Hemolysis of Erythrocytes by Antibacterial Preservatives III

Influence of Dimethyl Sulfoxide on the Hemolytic Activity of Phenol

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The influence of dimethyl sulfoxide (DMSO) on the hemolytic activity of phenol has been investigated *in vitro*. Although DMSO freely penetrates the erythrocytic membrane and, in high concentrations, is destructive to red cells, it did not enhance but greatly reduced phenol-induced hemolysis of both washed and unwashed erythrocytes. This interference may be manifest through chemical interaction with phenol or through a direct cellular effect.

DIMETHYL sulfoxide (DMSO) is currently the subject of numerous reports and investigations directed toward determining its efficacy as a pharmaceutical adjunct and therapeutic agent. In a review of the medicinal and pharmaceutical aspects of DMSO, Block (1) has collated preliminary reports that indicate the varied potential of DMSO as a primary pharmacologic agent in producing analgesia, tranquility, diuresis, and reduced inflammation.

DMSO has been the subject of conflicting reports concerning its ability to act as a penetrant carrier of drugs through biologic membranes. Some investigators (2–4) attribute greatly increased drug penetration to the presence of DMSO whereas others (5, 6) report effects much the same as those produced by more common vehicles and experimental controls.

DMSO has been employed as a solvent in certain biochemical procedures (3, 7) and as an endocellular cryophylactic agent for the protection of cells, including erythrocytes, and tissues against freeze damage during low-temperature preservation (3, 7, 8–14).

The present investigation represents a continuation of studies (15–17) to determine the hemolytic activity of antibacterial preservatives alone and in the presence of pharmaceutical adjuncts. The controversial reputation of DMSO as a penetrant carrier of drugs prompted the question of whether its presence would affect the hemolytic activity of an antibacterial preservative. The hemolytic activity of certain antibacterial preservatives including phenol has been shown to be indicative of their antimicrobial activity (15). An alteration in the hemolytic response of erythrocytes to phenol in the presence of DMSO would be suggestive of a like alteration in antimicrobial activity. This information would be useful should future pharmaceuticals containing DMSO as a solvent, penetrant carrier, or primary pharmacologic agent require the presence of an antibacterial preservative. Phenol was selected for the present study since its hemolytic activity has recently been characterized in this laboratory (15, 16).

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EXPERIMENTAL

Materials.—Dimethyl sulfoxide was experimental drug grade (Crown Zellerbach Corp., Camas, Wash.). Phenol and sodium chloride were reagent grade.

Blood Samples.—Rabbit blood, obtained by heart puncture in the manner described by Grosicki and Husa (18), was used throughout this study. Each blood sample was collected just prior to its use and was verified for osmotic normalcy during the course of each experiment (15).

Quantitative Determination of Per Cent Hemolysis.—The colorimetric method employed for the determination of the degree of hemolysis occurring in each test solution has recently been described (15). In brief, it involved the addition of 0.05 ml. of defibrinated blood to duplicate pairs of colorimeter tubes each containing 5 ml. of test solution. The test mixtures were incubated in a water bath for 45 min. at 37° after which the unhemolyzed cells were settled by centrifugation and the absorbance readings of the hemolysate determined with a Klett-Summerson photoelectric colorimeter. Each absorbance reading was compared with a total hemolysis reading obtained by laking red cells in distilled water. The degree of hemolysis occurring in each test solution was calculated as a per cent of total hemolysis. The data reported represent the average of a minimum of two like experiments.

During and after the 45-min. incubation period, each test mixture was macroscopically observed for color changes, precipitation, and other signs

of denaturation. Throughout the investigation signs of blood denaturation were observed in solutions of high DMSO concentration. The following experiment was conducted to determine which test solutions affected hemoglobin such that colorimetric determinations no longer remained an accurate measure of degree of hemolysis. Washed blood cells were laked in distilled water, and like amounts of the resulting hemoglobin solution added to 5-ml. volumes of water, to individual solutions of phenol, sodium chloride, and DMSO, and to combinations of these ingredients as employed in the hemolysis experiments. The absorbance of each test solution was determined prior to and after the addition of hemoglobin solution and the final readings adjusted by subtracting the blank readings. Variations in the final readings were indicative of alterations in the hemoglobin. Each solution was also macroscopically examined for discoloration and precipitation.

Experimental Solutions.—Aqueous solutions were employed throughout the investigation. Concentrations of DMSO are expressed as per cent v/v and phenol and sodium chloride as per cent w/v.

Solutions of DMSO ranging in concentration from 0.5 to 99.5% were prepared and their absorbance readings determined. These readings served as blank readings that were subtracted from the colorimetric readings of subsequent hemolysis experiments.

Solutions containing 0.5 to 90% DMSO in 0.6% sodium chloride were similarly handled. The sodium chloride was added to provide protection to the erythrocytes against osmotic hemolysis. Hemolysis which occurred could be attributed to the presence of DMSO.

The primary objective of this investigation was to determine the influence of DMSO on the hemolytic activity of phenol. Previous studies (16) showed that phenol in the presence of 0.6% sodium chloride induced trace hemolysis of unwashed rabbit erythrocytes at approximately 0.41% phenol concentration and caused total hemolysis when present at 0.47% concentration. In the present study 0.44% phenol was employed. At this concentration 50 to 90% hemolysis usually occurs and the effect of added DMSO, whether it be increased or decreased hemolysis, could be readily detected. Hemolysis studies were conducted on test solutions containing 0.6% sodium chloride, 0.44% phenol, and concentrations of DMSO varying from 0.5 to 80%. Solutions containing sodium chloride and phenol but not DMSO served as the controls.

Similar experiments utilizing washed red blood cells were conducted to reveal the influence of serum on the DMSO–phenol activity. Erythrocytes separated from defibrinated blood by centrifugation were washed approximately 5 times with 0.6% sodium chloride until the washings tested free of protein to 0.5 *N* mercuric chloride T.S. After the final washing, the cells were resuspended in 0.6% sodium chloride to the approximate volume of the original blood sample. The cell suspension was employed in the same manner as the unwashed blood samples.

Kinetic Studies.—The experimental design of this investigation was such that the hemolytic effect of the various test solutions was determined after a constant 45-min. incubation period. Test solutions that were fundamental to the experiments or in which the erythrocytes responded diversely were selected for a time study. The hemolytic activity of these solutions was determined at intervals of from 5 to 15 min. during a period of 60 min.

RESULTS AND DISCUSSION

DMSO Solutions.—Erythrocytes incubated in aqueous DMSO solution ranging in concentration from 0.5 to 25% completely hemolyzed (Fig. 1). The color of the hemolysate appeared normal and there were no macroscopic signs of blood denaturation. Thirty and 40% DMSO solutions also induced total hemolysis; however, the resulting hemoglobin solutions were slightly turbid. At 50 and 60% DMSO concentrations, the hemolysate was no longer red but amber. At 80% DMSO concentration the test mixtures contained bulky brown-colored sediments with no sign of intact red cells or hemoglobin.

DMSO is not capable of maintaining the integrity of erythrocytes, for it permeates the red cell membrane (19) and allows the influx of water and the hemolytic consequence. To prevent the osmotic hemolysis of erythrocytes, the presence of an extracellular ingredient to which the red-cell membrane is impermeable is required. It should be noted that in the successful preservation of blood against freeze damage, the optimal concentration of DMSO is 15 to 20% (20) and an agent that contributes to the tonicity of the solution is added (10, 19).

The deleterious effect of high concentrations of DMSO on blood was noticed throughout this investigation. The discoloration of blood, the flocculation of its components, and the precipitation of hemoglobin were observed consistently in test solutions containing more than 40% DMSO.

In a toxicologic study of DMSO, conducted to determine the feasibility of employing the material as the vehicle during the intravenous administration of water-insoluble antitumor agents, Willson *et al.* (21) attributed perivascular inflammatory reactions and intravascular thrombi in dogs to the use of undiluted DMSO injections. They found hemolytic anemia to occur in both rats and dogs subjected to repeated intravenous injections of DMSO. Both hematuria and hemoglobinuria were also noted. The latter claimed to be the result of intravascular hemolysis.

Similar observations were reported by DiStefano and Klahn (22) who studied the hematologic effects of DMSO on cat blood *in vivo* and *in vitro*. Their work indicated that DMSO is a potent hemolytic agent through direct action on the blood cells and that the degree of dilution of DMSO is an important determinant of its hemolytic activity.

DMSO–NaCl Solutions.—The presence of 0.6% sodium chloride protected erythrocytes against osmotic hemolysis in solutions containing less than 20% DMSO (Fig. 1). Trace hemolysis was detected in saline solutions containing 25% DMSO, and 25% hemolysis occurred when 40% DMSO was present. In sodium chloride solutions containing 50% DMSO and greater there was no evidence of intact cells but rather a brown flocculant sediment and a green amber-colored supernatant liquid. The results show that although sodium chloride is

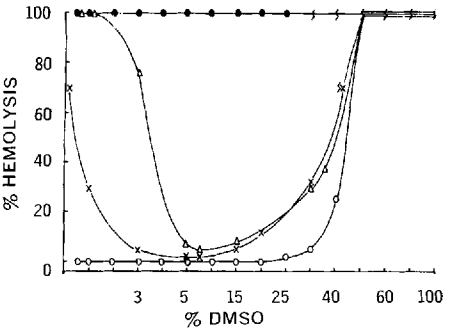

Fig. 1.—Hemolytic activity of solutions containing DMSO. Key: ●, aqueous DMSO; △, DMSO plus 0.6% NaCl and 0.44% phenol using washed erythrocytes; ×, DMSO plus 0.6% NaCl and 0.44% phenol using defibrinated blood; ○, DMSO plus 0.6% NaCl; ǂ, denaturation as color changes and/or flocculation.

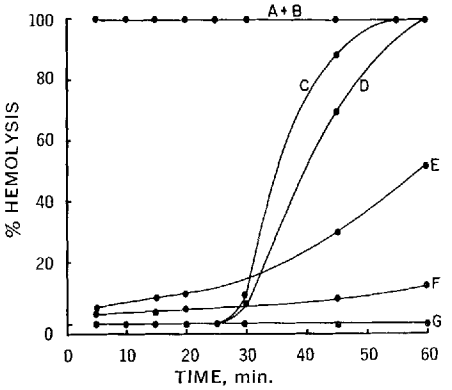

Fig. 2.—Kinetic study of the hemolytic activity of various test solutions. Key: A, distilled water; B, 30% DMSO; C, 0.6% NaCl plus 0.44% phenol; D, 0.6% NaCl plus 0.44% phenol and 0.5% DMSO; E, 0.6% NaCl plus 0.44% phenol and 30% DMSO; F, 0.6% NaCl plus 30% DMSO; G, 0.6% NaCl.

capable of preventing osmotic hemolysis in solutions of less than 20% DMSO strength, it is incapable of preventing the cytotoxic effects of higher concentrations of DMSO.

DMSO-NaCl-Phenol Solutions.—Figure 1 shows the influence of varying concentrations of DMSO on the hemolytic activity of 0.44% phenol in 0.6% sodium chloride. It should be noted that in the absence of DMSO, the phenol–salt solution induced 70% hemolysis of the erythrocytes. With the phenol and sodium chloride concentrations held constant, the addition of DMSO altered the hemolytic response. As the DMSO concentration was increased to 7%, phenol-induced hemolysis was virtually eliminated. With greater concentrations of DMSO, hemolysis increased steadily until denaturation ensued at 40% DMSO concentration and prevented further quantitation. At that concentration a red flocculant sediment was observed, indicating that a portion of the hemolysate was no longer in solution. At concentrations of DMSO exceeding 50%, the sediment was the often-seen brown flocculant material underlying a green-amber supernatant liquid.

Hemoglobin solution, prepared by lysing washed erythrocytes in distilled water, was increasingly discolored and precipitated by all test solutions containing DMSO in concentrations of 40% and greater; therefore, in experiments employing DMSO in high concentrations the colorimetric readings were no longer an accurate indication of the degree of hemolysis, for the hemoglobin released by laked cells was at least partially precipitated and colorimetrically undetectable.

Washed Erythrocytes.—Under the conditions of this study, erythrocytes were found to be more easily hemolyzed by phenol in the absence of blood serum. A standard test solution of 0.44% phenol in 0.6% sodium chloride consistently induced complete hemolysis of washed erythrocytes after 45 min., whereas an average of 70% of the unwashed cells lysed in one series of experiments (Fig. 1) and 90% in another (Fig. 2). The greater degree of lysis with washed cells could be attributed to the absence of the protective action of the serum proteins and to the increased fragility of the erythrocytes caused by the repeated washings. The influence of DMSO on phenol-induced hemolysis, however, followed a similar pattern for both the washed and unwashed red blood cells (Fig. 1). As the DMSO concentration in the phenol–salt test solution was increased to 7%, hemolysis decreased from complete hemolysis to about 5% hemolysis. As the DMSO concentration was further increased, hemolysis increased until denaturation occurred at 40% DMSO concentration.

Interpretation of Results.—The data seem to suggest the biologic inactivation of phenol as part of a chemical complex with DMSO or by virtue of increased cellular resistance to its hemolytic capabilities.

The observation that antimicrobial agents are inactivated by various chemical agents resulting in a loss of antimicrobial and hemolytic activities is not new (16, 23–28). Although phenol and DMSO have been shown (14) to react under certain laboratory conditions (dissimilar to those of the present study) the addition of small amounts of DMSO to solutions of a number of other organic chemicals has produced evidence of complex formation (3).

In a separate investigation (29) involving the hemolytic activity of the antimicrobial agent chlorhexidine diacetate, DMSO had an influence on hemolysis similar to that shown with phenol. Chlorhexidine diacetate was employed in hemolytic concentrations, as phenol had been, in the presence of 0.6% sodium chloride and varying amounts of DMSO. Curiously, the hemolytic activity of chlorhexidine diacetate was decreased with increasing amounts of DMSO with minimal hemolysis occurring in the presence of 9% DMSO; greater concentrations of DMSO resulted in increased hemolysis.

The possibility of a direct influence of DMSO on the erythrocyte rendering the phenol ineffective in its hemolytic activities was explored. In pursuing the premise that DMSO was directly affecting the red cell membrane, experiments were designed in which washed erythrocytes were incubated in saline and varying amounts of DMSO with the intention of later removing the DMSO and subjecting the cells

to phenol to determine whether the cells were permanently altered by the DMSO pretreatment. Erythrocytes were incubated with 1, 7, 20, and 30% concentrations of DMSO. Attempts to remove the DMSO by washing the cells with 0.6% sodium chloride were unsuccessful. The red cells pretreated with 20 and 30% DMSO solutions completely hemolyzed on the first attempted washing. Appreciable hemolysis occurred in the 7% DMSO pretreated cell sample and trace hemolysis in the 1% DMSO pretreated cells as washing was attempted. The same washing fluid was efficient in the concurrent washing of the control cells subjected to the same conditions except for the exposure to DMSO. In blood preservation work, the use of DMSO as an endocellular cryophylactic agent has stimulated investigators to seek an effective method of removing DMSO from within the cells prior to transfusion (20). As in the present work, efforts to wash the cells free of DMSO have resulted in hemolysis (20). It has been found possible to dialyze erythrocytes free of DMSO, but the required dialysis time has been found prohibitive to practical application (20).

Kinetic Studies.—As can be seen in Fig. 2, both distilled water and 30% DMSO induced total hemolysis within the first 5 min. of the experiment. Hemolysis occurring in the 30% DMSO test solutions can be largely attributed to osmotic hemolysis resulting from the penetration of this hygroscopic material (3, 14) through the erythrocytic membrane (19). It is not unlikely, however, that the high concentration of DMSO also exerted damaging changes to the cell structure.

The addition of 0.6% sodium chloride to 30% DMSO reduced the hemolytic effect of the latter such that at the conclusion of the experiment, only 12% hemolysis had occurred. Since 0.6% sodium chloride is sufficient to prevent osmotic hemolysis, the hemolysis that did occur can be attributed to the cytotoxic activity of DMSO.

Phenol at a concentration of 0.44% in the presence of 0.6% sodium chloride induced trace hemolysis after 25 min. of incubation and total hemolysis after 55 min. The inclusion of 0.5% DMSO to the phenol–sodium chloride solution slightly decreased the rate of hemolysis. The inclusion of 30% DMSO to the phenol–sodium chloride solution prompted an initial hemolytic response that progressed steadily throughout the duration of the experiment. At the conclusion of the study, however, only about half of the erythrocytes were lysed in contrast to total hemolysis which occurred in the other solutions containing phenol.

In summary, DMSO has been shown to interfere with the hemolytic activity of phenol *in vitro*. This interference may be manifest through chemical interaction or through a direct cellular effect. It would be interesting to investigate the antimicrobial activity of phenol and other preservatives in the presence of DMSO. Such a study would further elucidate the analytical value of erythrocytes in predicting microbial responses to preservatives. It would also provide useful information relative to the preservation of pharmaceuticals containing DMSO.

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