Influence of Polyethylene Glycols on the Hemolytic Activity of Phenolic Preservatives

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The influence of polyethylene glycols on the response of rabbit erythrocytes to hemolytic concentrations of phenol, *m*-cresol, and *p*-chlorophenol has been determined. Through their interactions with each of these preservative agents, the polyethylene glycols were capable of preventing hemolysis. The higher molecular weight polymers were more effective complexing agents than those with lower degrees of polymerization. The ability of phenol and polyethylene glycols to interact was enhanced by the presence of sodium chloride. The hemolytic method represents a new approach in appraising phenolic–polyethylene glycol interactions and consequent preservative availability.

NUMEROUS reports have appeared in recent years concerning the inactivation of various antibacterial preservatives when in the presence of pharmaceutically employed materials such as vegetable gums (1–5) and nonionic surfactants (6–18). Several of these studies have included data and hypotheses on the nature of the interactions between phenolic compounds and polymers, as polyethylene glycol and polysorbate 80 (13–15). Such interactions have resulted in pharmaceutical incompatibilities (19, 20) or in the reduction of antibacterial activity (17, 21, 22).

In view of the possibility of preservative interactions with other formulative materials, it is recognized that chemical assays of preservative concentration may not always be a true measure of effective antimicrobial concentration (22). It is not unlikely that biologically inactive complexes of preservative and interacting substance may be broken down during the course of a chemical analysis so that most, if not all, of the original preservative may be recovered and measured as such. It has been suggested, therefore, that a biological assay method for determining effective preservative content may be more meaningful than chemical analysis (22).

The interactions of preservatives with other substances have been studied by such physical chemical methods as equilibrium dialysis (7–10, 14, 15) and phase analysis (13, 14), and biologically by a manometric method (23) utilizing baker's yeast and a turbidimetric method (22, 24) in which the growth of microorganisms reflects preservative inactivity. An earlier report (25) indicated microorganism killing and erythrocyte hemolysis by certain antibacterial preservatives to be quantitatively comparable. The present investigation was undertaken to determine whether erythrocytes could effectively serve as test cells in detecting preservative interactions with interfering agents. The phenolic–polyethylene glycol system was selected for this initial study primarily because of the demonstrated interactions between these types of compounds in aqueous solution (13, 14).

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EXPERIMENTAL

Materials.—The polyethylene glycols (PEG's) employed in this investigation were commercial grade; all other chemicals were reagent grade.

Blood Samples.—Rabbit blood, obtained by heart puncture in the manner described by Grosicki and Husa (26), was used in each of the hemolysis experiments. Each blood sample was collected just prior to its use and was verified for osmotic normalcy (25) during the course of each experiment.

Quantitative Determination of Per Cent Hemolysis.—The colorimetric method employed in this study for the determination of the degree of hemolysis has recently been described (25). The only variation in the procedure was that blood in the presence of 0.6% rather than 0.9% sodium chloride served as the colorimetric blank.

Determinations of pH.—The pH of each test solution was determined prior to and after its admixture with blood with a Beckman Zeromatic pH meter. Each phenolic test solution had an initial pH within the range pH 5.6–5.8. The addition of blood changed the pH values to approximately 7.0.

Experimental Solutions.—Aqueous solutions were employed throughout the investigation. Sodium chloride solutions ranging in concentration from 0.32 to 0.52% were used to verify the osmotic normalcy of each blood sample during the course of its use (25).

Solutions of varying concentrations were prepared for each phenolic in the presence of 0.6% sodium chloride to determine their hemolytic activities. The sodium chloride offered the erythrocytes protection against osmotic hemolysis, and therefore any hemolysis which occurred could be attributed to the cytotoxicity of the preservative agent (25). The data obtained were used to establish the minimum concentration of each preservative required to induce total hemolysis. These concentrations were sub-

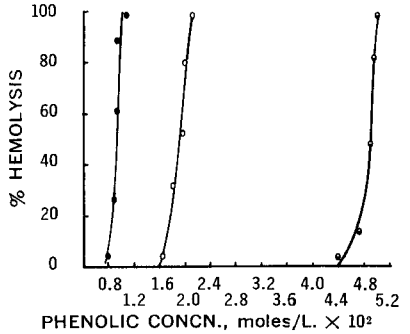

Fig. 1.—Hemolysis of rabbit erythrocytes in the presence of 0.6% sodium chloride at 37°. Key: ●, *p*-chlorophenol; ○, *m*-cresol; ◒, phenol.

sequently employed in experiments designed to detect preservative interactions with polyethylene glycols.

Test solutions were prepared to contain varying amounts of a polyethylene glycol, the hemolytic concentration of a phenolic, and 0.6% sodium chloride. The ability of the polyethylene glycol to interact with the preservative, rendering it ineffective in its cytotoxic activity, would be reflected by decreased hemolytic response.

The phenolic–PEG–sodium chloride solutions were allowed to equilibrate overnight at ambient room temperatures prior to conductance of hemolysis experiments at 37°. Solutions containing the hemolytic concentration of phenolic and 0.6% sodium chloride were concurrently employed to affirm the total hemolytic response in the absence of polyethylene glycol.

The influence of sodium chloride on the interaction between phenol and PEG 4000 was determined by varying the salt concentration as well as the polymer concentration of solutions of constant phenol strength. The sodium chloride level could not be reduced below 0.5%, that amount being necessary to protect against osmotic hemolysis.

Preliminary studies indicated that polyethylene glycols, at concentrations employed in this investigation, were inconsequential to the stability of the erythrocytes in the absence of hemolytic phenolics.

The data presented in each hemolysis study represent the average of a minimum of three like experiments.

Phase Analyses of Phenol–Polyethylene Glycol Interactions.—In an earlier paper, Higuchi and Lach (13) studied the interaction of phenol and polyethylene glycol 6000 by a method involving the titration of the glycol with aqueous phenol to a cloud point, the relative amounts of the reactants being indicative of the stoichiometric dependency of the reaction. This method was used to provide additional data on the interaction of phenol and each polyethylene glycol employed in the hemolysis experiments. A correlation between the relative capability of each PEG to interact with phenol as determined titrimetrically and hemolytically was thus facilitated.

RESULTS AND DISCUSSION

Hemolytic Activity of Phenol, m-Cresol, and p-Chlorophenol.—Initial experiments conducted to determine the hemolytic activities of the phenolic preservatives in the presence of 0.6% sodium chloride showed *p*-chlorophenol to have about twice the hemolytic capability of *m*-cresol and five times that of phenol (Fig. 1). These results closely parallel those of an earlier study conducted in the presence of 0.9% sodium chloride (25). The hemolytic activity of phenolic compounds is attributed, in part, to their ability to cause physical damage to the cell membrane, resulting in destruction of permeability barriers and subsequent cellular leakage. The increased activity of methylated and chlorinated derivatives is attributed to their increased lipid solubility, permitting freer penetration of the lipid structure of the cell membrane (25).

The concentration of each phenolic just sufficient to cause total hemolysis was employed in experiments involving the polyethylene glycols. As shown in Fig. 1, total hemolysis was induced by 5.0×10^{-2} *M* phenol, 2.1×10^{-2} *M* *m*-cresol, and 1.0×10^{-2} *M* *p*-chlorophenol in the presence of 0.6% sodium chloride. The use of these concentrations together with varying proportions of the PEG's permitted the observation of phenolic–PEG interactions since a decreasing degree of hemolysis occurred as the hemolytic agents became more inactivated by the polymer.

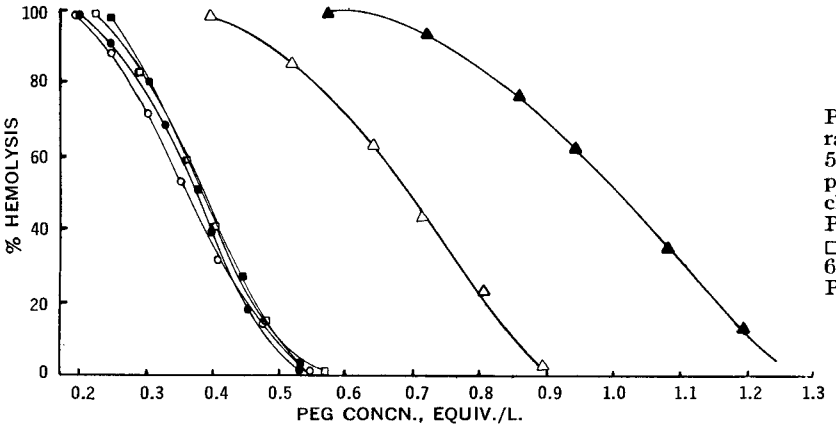

Fig. 2.—Influence of PEG's on hemolysis of rabbit erythrocytes by 5×10^{-2} *M* phenol in the presence of 0.6% sodium chloride at 37°. Key: ○, PEG 1000; ●, PEG 4000; □, PEG 1540, ■, PEG 6000; △, PEG 400; ▲, PEG 200.

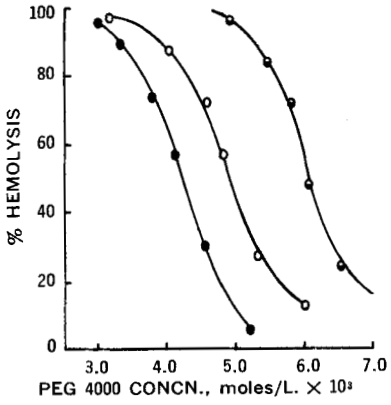

Fig. 3.—Influence of sodium chloride on the hemolytic activity of 5×10^{-2} *M* phenol in the presence of PEG 4000 at 37°. Key: ●, 0.9% sodium chloride; ○, 0.7% sodium chloride; ◒, 0.5% sodium chloride.

Phenolic–PEG Interactions.—As can be seen in Fig. 2, the hemolytic activity of 5.0×10^{-2} *M* phenol decreased with increased concentrations of polyethylene glycols. The PEG concentrations were calculated on a repeating unit basis, this is, gram equivalents of ethylene oxide linkage (O—CH_2—CH_2) per liter of solution (13). Polyethylene glycols 1000, 1540, 4000, and 6000 were similarly effective in interacting with phenol, thus preventing hemolysis. The curve drawn for each of the higher molecular weight polymers represents the average data of three like experiments. The data, varying only slightly for each PEG and between each PEG, resulted in individual curves for the four higher molecular weight polymers which were suggestive of similarity of activity rather than an order of activity.

Although polyethylene glycols 200 and 400 were less effective than the higher molecular weight polymers at equivalent concentrations, they did demonstrate a binding capability for phenol.

Figure 1 shows that hemolysis induced by phenolic compounds is concentration dependent but proceeds rather sharply once the initial lytic concentration is reached. Phenol, for instance, first induced hemolysis at a concentration of 4.4×10^{-2} *M* and caused complete hemolysis at a concentration of 5.0×10^{-2} *M*. The concentration of phenol which is responsible for the increase from initial to total hemolysis is therefore 6.0×10^{-3} moles/L. The elimination or inactivation of all or a part of this amount from solutions of 5.0×10^{-2} *M* strength would result in diminished hemolytic activity. Figure 2 shows the hemolytic response of erythrocytes to 5.0×10^{-2} *M* concentrations of phenol in the presence of varying proportions of the various PEG's. The progressive reduction in hemolysis by increased concentrations of the PEG's indicated that phenol was being interfered with such that its activity as a hemolytic agent was impaired. If one can assume that the PEG's had interacted with phenol and that the interaction was solely responsible for decreased hemolytic response, the amounts of each material involved in the reduction of hemolysis from 100 to 0% can be determined. As stated above, the reduction of phenol-induced hemolysis from 100 to 0% would require the elimination or inactivation of 6×10^{-3} moles/L. of phenol from the test solutions. According to Fig. 2, the higher molecular weight polymers initiated the reduction of phenol-induced hemolysis at a concentration of approximately 0.22 equiv./L. (about 1% PEG) and eliminated the hemolytic response at a concentration of approximately 0.55 equiv./L. (about 2.5% PEG). Thus, the concentration of the higher molecular weight PEG's required to reduce phenol-induced hemolysis from 100 to 0%, thereby inactivating 6×10^{-3} moles/L. of phenol, was approximately 0.33 equiv./L.

The concentration 0.33 equiv./L., with respect to the four higher molecular weight polymers, reflects the following polymer concentrations in moles/L.: PEG 1000, 1.5×10^{-2}; PEG 1540, 1.0×10^{-2}; PEG 4000, 3.7×10^{-3}; and PEG 6000, 2.5×10^{-3}. On a mole for mole basis, 1 mole of phenol had apparently interacted with 2.5 moles of PEG 1000, 1.7 moles of PEG 1540, 0.6 mole of PEG 4000, and 0.4 mole of PEG 6000. It appears, therefore, that the higher molecular weight polymers, with their greater number of ethylene oxide linkages per molecule, interacted with phenol more capably with increasing molecular weight.

The interactions of phenols and macromolecules of the polyoxyethylene type have generally been attributed to hydrogen bond formation between the hydrogen of the phenolic hydroxyl group and the oxygen of the ether linkage (13–15). The inactivation of phenols in micelles has also advanced as a possible mechanism of the interaction (16).

Higuchi and co-workers (13, 14) noted that a significant characteristic of phenol–PEG interactions is a dependence on the concentration of phenol and independence of the concentration of the polymer. They suggested that the individual tendency of phenol molecules to attach to a polyether molecule is small, but when several of them do so, a favorable environment for additional combining is created.

Influence of NaCl on the Phenol–PEG 4000 Interaction.—The hemolytic studies of this investigation required the presence of adequate amounts of sodium chloride to prevent spontaneous osmotic hemolysis. The minimum sodium chloride concentration found so effective was 0.50%. Experiments were conducted, using that salt concentration and greater, to determine its influence on the phenol–PEG 4000 interaction. The results are shown in Fig. 3. The interaction was enhanced by increasing salt concentration, as indicated by decreasing hemolysis at constant PEG 4000 concentration. At 5×10^{-3} *M* PEG 4000 concentration, for example, 5×10^{-2} *M* phenol induced 95% hemolysis in the presence of 0.5% sodium chloride, 40% hemolysis with 0.7% salt, and 10% hemolysis in the presence of 0.9% sodium chloride.

In their titrimetric studies, Guttman and Higuchi (14) found several neutral salts, including sodium chloride, effective in enhancing the interaction between phenol and polyethylene glycols. This activity was attributed to the dehydrating effect of sodium chloride, making the water molecules less available for hydrogen bonding with the ether oxygens of the PEG, thereby decreasing the aqueous solvation of the polymer and favoring the competing phenol–polymer interaction.

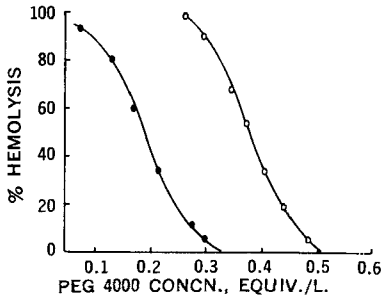

Fig. 4.—Influence of PEG 4000 on hemolysis of rabbit erythrocytes by *m*-cresol and *p*-chlorophenol in the presence of 0.6% sodium chloride at 37°. Key: ●, 2.1×10^{-2} *M* *m*-cresol; ○, 1.0×10^{-2} *M* *p*-chlorophenol.

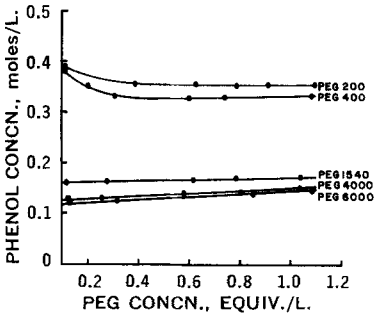

Fig. 5.—Phase diagram showing the interaction of phenol with various PEG'S in water. Lines below are conditions of solubility; lines above are conditions of immiscibility.

m-Cresol and p-Chlorophenol Interactions with PEG 4000.—The response of erythrocytes to hemolytic concentrations of *m*-cresol and *p*-chlorophenol in varying concentrations of PEG 4000 is shown in Fig. 4. In each case, the polymer was effective in interacting with the phenolic such to prevent hemolysis. According to Fig. 1, the reduction in hemolysis from 100 to 0% would require the elimination or inactivation of 5×10^{-3} moles/L. of *m*-cresol and 3×10^{-3} moles/L. of *p*-chlorophenol. Since a lesser amount of PEG 4000 was required to prevent *m*-cresol-induced hemolysis than *p*-chlorophenol-induced hemolysis and since a greater proportion of *m*-cresol was involved in the interaction, the data indicate that PEG 4000 has a greater affinity for *m*-cresol than *p*-chlorophenol.

Titrimetric Studies.—Although the hemolytic studies showed that higher molecular weight polyethylene glycols have a greater affinity for phenol than those with lower degrees of polymerization, the order of activity for PEG's 1000, 1540, 4000, and 6000 was not clear-cut (Fig. 2). For comparative purposes, the work of Higuchi and Lach (13), involving the titrimetric analysis of the interaction between phenol and PEG 6000, was extended to include the other polymers

Titrations were conducted at ambient room temperatures. Comparison with hemolysis data collected at 37° was justified, however, since the phenol–PEG interaction has been shown to be largely temperature independent (13, 14). The titrimetric data obtained are presented in Fig. 5. The areas below the lines indicate conditions of solubility and those above the lines conditions of immiscibility. A comparison of Fig. 2 and Fig. 5 shows that the titrimetric method more clearly defined the order of PEG activity than did the hemolytic method, although the over-all data are quite similar. Each method showed that the interaction capability of the higher molecular weight PEG's was pronounced and practically equivalent, and that the lower molecular weight polymers were capable of interaction, but to a much lesser extent. As can be seen through Fig. 5, the 0.05 *M* phenol concentration employed in the hemolysis experiments is well within the area of solubility for each polymer studied.

The hemolytic method has been shown to be useful in detecting the interactions between phenolic preservatives and polyethylene glycols.

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