as in the case of the metabolites of taurinophenetidine in rabbit urine.

In conclusion, it was ascertained that the main metabolite of taurinophenetidine is the unchanged taurinophenetidine which is different from the main metabolite of acetophenetidine, that is, *p*-acetamidophenol. Nevertheless, the structure of taurinophenetidine is similar to that of acetophenetidine. Therefore, it became possible to predict that taurinophenetidine might have characteristic pharmacological action comparing with acetophenetidine. From the results of the basic experiments in the present work, biopharmaceutical and pharmacological characteristics of taurinophenetidine will be discussed in the next paper.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 29, 1969, from the *Kyoto College of Pharmacy Misasagi Yamashina, Higashiyama-ku, Kyoto, Japan*

Accepted for publication July 15, 1969.

This constitutes Part XXXVII of a series entitled "Studies on Absorption and Excretion of Drugs" and also Part III of a series entitled "Pharmaceutical Studies on 2-Aminoethanesulfonic Acid Derivatives," by S. Naito.

Behavior of Erythrocytes in Various Solvent Systems V: Water-Liquid Amides

DONALD E. CADWALLADER and JANIS R. PHILLIPS

Abstract □ Hemolytic behavior of human erythrocytes in water-amide solutions was investigated. Complete hemolysis of erythrocytes occurred in all formamide, DMF, DMA, DEA, and DEF solutions. Sodium chloride was effective in preventing complete hemolysis in solutions containing up to 10% formamide, 20% DMF, 20% DMA, 8% DEA, and 0.2% DEF. The addition of sodium chloride to solutions containing more than the above concentrations of amide did not prevent hemolysis, discoloration, and/or precipitation of human red blood cells. The addition of isotonic phosphate buffer (pH 7) to solutions increased the critical concentration at which the amide solutions damaged red cells. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in the various amide solutions.

Keyphrases □ Erythrocytes behavior—water-amide solutions □ Hemolysis, erythrocytes—water-amide solutions □ Sodium chloride effect—erythrocyte hemolysis □ Phosphate buffer effect—erythrocyte hemolysis

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 hemolytic effects of aqueous dimethylsulfoxide on human erythrocytes (1–4).

This report is concerned with the investigation of various water-liquid amide systems. The amides included in this investigation are formamide, dimethylformamide (DMF), dimethylacetamide (DMA), diethylactamide (DEA), and diethylformamide (DEF), Knazko (5) reported that pure formamide and dimethylformamide are satisfactory solvents for certain drugs, preferable for those which are hydrophobic and have an alicyclic structure and less favorable for compounds with heterocyclic structures. Very good solubility in DMF was shown by menthol, camphor, phenols, and sulfonamides. The solubility of sulfadiazine in water-DMF mixtures has been investigated (6). Spiegel and Noseworthy (7) in their extensive article on nonaqueous solvents for use in parenteral products surveyed the physical properties, toxicities, and parenteral applications of dimethylacetamide. They found this solvent to be miscible in all proportions with water and alcohol and very soluble in organic solvents and mineral oil. Waaler (8) demonstrated that DMA is extremely stable in 1 *M* aqueous solutions. For instance, the decomposition at pH 4.2 was about 1% after 600 hr. A study of the acute toxicities of DMA, DMF, and propylene glycol was made by Davis and Jenner (9) after single doses were administered intraperitoneally to mice. Results were as follows: LD_{50} for DMF was 1122 mg./kg., for DMA 3236 mg./kg., and propylene glycol 11,400 mg./kg. The LD_{100} for DMA was 5012 mg./kg.

The purpose of this investigation was to observe the behavior of human erythrocytes in aqueous formamide, DMF, DMA, DEA, and DEF solutions. In each experiment, the hemolytic method was utilized. By comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium chloride-water-amide solutions, it was possible to calculate hemolytic isotonic coefficients for sodium chloride in various water-amide solutions.

EXPERIMENTAL

Materials—Formamide, purified, and reagent grade dimethylformamide (J. T. Baker Chemical Co.) and reagent grade dimethylacetamide, diethylformamide, and diethylacetamide (Eastman

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

Solvent	Hemolysis 25%	50%	75%	Av.
Formamide, % v/v				
5	1.73	1.63	1.59	1.65
10	1.62	1.66	1.58	1.62
15	—	1.50	1.50	1.50
Dimethylformamide, % v/v				
5	1.88	1.89	1.86	1.88
10	1.87	1.88	1.94	1.90
15	1.96	1.94	1.92	1.94
Dimethylacetamide, % v/v				
5	1.82	1.82	1.80	1.81
10	1.82	1.82	1.80	1.81
15	1.78	1.78	1.75	1.77
Diethylacetamide, % v/v				
5	1.78	1.78	1.78	1.78

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

Organic Chemicals) were used. All electrolytes and nonelectrolytes employed in this study were reagent grade.

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of several 20–25-year-old Caucasian donors. 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 decanted into a 50-ml. conical flask and aerated by swirling the flask gently for about 5 min.

Preparation of Solutions—All of the amide solutions were volume-in-volume percentage preparations. Sodium chloride solutions were prepared on a weight-in-volume basis. Data from a previous paper [*viz.*, Table I (2)] was used in preparation of isotonic solutions of $CaCl_2$, $MgSO_4$, dextrose, sucrose, KBr, KCl, and Na_2SO_4. All pH adjustments were made using Sorensen isotonic buffer systems. Distilled water was used to prepare all solutions.

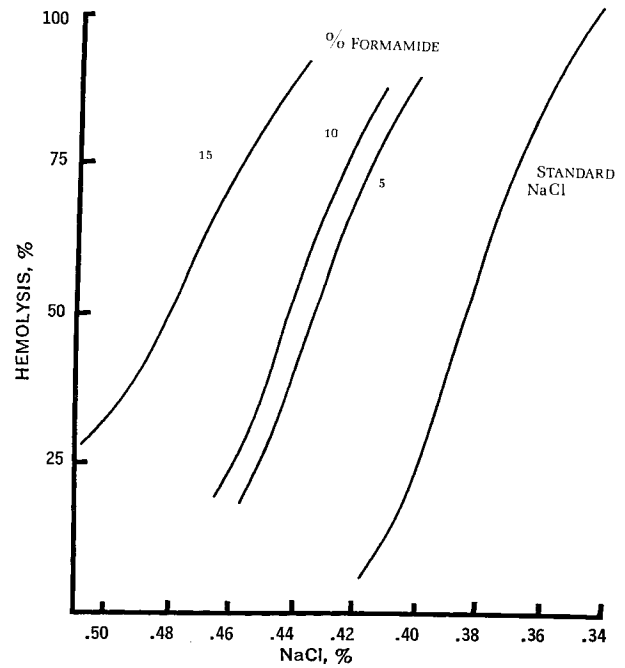

Figure 1—*Hemolysis of human erythrocytes after 45 min. at 37° in various formamide—saline solutions.*

Quantitative Determination of Percent Hemolysis—In each experiment, the hemolytic method was used to determine the degree of hemolysis of erythrocytes in the various amide 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 (0.36, 0.37 ... 0.41, 0.42%) and 5 ml. of the mixed solvent system being tested. After the test tubes were brought to a constant temperature by placing in a water bath (37 ± 0.5°), 0.05 ml. of blood was pipeted into each tube. Each tube was then inverted several times to insure thorough mixing and allowed to remain 45 min. at 37°. The tubes were centrifuged at approximately 2500 r.p.m., and the light absorbance of the supernatant liquid was measured using a photoelectric colorimeter (Klett-Summerson) equipped with a no. 54 filter. To find the percent hemolysis, these absorbance readings were divided by the absorbance readings for 0.05 ml. of blood in 5 ml. of distilled water (standard for 100% hemolysis) and multiplied by 100. A blank, made by placing 0.05 ml. of blood in 5 ml. of 0.9% sodium chloride solution, was used to cancel any light absorbance inherent to the blood sample. Both the standard and the blank were subjected to the same conditions of standing for 45 min. at 37° followed by centrifuging. A pH meter (Corning model 7) was used for all pH measurements.

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

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

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.9% sodium chloride in water (10).

Curves showing the degree of hemolysis in sodium chloride-water solutions and sodium chloride-water-amide 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 amide solvent causing 25, 50, and 75% hemolysis. These values were inserted into Eq. 1, thereby giving the values of *i* for sodium chloride in a particular water-amide solution at concentrations producing 25, 50, and 75% hemolysis. The various *i* values for sodium chloride in aqueous formamide, dimethylformamide, dimethylacetamide, and diethylacetamide solutions are shown in Table I.

Preparation of Hemolysis Curves—Approximately 30 experiments employing human blood were carried out. A standard hemolysis curve (right-hand side of Fig. 1) was constructed from the average readings of these experiments. Hemolysis curves of the various amide solutions (Figs. 1–4) were constructed using the *i* values previously calculated from Eq. 1 and shown in Table I. Through a rearrangement of this equation

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

the grams of sodium chloride per 100 ml. in an amide solution causing 25% hemolysis was calculated. Similar calculations were carried out at 50 and 75% hemolysis. By plotting these three points, the hemolysis curves for the various amide solutions were constructed.

RESULTS

Water-Formamide Solutions—Complete hemolysis of human erythrocytes occurred in 0.0 to 100% formamide solutions after 45 min. at 37°. Hemolysis in aqueous solutions containing 0.0 to 23% formamide gave clear, normal red solutions. However, in solutions containing 24 to 35% formamide, red-brown solutions were observed. Red-brown solutions containing a brown precipitate resulted when blood was placed in solutions containing 40% or

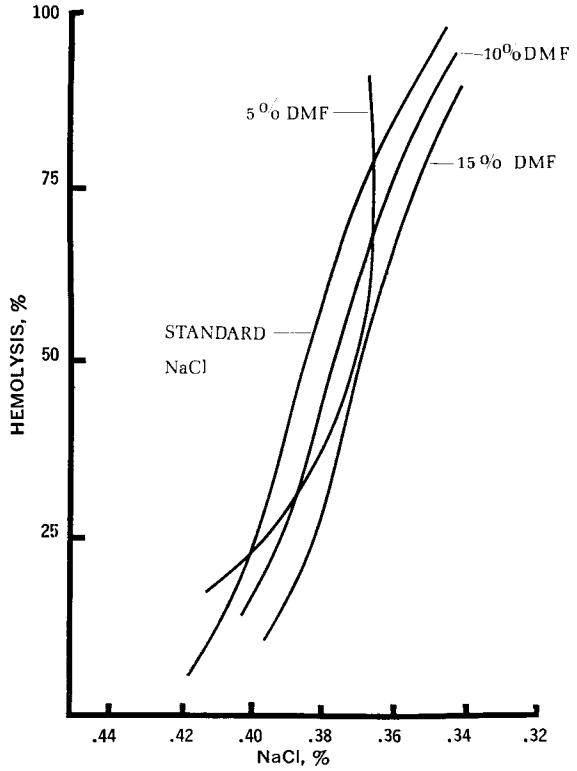

Figure 2—*Hemolysis of human erythrocytes after 45 min. at 37° in various dimethylformamide-saline solutions.*

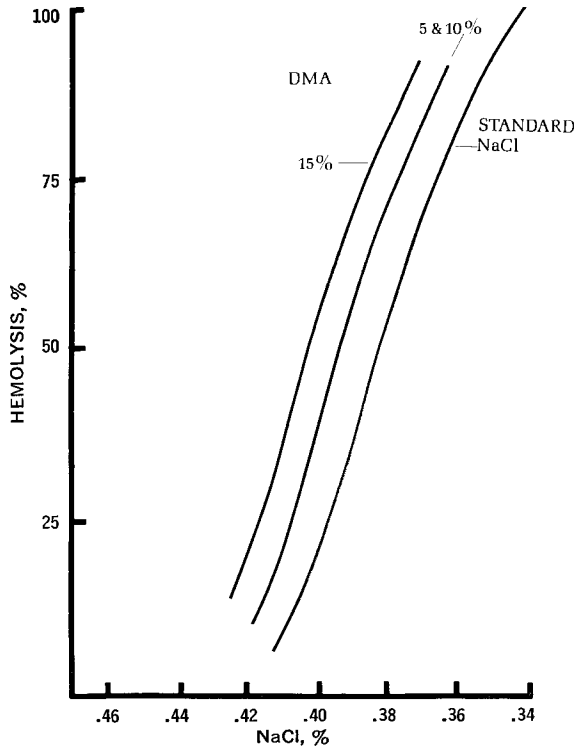

Figure 3—*Hemolysis of human erythrocytes after 45 min. at 37° in various dimethylacetamide-saline solutions.*

more formamide. The red-brown color gradually changed to a brown color at 50% becoming green-brown with heavy precipitation at 80% formamide concentrations.

Upon addition of 0.9% sodium chloride, hemolysis of human erythrocytes was essentially prevented (less than 5%) in solutions containing 0.0 to 10% formamide. Increased hemolysis was noted in solutions containing 10 to 23% formamide, and red-brown solutions resulted when blood was added to saline solutions containing 24% formamide. More pronounced discoloration and precipitation occurred in solutions containing higher concentrations of formamide.

Through the addition of hypotonic quantities of sodium chloride (0.36, 0.38 . . . 0.48, 0.50%) to various water-formamide solutions, it was possible to modify the fragility of human erythrocytes. When blood was added to the saline solutions containing 0.0 to 10% formamide, typical sigmoid hemolysis curves resulted (*viz.*, Fig. 1). These curves were constructed in the manner described in the *Experimental* section of this report utilizing the data presented in Table I.

Calculation of *i* values for sodium chloride in various water-formamide solutions was accomplished through use of Eq. 1. The average *i* values for sodium chloride in these systems (Table I) was less than 1.86, the accepted value for 0.9% sodium chloride in water.

The pH readings for formamide solutions were within a range of 5.6–9.7. Upon addition of isotonic phosphate buffer to limit this pH range, the first evident red-brown color appeared at 30% rather than 24% formamide.

Water-Dimethylformamide Solutions—All of the DMF solutions void of sodium chloride caused the complete hemolysis of human erythrocytes after 45 min. at 37°.

The addition of 0.9% sodium chloride to solutions containing 20% or less DMF afforded some protection to human erythrocytes producing only trace hemolysis (less than 5%); however, at the critical concentration (26%), damage of red blood cells occurred as in DMF solutions void of sodium chloride.

Hemolysis curves were constructed for DMA in the same manner as for formamide (Fig. 2) and *i* values calculated (Table I).

Dimethylformamide solutions gave pH readings of 6.5–7.5. Use of isotonic phosphate buffer gave the first evident color change at 28% instead of 26% DMF.

Water-Dimethylacetamide Solutions—All aqueous DMA solutions ranging in concentration from 0.0 to 100% caused complete hemolysis of human erythrocytes at 37°. Normal red solutions were evident up to concentrations of 28%, but at 29% a red-brown color became apparent. This color gradually deepened to a green-brown solution at 40% DMA indicating complete destruction of cells.

The inclusion of sodium chloride in aqueous solutions containing 0.0 to 20% DMA was effective in preventing hemolysis in human erythrocytes (less than 5%). However, the addition of sodium chloride in solutions containing 28% and more DMA did not prevent damage to blood cells. Above this critical concentration, the red blood cells were destroyed resulting in green-brown solutions with brown-black precipitates.

It was possible to calculate *i* values for sodium chloride in the various water-DMA solutions. The average *i* values for sodium chloride in 5, 10, and 15% DMA solutions were shown in Table I. For DMA hemolysis curves, see Fig. 3.

The pH readings for DMA solutions were approximately 5.5–7.0. When tested with the isotonic phosphate buffer, the first red-brown color appeared at 28%.

Water-Diethylacetamide Solutions—Hemolysis in all aqueous DEA solutions 0.0 to 100% was complete after 45 min. at 37°.

Addition of 0.9% sodium chloride produced only trace hemolysis in concentrations of 0.0 to 8% DEA. Increased hemolysis occurred in solutions containing 8.5 to 10% DEA, and a cloudy brown liquid resulted when blood was added to saline solutions containing 15% DEA.

Only the *i* value for 5% DEA solution could be calculated (Table I). This value was less than 1.86. Hemolysis curves are shown in Fig. 4.

The pH readings for the various DEA solutions were between 6.0–7.0. The first evident color change using the isotonic phosphate buffer system occurred at 14% DEA.

Water-Diethylformamide Solutions—Solutions of DEF proved to be very destructive to human erythrocytes at 37°. Hemolysis and destruction of blood cells in solutions containing 0.0 to 100% DEF was complete as evidenced by the green-brown color in solutions containing as little as 0.5% DEF.

Inclusion of 0.9% sodium chloride did very little to alter this destructive effect on blood cells. There was complete protection (no hemolysis) of cells up to 0.1% DEF; hemolysis increased through 0.2 to 0.3% DEF, and cells were completely destroyed at

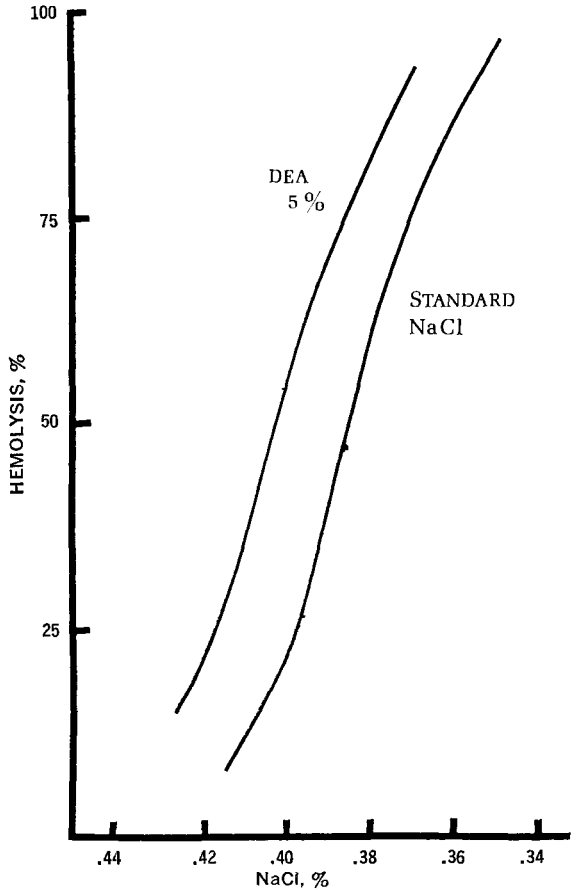

Figure 4—*Hemolysis of human erythrocytes after 45 min. at 37° in various diethylacetamide–saline solutions.*

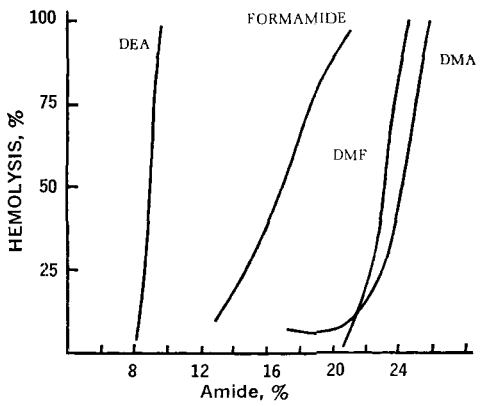

Figure 5—*Hemolysis of human erythrocytes after 45 min. at 37° in various liquid amide solutions containing 0.9% sodium chloride.*

0.4%, the resulting solution being brown with a dark brown precipitate.

Aqueous DEF solutions containing isotonic concentrations of various substances (1.15% calcium chloride, 9.39% dextrose, 7.16% sucrose, 1.73% magnesium sulfate, 1.2% potassium chloride, 1.91% potassium bromide, and 1.27% sodium sulfate) gave results similar to those described above for DEF-saline solutions. These compounds were no more effective in preventing hemolysis than the 0.9% sodium chloride.

The pH readings of DEF solutions were very acidic having a range of 2.5–4.0. With the addition of isotonic phosphate buffer to DEF solutions, hemolysis was prevented in solutions containing up to 8% DEF. At higher DEF concentrations complete hemolysis and discoloration occurred.

DISCUSSION

The experimental data showed that the liquid amides studied freely penetrate human red blood cells and offer no protection to these cells from hemolysis. Addition of 0.9% sodium chloride can be used to prevent complete hemolysis in the amide solutions containing up to 10% formamide, 20% DMF, 20% DMA, 8% DEA, and 0.2% DEF. Hemolysis in these or lower concentrations of amide is an osmotic phenomenon since the addition of 0.9% sodium chloride to these solutions prevents hemolysis and addition of hypotonic quantities of sodium chloride to these solutions (and amide solutions of lower concentration) prevents complete hemolysis.

There is a critical concentration for each amide solution (Table II) where addition of 0.9% sodium chloride does not prevent hemolysis and discoloration and/or precipitation of blood components. The transition from nonhemolytic concentrations to destructive concentrations is rather abrupt as evidenced from the hemolysis curves (Fig. 5).

Above the critical concentrations for each amide solution, further destruction of red cells occurs as amide concentrations are increased. This is apparent as the colors change from brownish-red solutions with brown precipitates ultimately to greenish solutions with green-black precipitates.

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 (11). The isotonic coefficients (i values) for aqueous solutions of formamide, DMA, and DEA were found to be slightly less than 1.86. This is evidence that these solvents offer no protection to human erythrocytes against osmotic hemolysis. On the other hand, those i values calculated for DMF were slightly higher than 1.86 indicating that this solvent contributes slightly to the tonicity of the extracellular aqueous solutions. It would appear from this data that the order of protection to human erythrocytes by the amide solutions would be DMA = DMF > formamide > DEA > DEF. These results, as they pertain to the formamides, are substantiated by the findings of Nash (12) who, in determining the protective action of neutral water-miscible compounds against freezing damage to human red blood cells, showed that formamide is a nonprotector, but progresses to a complete protector with successive methylation of each of its three

Table II—Critical Concentrations and pH of Aqueous Amide Solutions at which Discoloration[a] and/or Precipitation[b] of Blood Components Occurred[c]

Amide	Solutions Without Sodium Chloride: Critical Concn., % v/v	Solutions Without Sodium Chloride: pH of Solution	Solutions with 0.9% Sodium Chloride: Critical Concn., % v/v	Solutions with 0.9% Sodium Chloride: pH of Solution	Solutions with Isotonic Phosphate Buffer: Critical Concn., % v/v	Solutions with Isotonic Phosphate Buffer: pH of Solution
Formamide	24	9.0	24	9.1	30	7.6
DMF	26	7.8	26	7.25	28	7.7
DMA	29	5.15	28	7.0	28	7.6
DEA	14	7.1	12	6.25	14	7.0
DEF	0.1	3.8	0.4	3.75	10	6.3

[a] Discoloration was indicated by a dark amber to red-brown color. [b] Precipitation was usually indicated by light brown suspension and/or sediment. [c] Each value represents an average of at least two blood samples.

hydrogens. These changes were attributed to increase in basic strength and hydrogen-bonding ability on methylation.

The pH of the amide solution did have some effect upon the particular amide solution's tendency to damage human blood cells. The low pH of the DEF solutions appears to be the reason for the total destruction of cells at very low amide concentrations. When buffered to a near-neutral pH, the critical concentration was raised from 0.4 to 10% (Table II). With the other amide solvents, there was little change in the critical concentration by the addition of an isotonic phosphate buffer since none of these amide solutions had inherently high or low pH values.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 5, 1969, from the *School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication June 24, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Previous paper: D. E. Cadwallader and J. P. Drinkard, *J. Pharm. Sci.*, **56**, 351(1967).

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

Spectrophotometric Analysis of Glucose and Mixtures of Glucose, Fructose, and Sucrose

EDWARD R. GARRETT and JOHN F. YOUNG

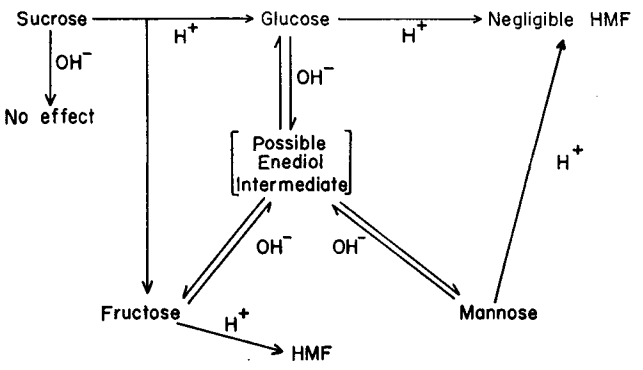
Abstract □ The aldose-ketose equilibrium in dilute alkali is utilized to transform a reproducible fraction of glucose to fructose; *e.g.*, 0.20 *N* NaOH, 40°, 3.5 hr. Acid treatment, *e.g.*, 1.0 *N* HCl, 80°, 10 hr., of this alkaline equilibrium solution results in the production of spectrophotometrically assayable hydroxymethylfurfural (HMF) ($\lambda_{max.}$ = 283 mμ) from the fructose produced. Glucose yields negligible HMF under these acid conditions prior to alkaline treatment. These two techniques permit the assay of fructose and glucose in mixtures. After 30 hr. of alkaline treatment under the stated conditions, fructose and glucose do not yield any HMF on acidification. However sucrose is stable under these conditions and on acidification hydrolyzes to fructose which yields a proportional amount of HMF. These facts permit the assay of fructose, glucose, and sucrose in mixtures. This assay is sensitive to concentrations for all three sugars as low as 10^{-4} *M*.

Keyphrases □ Glucose—analysis □ Fructose, glucose, sucrose mixture—analysis □ Aldose-ketose equilibrium, dilute alkali—glucose transformation to fructose □ Hydroxymethylfurfural formation—acid treatment aldose-ketose equilibrium □ UV spectrophotometry—analysis

The primary chemical methods for the analysis of glucose have been based on general methods for reducing sugars, such as oxidation by alkaline copper or ferricyanide solutions (1–5). Glucose has been analyzed in effluents after paper or column chromatographic separations of mixtures of sugars (6). Glucose has also been analyzed by GC after derivatization of sugar mixtures (7). Carbazole in sulfuric acid reactions (8), differential reaction-rate techniques (9), and dialysis based on differential kinetics (10) have also been applied to the determination of sugar mixtures.

Haworth and Jones (11) showed that acidification of an alkaline glucose solution could result in the production of hydroxymethylfurfural (HMF). Thus it is anticipated that with selective conditions of sequential alkali and acid treatment, a quantitative chemical analysis could be established for glucose based on the spectrophotometric assay of the HMF produced.

The Lobry de Bruyn-Alberda van Ekenstein transformation (12, 13) is the alkaline catalyzed equilibration of aldoses and ketoses through a possible enediol intermediate. The hexoses, mannose, glucose, and fructose, undergo such an alkaline equilibration (Scheme I).



Scheme I—Acid and base transformations of sugars