the rabbit following the intravenous injection of aqueous solutions containing different concentrations of propylene glycol in normal saline. Although the fragility of the red blood cells was not affected by different concentrations of propylene glycol, there was a marked decrease in blood clotting time with a corresponding increase in platelet count after the injection of 50% propylene glycol. The effect of 25% propylene glycol was considerably less. In this investigation the addition of sodium chloride to 30–40% propylene glycol solutions prevented hemolysis of rabbit and human erythrocytes, while the addition of sodium chloride to 50% propylene glycol solutions did not prevent complete hemolysis of these erythrocytes.

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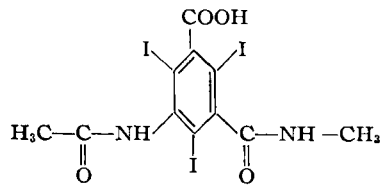
Drug Standards

Qualitative and Quantitative Tests for Iothalamic Acid

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drugs concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

5 - Acetamido - 2,4,6 - triiodo - *N* - methylisophthalamic acid, $C_{11}H_9I_3N_2O_4$; M.W. 613.92; 62.01% iodine. The structural formula of iothalamic acid may be represented as follows:



Received August 27, 1963, from the Drug Standards Laboratory, American Pharmaceutical Association Foundation, Washington, D. C. 20037.
Accepted for publication October 2, 1963.
This monograph was developed by Robert R. Stark. Mallinckrodt Chemical Works has cooperated by furnishing samples and data to aid in its development and preparation.

Physical Properties.—Iothalamic acid occurs as a white, odorless, bulky crystalline powder. In a