Behavior of Erythrocytes in Various Solvent Systems VII: Water–Monohydric Alcohols

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Abstract □ The hemolytic behavior of human erythrocytes in water–monohydric alcohol solutions was investigated. Complete hemolysis of erythrocytes occurred in all methanol, ethanol, *n*-propanol, and isopropanol solutions. Sodium chloride was effective in preventing complete hemolysis in solutions containing up to 18% methanol, 11% ethanol, 4% *n*-propanol, and 8% isopropanol. The addition of sodium chloride to solutions containing more than these concentrations of alcohol did not prevent hemolysis, discoloration, and/or precipitation of human red blood cells. The addition of 2% sodium chloride did not appreciably influence the hemolytic action of methanol, ethanol, *n*-propanol, or isopropanol, while 5% sodium chloride lowered the alcohol concentrations needed to cause 100% hemolysis. The data were used to calculate Van't Hoff *i* values for sodium chloride in the various alcohol solutions. Studies in aqueous ternary systems containing two alcohols (in 1:1 proportion) indicated an additive effect for the hemolytic action of alcohols.

Keyphrases □ Erythrocytes, human—hemolysis in water–monohydric alcohol solutions □ Hemolysis of human erythrocytes—water–monohydric alcohol solutions □ Alcohol (monohydric)–water solutions—effect on hemolysis of human erythrocytes

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 solutions of dimethyl sulfoxide, liquid amides, and tetramethylurea on human erythrocytes (1–6).

This report is concerned with the investigation of water–monohydric alcohol systems. The alcohols studied were methanol, ethanol, *n*-propanol, and isopropanol. Next to water, the monohydric alcohols are probably the most widely used pharmaceutical solvents. One extensive article (7) on nonaqueous solvents for use in parenteral products surveyed the physical properties, toxicities, and parenteral application of ethanol. It was reported that this solvent had occasional use in parenteral products, particularly the digitalis glycosides. USP XVIII (8) states that a digoxin preparation containing 9–11% alcohol may be used for intramuscular or intravenous administration.

The purpose of this investigation was to observe the behavior of human erythrocytes in various water–monohydric alcohol solutions utilizing the hemolytic method. By comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium chloride–water–monohydric alcohol solution, it was possible to calculate the hemolytic isotonic coefficient for sodium chloride in various water–alcohol solutions.

EXPERIMENTAL

Materials—Ethanol, absolute[1], and reagent grades of methanol[2], *n*-propanol[3], isopropanol[2], and sodium chloride[2] were used.

Preparation of Solutions—All alcohol solutions were volume-in-volume percentage preparations. Sodium chloride solutions were prepared on a weight-in-volume basis. Necessary dilutions were made from stock solutions, and distilled water was used to prepare all solutions.

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of several 20–25-year-old Caucasian and Oriental subjects. Fresh blood samples were used in all experiments. Approximately 10 ml of blood was obtained from the donors and placed in a 50-ml round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min, and then the blood was decanted into a 50-ml conical flask and aerated by swirling the flask gently for about 5 min.

Quantitative Determination of Percent Hemolysis—In each experiment, the hemolytic method was used to determine the degree of hemolysis of erythrocytes in the various alcohol 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 (0.32, 0.34, . . ., 0.46, 0.48%) or 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 ± 0.5°), 0.05 ml of blood was pipeted into each tube. Each tube was shaken on a vibratory mixer for approximately 5 sec to ensure thorough mixing and then was allowed to stand 45 min at 37°. The tubes were centrifuged at approximately 2500 rpm, and the light absorbance of the supernatant liquid was measured using a photoelectric colorimeter[4] 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% NaCl solution, was used to cancel any light absorbance inherent to the blood sample. Both the standard and the blank were subjected to the same conditions of standing for 45 min at 37° followed by centrifuging.

Calculations of *i* Values—Through use of the hemolytic method, concentrations of sodium chloride and the alcohol 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:

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

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.9% NaCl in water (9).

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–alcohol 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 alcohol solvent causing 25,

[1] U.S. Industrial Chemical Co.
[2] J. T. Baker Chemical Co.
[3] Mallinckrodt Chemical Works.
[4] Klett-Summerson.

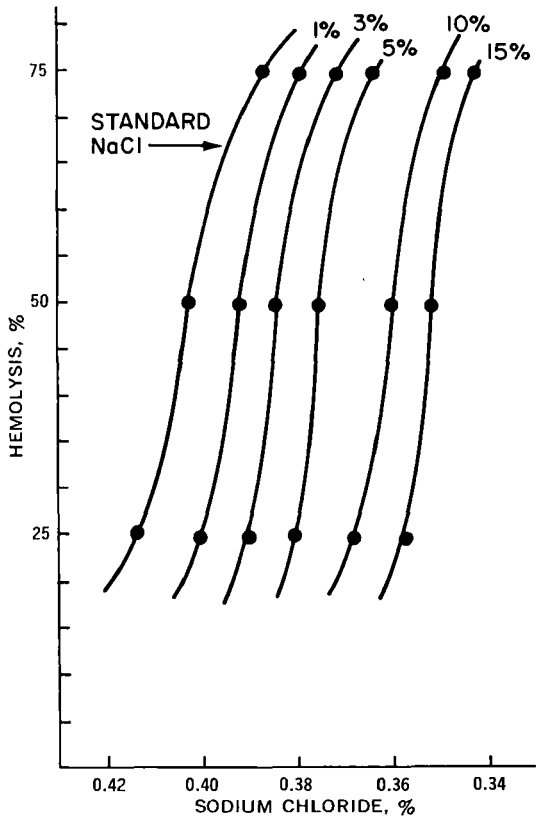

Figure 1—*Hemolysis of human erythrocytes after 45 min at 37° in various methanol solutions.*

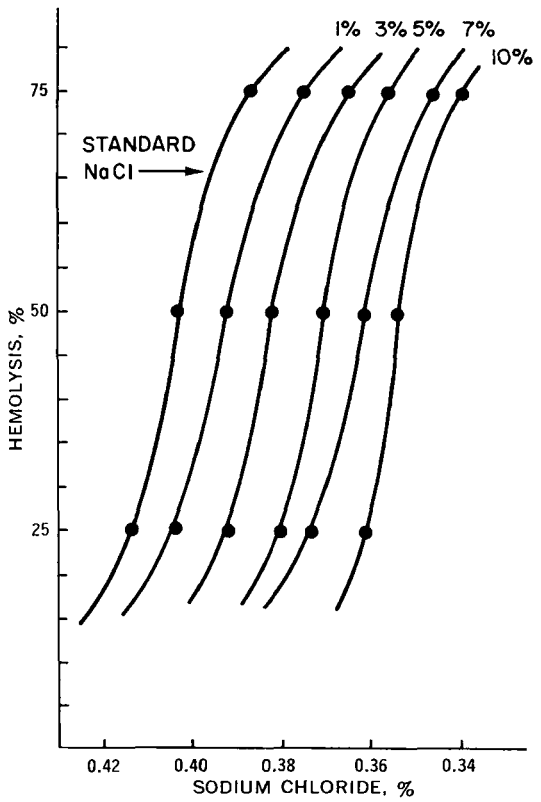

Figure 2—*Hemolysis of human erythrocytes after 45 min at 37° in various ethanol solutions.*

50, and 75% hemolysis. These values were inserted into Eq. 1, thereby giving the values of i for sodium chloride in a particular water-alcohol solution at concentrations producing 25, 50, and 75% hemolysis. The various i values for sodium chloride in aqueous methanol, ethanol, *n*-propanol, and isopropanol solutions are shown in Table I.

Preparation of Hemolysis Curves—Approximately 17 experiments employing human blood were carried out. A standard hemolysis curve (left-hand side of Figs. 1–4) was constructed from the average readings of these experiments. Hemolysis curves of the various alcohol solutions (Figs. 1–4) were constructed using the i values previously calculated from Eq. 1 and shown in Table I. Through a rearrangement of Eq. 1, the grams of sodium chloride/100 ml in an alcohol solution causing 25% hemolysis were calculated using Eq. 2:

$$\begin{pmatrix}\text{grams of NaCl in}\\ \text{100 ml of}\\ \text{alcohol solution}\\ \text{causing 25%}\\ \text{hemolysis}\end{pmatrix} = \frac{\begin{pmatrix}\text{1.86-}i\text{ value for}\\ \text{NaCl in water}\end{pmatrix}\begin{pmatrix}\text{grams of NaCl in 100 ml of water}\\ \text{causing 25\% hemolysis}\end{pmatrix}}{i\text{ value for NaCl in alcohol solution}} \quad \text{(Eq. 2)}$$

Similar calculations were carried out at 50 and 75% hemolysis. By plotting these three points, the hemolysis curves for the various alcohol solutions were constructed (Figs. 1–4).

RESULTS

Water–Monohydric Alcohol Solutions—Quantitative hemolytic determinations were carried out with methanol, ethanol, *n*-propanol, and isopropanol employing human blood. Complete hemolysis and/or denaturation of human erythrocytes occurred in all aqueous alcohol test solutions ranging in concentration from 0.0 to 100% after 45 min at 37°. Normal red solutions were evident up to concentrations of 35% methanol, 25% ethanol, 12% *n*-propanol, and 17% isopropanol; however, red-brown discolorations were observed in solutions containing 36–40% methanol, 26–28% ethanol, 13–14% *n*-propanol, and 18–20% isopropanol. Very heavy pinkish-red to pinkish-brown turbidity and/or precipitates occurred when blood was placed in solutions containing 50, 60, and 70% methanol; 30, 40, and 50% ethanol; 15–30% *n*-propanol; and 25–40% isopropanol. In solutions containing 80% or more metha-

Table I—Values of i for Sodium Chloride in Various Water–Alcohol Solutions, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes at 37° [a]

Percent (v/v) Alcohol	Percent Hemolysis			Average i
	25	50	75	
Methanol				
1	1.92	1.91	1.90	1.91
3	1.97	1.95	1.94	1.95
5	2.02	2.00	1.98	2.00
10	2.09	2.08	2.06	2.08
15	2.15	2.13	2.10	2.13
Ethanol				
1	1.90	1.91	1.92	1.91
3	1.96	1.96	1.97	1.96
5	2.02	2.02	2.02	2.02
7	2.06	2.07	2.08	2.07
10	2.13	2.12	2.12	2.12
n-Propanol				
1	1.91	1.92	1.89	1.91
3	1.97	1.99	1.95	1.97
Isopropanol				
1	1.92	1.91	1.91	1.91
3	1.95	1.97	1.95	1.96
5	2.01	2.01	1.99	2.00
7	2.02	2.01	2.00	2.01

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

Table II—Critical Concentrations (Percent v/v) of Aqueous Alcohol Solutions at which Hemolysis, Complete Hemolysis, Discoloration, and/or Precipitation of Blood Components Occurred and the Effect of the Addition of Sodium Chloride to These Solutions[a]

Alcohol	Maximum Concentration Allowing No Hemolysis[b], %	Complete Hemolysis[c], %			Discoloration[d] and/or Precipitation[e], %			
	0.9% NaCl	0.9% NaCl	2% NaCl	5% NaCl	No NaCl	0.9% NaCl	2% NaCl	5% NaCl
Methanol	18	22	22.5	20	40	35	35	30
Ethanol	11	14	14	12	29	25	25	18
n-Propanol	4	6	6	5	13.5	12	10	8
Isopropanol	8	10	10	8	19	15	15	12

[a] Each value represents an average of at least two blood samples. [b] These alcohol concentrations represent the critical concentrations above which hemolysis occurred (greater than 5%) and below which there was complete protection of human red cells. [c] Essentially 100% hemolysis as indicated by hemolysis readings of 95–105% of 100% hemolysis standard. [d] Discoloration occurred which resulted in brownish-red color. [e] Turbidity occurred which resulted in brick-red precipitate after centrifuging.

nol, 60% or more ethanol, 35% or more *n*-propanol, and 45% or more isopropanol, hemagglutination occurred immediately, which resulted in a very deep red-brown clumped precipitate and a very pale greenish-yellow supernate.

Aqueous Monohydric Alcohol Solutions Containing 0.9% NaCl—Upon addition of 0.9% NaCl, hemolysis of human erythrocytes was essentially prevented (less than 5%) in solutions containing 0.0–18% methanol, 0.0–11% ethanol, 0.0–4% *n*-propanol, and 0.0–8% isopropanol. Hemolysis occurred in solutions containing 19–22% methanol, 12–14% ethanol, 5–6% *n*-propanol, and 9–10% isopropanol (Fig. 5). Red-brown solutions resulted when blood was added to saline solutions containing 35% methanol, 25% ethanol, 12% *n*-propanol, and 15% isopropanol. More pronounced discoloration and precipitation occurred in solutions containing higher concentrations of alcohols.

Values of *i* for Sodium Chloride—Through the addition of hypotonic quantities of sodium chloride (0.32, 0.34, ..., 0.46, 0.48%) to various water-alcohol solutions, it was possible to modify the fragility of human erythrocytes. When blood was added to the saline solutions containing 0.0–15% methanol, 0.0–10% ethanol, 0.0–3% *n*-propanol, and 0.0–7% isopropanol, typical sigmoid hemolysis curves resulted (*viz.*, Figs. 1–4). These curves were constructed in the manner described in the *Experimental* section. The *i* values for sodium chloride in various water-alcohol solutions were calculated using Eq. 1 and are presented in Table I.

Aqueous Monohydric Alcohol Solutions Containing 2 or 5% NaCl—The addition of 2% NaCl did not appreciably influence the hemolytic action of methanol, ethanol, *n*-propanol, or isopropanol, while 5% NaCl lowered the alcohol concentrations needed to cause 100% hemolysis (Table II).

Alcohol-Alcohol (1:1) Combinations—Quantitative hemolytic determinations were also carried out in ternary systems containing two alcohols (in 1:1 proportion) in water containing 0.9% NaCl (Table III).

DISCUSSION

The concentrations of methanol, ethanol, *n*-propanol, and isopropanol in water that are isoosmotic to 0.15 *M* NaCl and human blood, according to osmotic calculations (10)[5], are approximately 0.93, 1.33, 1.74, and 1.74%, respectively. Regardless of the alcohol content in aqueous solution, all monohydric alcohol solutions studied failed to prevent hemolysis of human erythrocytes. These experimental data point out that when water-alcohol systems are used as vehicles for intravenous solutions, the finished product should not be assumed isotonic or hypertonic with respect to blood, even when there is a high concentration of alcohol present. All alcohol solutions investigated freely penetrated human red blood cells and caused complete hemolysis and/or denaturation of human erythrocytes in solutions void of sodium chloride after 45 min at 37°.

$$[5]\text{osmotic factor} = \frac{(\text{number of particles from one molecule of solute})\ (\text{grams of solute in 100 ml of solution})}{(\text{grams} - \text{molecular weight of solute})}$$

Zanowiak and Husa (11) reported that complete hemolysis of human blood cells occurred in 7% *n*-propanol and in 10% methanol, ethanol, or isopropanol solutions even in the presence of 0.2% (0.03 *M*) NaCl; addition of 0.6% (0.1 *M*) NaCl offered complete protection to human erythrocytes from hemolysis in 10% methanol and allowed approximately 70% hemolysis in 10% ethanol and 100% hemolysis in 7% *n*-propanol. In their investigations, only the highest practical concentrations of certain monohydric alcohols were employed.

In this report, the critical concentration for an individual solvent is defined as that concentration above which hemolysis (more than 5%) is not prevented by the addition of 0.9% NaCl and below which there is complete protection of human red cells. The present investigations showed that the inclusion of 0.9% (0.15 *M*) NaCl was effective in preventing hemolysis of human erythrocytes in aqueous alcohol solutions containing up to 18% methanol, 11% ethanol, 4% *n*-propanol, and 8% isopropanol. These concen-

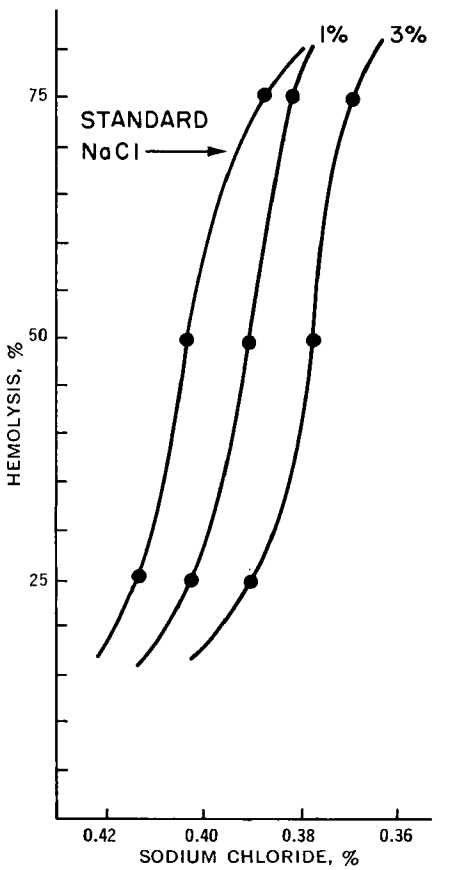

Figure 3—*Hemolysis of human erythrocytes after 45 min at 37° in various* n-*propanol solutions.*

Table III—Critical Concentrations (Percent v/v) of Aqueous Alcohol–Alcohol (1:1) Solutions Containing 0.9% Sodium Chloride Affecting Human Erythrocytes[a]

Solvent System	Critical Concentrations[b], %		Concentration Range Causing 5–100% Hemolysis, %
	Theoretical[c]	Experimental	
Methanol–ethanol (1:1)	14.5	15	16–20
Methanol–*n*-propanol (1:1)	11	7	8–12
Methanol–isopropanol (1:1)	13	12	13–16
Ethanol–*n*-propanol (1:1)	7.5	7	8–10
Ethanol–isopropanol (1:1)	9.5	10	11–13
n-Propanol–isopropanol (1:1)	6	6	7–10

[a] Each value is an average of at least two blood samples. [b] These total alcohol concentrations represent the critical concentrations above which hemolysis occurred (greater than 5%) and below which there was complete protection of human red cells. [c] These concentrations obtained by taking one-half the summation of the critical concentrations (see Table II) of the two alcohols in a particular ternary system. For example, the critical concentrations of ethanol and methanol are 11 and 18%, respectively. The theoretical critical concentration would be 29%/2 or 14.5%.

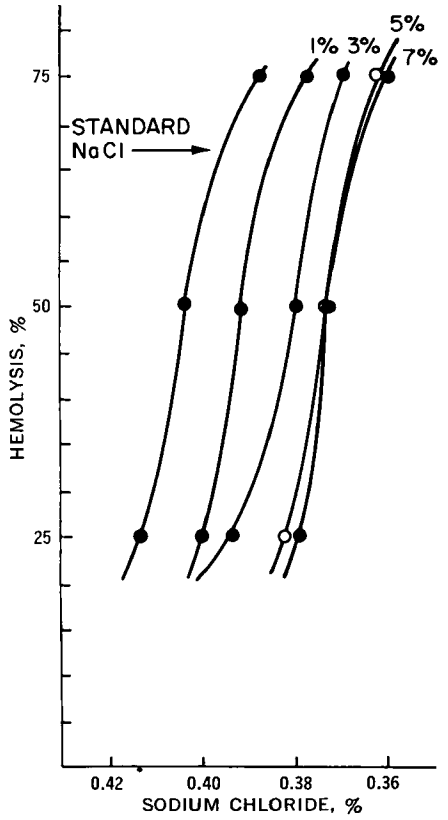

Figure 4—*Hemolysis of human erythrocytes after 45 min at 37° in various isopropanol solutions.*

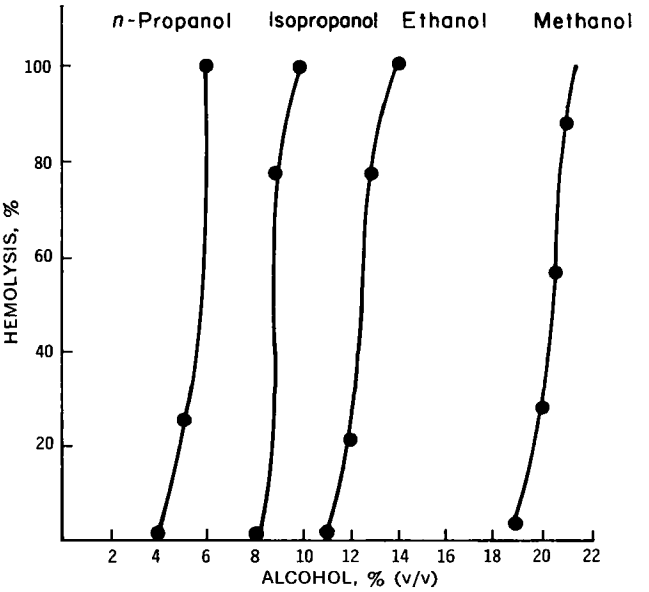

Figure 5—*Hemolysis of human erythrocytes after 45 min at 37° in various water–alcohol solutions containing 0.9% NaCl.*

trations represent the critical concentrations of the alcohols. Hemolysis in these or lower concentrations of alcohol is an osmotic phenomenon since the presence of an isotonic amount of sodium chloride in these solutions prevented hemolysis. Above these critical concentrations, a drastic increase in hemolysis occurred. As alcohol concentrations increased (Table II), further destruction of blood cells occurred resulting in brown-red discoloration and/or precipitation of blood components. The transition from nonhemolytic concentration to destructive concentrations was very abrupt, as evidenced by the hemolysis curves in Fig. 5.

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 (12). The isotonic coefficients (*i* values) for sodium chloride in aqueous solutions of methanol, ethanol, *n*-propanol, and isopropanol were found to be in the range of 1.91–2.13 (Table I) for human blood. An *i* value greater than 1.86 would be an indication that the alcohol contributed slightly to the tonicity of the extracellular aqueous solutions. The slightly higher *i* values obtained in this study indicated that alcohols themselves offered very little protection to red blood cells against osmotic hemolysis. From these experimental *i* values, it appears that the order in which the alcohols, in the presence of sodium chloride, protect human erythrocytes against hemolysis is: methanol > ethanol > isopropanol > *n*-propanol. This is in agreement with Hober and Orakov (13) who found that the permeability of erythrocytes decreased in the order: methanol < ethanol < propanol < butanol. It would appear from these data that the ability of alcohols to contribute to the tonicity of aqueous solutions is dependent on the molecular weight and the isomeric structure present. All monohydric alcohols investigated apparently were able to penetrate the erythrocyte membrane and therefore have little or no effective concentration in the extracellular solution. Hemolytic activity increases with an increase in molecular weight of the monohydric alcohols. The isomeric alcohol was noted to possess weaker hemolytic activity than the corresponding normal alcohol.

The addition of 2% NaCl did not appreciably influence the hemolytic action of methanol, ethanol, *n*-propanol, and isopropanol at their critical concentrations, while 5% NaCl even lowered the alcoholic concentrations needed to cause 100% hemolysis (Table II). This indicated that at the critical concentration the particular alcohol was having a direct effect on the cell membrane which was not inhibited by increased tonicity; in fact, the higher salt concentration appeared to have an additional weakening effect on the membrane.

When two alcohols were combined to make an aqueous ternary solvent system, the critical concentration at which hemolysis decreased was very close to the concentration predicted by taking

one-half the summation of the critical concentrations of the two individual alcohols (Table III). The effect of the alcohols on red cells appeared to be strictly an additive one and did not depend on one of the alcohols being at or near its critical concentration.

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Influence of Cetrimonium Bromide on Base-Catalyzed Hydrolysis of *p*-Substituted Ethyl Benzoates

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Abstract □ The effect of cetrimonium bromide at a concentration above its CMC on the base-catalyzed hydrolysis of eight *p*-substituted ethyl benzoates was investigated. The *p*-substituents were chosen on the basis of their Hammett substituent constants and included nitro, cyano, acetyl, fluoro, hydrogen, methoxy, amino, and dimethylamino. Of these, the nitro and cyano esters showed an increase in rate in the presence of cetrimonium bromide whereas all the others showed a decrease in rate. Micellar rate constants were calculated from both kinetic and gel filtration data. Treatment of the results according to the Hammett relationship for the effect of *p*-substituents on the rate of aromatic side-chain reactions led to the hypothesis that the observed rate modifications are dependent not only on the *p*-substituent and the surface pH of the micelle but also on the dielectric constant at the surface of the micelle.

Keyphrases □ Cetrimonium bromide—effect on base-catalyzed hydrolysis of eight *p*-substituted ethyl benzoates, Hammett considerations □ Benzoates, ethyl, *p*-substituted—effect of cetrimonium bromide on base-catalyzed hydrolysis, Hammett considerations □ Hydrolysis, base catalyzed, *p*-substituted ethyl benzoates—effect of cetrimonium bromide, Hammett considerations

In recent years, there have been a number of investigations into the modifying effect of surfactants on the rates of organic reactions, both with regard to their influence on drug stability and as models for enzymatic and other biological reactions, and several reviews have been written on the subject (1–3). Much published work, however, is not easily interpreted and the mechanisms by which surfactants exert their influences are not fully understood.

The modifying effect of surfactants on the kinetics of hydrolysis reactions results from the fact that the rate of reaction for the substrate, which is often an ester associated with the micelles, differs from that in the bulk phase. The observed rate constant is, therefore, determined by the aqueous and micellar rate constants and the fraction of the substrate associated with the micelles, as represented by Eq. 1:

$$k_{obs} = k_m F_m + k_w F_w \qquad \text{(Eq. 1)}$$

where k_{obs}, k_m, and k_w are the observed, micellar, and aqueous reaction rate constants, respectively, and F_m and F_w are the fractions of the ester associated with the micelles and the aqueous phase, respectively.

The rate of the micellar reaction depends upon the site of association of the drug with the micelle. Penetration of the substrate into the hydrocarbon interior would result in protection from attack and would reduce the rate considerably. NMR (4) and UV (5) spectroscopic measurements, however, indicate that simple aromatic esters are solubilized at, or close enough to, the micellar surface for electrostatic interactions between the surface and attacking species to have an effect; therefore, the ionic nature of the surfactant is of importance. Simple electrostatic theory (6) would predict that the base-catalyzed hydrolysis of an uncharged ester will be enhanced in the presence of cationic micelles and retarded in the presence of anionic micelles; conversely, the acid-catalyzed hydrolysis will be enhanced by anionic and retarded by cationic micelles.

Contrary to this theory, it was previously reported that the presence of cetrimonium bromide (I) at concentrations above its critical micelle concentration (CMC) increases the rate of base-catalyzed hydroly-