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Hemolysis of Erythrocytes by Antibacterial Preservatives

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A study has been made of the hemolytic activity of antibacterial preservatives on human and rabbit erythrocytes in the presence of 0.9 per cent sodium chloride. The hemolytic potency of phenol increased with methyl and/or chlorine substitution. The order of activity was: *p*-chloro-*m*-cresol ⟩ *p*-chlorophenol ⟩ tricresol and *m*-cresol ⟩ phenol. Chlorinated alcohols were similarly more hemolytic than the non-halogenated ones: *p*-chloro-β-phenylethyl alcohol ⟩ chlorobutanol ⟩ phenylethyl alcohol ⟩ benzyl alcohol. Erythrocytes were highly sensitive to benzalkonium chloride, benzethonium chloride, and phenylmercuric nitrate. Thimerosal, butylparaben, and sodium formaldehyde sulfoxylate also induced hemolysis. The data indicate that the hemolytic and antimicrobial mechanisms of action may be identical for many compounds.

THE U.S.P. XVI (1) states that suitable substances may be added to preparations intended for parenteral administration to increase the stability of the product, provided they are harmless in the amounts administered and do not interfere with the therapeutic efficacy or the assay procedures. Limited amounts of various antibacterial preservatives are recommended for multiple-dose parenteral products regardless of the method of sterilization. A personal survey of currently marketed parenteral products indicated that a variety of antibacterial agents are used in preparations intended for each route of administration, including intravenous administration.

It has been shown, *in vitro* and *in vivo*, that certain chemicals in amounts calculated to be isoosmotic to the red blood cell according to physicochemical data may pass through the erythrocytic membrane and fail to prevent osmotic hemolysis (2–9). In addition, certain substances may be cytotoxic to the erythrocyte, thus altering the integrity of the membrane and resulting in the escape and/or denaturation of the cell contents.

It was the purpose of this investigation to study the hemolytic activity of various antibacterial preservatives on human and rabbit erythrocytes in the presence of isotonic media. By preparing the preservative solutions in 0.9% sodium chloride it was felt that any alteration in the integrity of the erythrocytes could be directly attributed to the action of the preservative and not to the original osmotic pressure of the external solution.

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EXPERIMENTAL

Collection of Blood.—The rabbit and human (Caucasian) blood samples employed in this investigation were obtained in the manner described by Grosicki and Husa (9). Caucasian blood was consistently employed to avoid the osmotic differences found in blood samples from Negro donors (8). Fresh blood samples from healthy donors were used throughout the study.

Materials.—The following chemicals used in this study were supplied gratuitously by their respective manufacturers: chlorobutanol U.S.P. and benzyl alcohol N.F., Benzol Products Co.; methylparaben U.S.P., propylparaben U.S.P., ethylparaben, and butylparaben, Heyden Newport Chemical Corp.; phenylmercuric nitrate, Metalsalts Corp.; *p*-chlorophenol, Dow Chemical Co.; *p*-chloro-*m*-cresol, Burroughs Wellcome and Co.; thimerosal N.F., Eli Lilly and Co.; sodium formaldehyde sulfoxylate, Rohm and Haas; and *p*-chloro-β-phenylethyl

alcohol, Ciba Pharmaceutical Co. Cresol U.S.P., *m*-cresol, phenol U.S.P., sodium chloride reagent, and phenylethyl alcohol were obtained commercially.

Preparation of Solutions.—The various solutions of each preservative contained the antibacterial agent and 0.9% sodium chloride in purified water. All solutions were prepared on a per cent w/v basis, except for benzyl alcohol, (tri)cresol, *m*-cresol, phenylethyl alcohol, and *p*-chloro-β-phenylethyl alcohol, which were per cent v/v.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis, essentially that of Hunter (10), is based on the fact that the quantity of oxyhemoglobin exuded from the erythrocytes of a blood sample is a direct function of the proportion of red blood corpuscles hemolyzed.

Five-milliliter volumes of each preservative-saline solution were pipeted into each of two colorimeter tubes. Similarly, 5-ml. portions of purified water, 0.9% sodium chloride, and sodium chloride solutions ranging in concentrations from 0.32 to 0.52% were each transferred into duplicate tubes. Exactly 0.05 ml. of fresh defibrinated and aereated blood was added to each tube and the contents mixed by gentle inversion. The tubes were then placed in a water bath at 37° for 45 minutes, then centrifuged for 4 minutes at approximately 2000 r.p.m. A Klett-Summerson photoelectric colorimeter with a No. 54 green filter was adjusted to read zero absorbance with the tubes containing the blood in 0.9% sodium chloride. Each of the other tubes was read for its absorbance and observed for signs of denaturation. The average colorimeter reading of the two tubes containing the blood in purified water was taken to be the total hemolysis value. The per cent hemolysis in each duplicate pair of tubes was calculated by dividing their average absorbance reading by the total hemolysis value and multiplying by 100%. Trace hemolysis was considered to be the first colorimetric detection of exuded hemoglobin in a preservative test series; in all cases it represented less than 10% hemolysis. A minimum of six preservative concentrations intermediate to those inducing trace and total hemolysis were employed in each test series.

The data obtained on the response of erythrocytes to the various concentrations of sodium chloride were used to plot the characteristic sigmoid curves (11) for the purpose of verifying the osmotic normalcy of each blood sample.

Colorimetric readings indicating that well over 100% hemolysis had occurred in certain test solutions (notably the cationic surfactants) were attributed to an alteration of the normal color of exuded cellular material. Procedural modifications were made according to the method previously described (8) for the quantitative determination of hemolysis in colored solutions.

DISCUSSION AND RESULTS

All of the preservatives studied, except for the parabens, sodium formaldehyde sulfoxylate, and thimerosal, caused total hemolysis of rabbit and human erythrocytes within concentrations normally employed in parenteral solutions (see Table I). It is interesting to compare the reported bactericidal concentrations of many of these agents with the amounts found to induce hemolysis. The antibacterial mechanism of action of a preservative which promotes bacteriolysis may be similar to that which is responsible for hemolysis.

A widely accepted conception is that the erythrocyte is bounded by a lipid-like membrane which is perforated with positively charged aqueous pores or channels of various diameters (12–14). Schanker, *et al.* (15), suggest that small lipid-soluble molecules might penetrate the cells by dissolving in the lipoid phase and by diffusing through the aqueous channels; larger lipid-soluble molecules would enter mainly through the lipoid phase. Lipid-insoluble molecules and anions would enter the cell if they were small enough to pass through the pores, whereas cations would be largely excluded because of their lipid insolubility and their inability to pass through the positively charged pores. Wintrobe (16) agrees that the ultrastructure may act as a preferential barrier and help to determine the internal contents of the cell, but he points out that some chemical agents which have been shown to cause hemolysis *in vitro* do not do so *in vivo*.

TABLE I.—PER CENT CONCENTRATION OF PRESERVATIVE CAUSING TRACE AND TOTAL HEMOLYSIS OF RABBIT AND HUMAN ERYTHROCYTES IN THE PRESENCE OF 0.9% SODIUM CHLORIDE

Preservative	Concn. of Preservative Causing Hemolysis, %[a]			
	Rabbit Erythrocytes		Human Erythrocytes	
	Trace	Total	Trace	Total
Phenol	0.43	0.48	0.49	0.54
Cresol	0.16	0.225	0.225	0.25
m-Cresol	0.20	0.225	0.26	0.30
p-Chlorophenol	0.11	0.125	0.11	0.15
p-Chloro-*m*-cresol	0.047	0.055	0.052	0.060
Chlorobutanol	0.26	0.32	0.37	0.50
Benzyl alcohol	0.81	0.94	1.05	1.20
Phenylethyl alcohol	0.52	0.62	0.65	0.80
p-Chloro-β-phenylethyl alcohol	0.10	0.14	0.12	0.18
Thimerosal	0.050	0.50	1.50	2.00[b]
Phenylmercuric NO_3	0.0010	0.0030	0.0040	...[c]
Benzethonium Cl	0.0013	0.0023	0.0015	0.0022
Benzalkonium Cl	0.0010	0.0025	0.0017	0.0027
Na-Formaldehyde sulfoxylate	1.0[d]	5.0[e]	...[f]	...[f]
Methylparaben	...[c]	...[c]	...[c]	...[c]
Ethylparaben	...[c]	...[c]	...[c]	...[c]
Propylparaben	...[c]	...[c]	...[c]	...[c]
Butylparaben	0.02	...[c]	...[c]	...[c]

[a] Average of a minimum of two experiments. [b] 98% hemolysis. [c] Data unobtainable because of solubility limitations. [d] Black-red denaturation of cells. [e] Black-red hemoglobin color. [f] Data unobtainable prior to denaturation.

Phenolics.—Phenol, a protoplasmic poison, is toxic to all types of cells; high concentrations precipitate proteins and low concentrations denature them without coagulation. This denaturation process does not bind phenol but leaves it free to penetrate the tissues (17).

The hemolytic activity of the phenolics in order of increasing effectiveness was: phenol, *m*-cresol and cresol, *p*-chlorophenol, and *p*-chloro-*m*-cresol. Hemolysis induced by these compounds probably resulted from membrane damage and/or penetration of the compounds into the cells' interiors with the subsequent influx of water and the hemolytic consequence. It is interesting that the cresols were about twice as hemolytic as phenol and the *p*-chloro-derivatives of *m*-cresol and phenol were each about four times as hemolytic as the parent compounds.

A direct relationship seems to exist between the antibacterial and hemolytic activities of the phenolic compounds studied. Recent studies (18) indicate phenolic antibacterial agents exert lethal action by physical damage to the bacterial cell membrane or to mechanisms which control the permeability of the cell membrane. Phenolic agents promote the leakage of bacterial cellular constituents (19).

A comparison of some literature-reported antibacterial phenol coefficients with a similarly calculated "hemolytic phenol coefficient" strongly indicates a like mechanism of action for the two activities. "Hemolytic phenol coefficient," a term hereby fabricated to facilitate the desired comparison, was calculated by dividing the lowest per cent concentration of phenol found to cause total hemolysis (rather than killing of microorganisms) by the lowest per cent concentration of another phenolic chemical also causing total hemolysis under the same conditions. The two coefficients are presented in Table II.

The data obtained indicate that the methyl and/or chlorine substitution of phenol intensifies its hemolytic activity just as it increases its antibacterial potency (20).

Alcohols.—The hemolytic activity of the alcohols in order of increasing effectiveness was: benzyl alcohol, phenylethyl alcohol, chlorobutanol, and *p*-chloro-*β*-phenylethyl alcohol. As in the case of the phenolics, the chlorinated molecules were the most hemolytic. Lachman, *et al.* (21), in reporting on the antibacterial activity of the same alcoholic preservatives, noted that the chloro compounds were more active than the nonchlorinated ones. The greater activity was attributed to the inherent ability of the halogenated molecules to distribute more readily into nonpolar solvents and thus to penetrate the lipid barrier of the microorganisms more readily. The same may apply to their activity on the erythrocyte. The similarity of Lachman's data on bactericidal activity with that on the hemolytic activity obtained in the present study is shown in Table III.

TABLE II.—COMPARISON OF THE MICROBICIDAL AND HEMOLYTIC ACTIVITIES OF PHENOLIC COMPOUNDS

Compd.	Phenol Coefficient	Hemolytic Phenol Coefficient[a] Human Blood	Rabbit Blood
Phenol	1.0	1.0	1.0
Cresol	2.5[b]	2.2	2.2
p-Chlorophenol	3.9[b]	3.6	3.8
p-Chloro-*m*-cresol	10.7[c]	9.0	8.7

[a] Calculated from data in Table I. [b] Data from Wilson and Gisvold (17) using *E. typhosa*. [c] Data from Klarmann and Wright (20) using *S. typhosa*.

Mercurials.—The maximum amount of a mercurial preservative allowed in an official parenteral product is 0.01%. There was no hemolysis of rabbit or human erythrocytes in saline solutions containing 0.01% thimerosal. Trace hemolysis of rabbit erythrocytes was detected in 0.05% thimerosal, with total hemolysis occurring in concentrations of 0.5%. Trace hemolysis of human erythrocytes was observed in 1.5% thimerosal solutions, and near-total hemolysis was effected in 2.0% solutions. Although there was no macroscopic evidence of protein denaturation in this study, the use of an electron microscope by another investigator (22) has shown mercury to produce changes in the human red blood cell membrane in the form of folds, granulation, and coagulation.

Phenylmercuric compounds and mersalyl were reported (23) to be hemolytic agents due to the interaction of the organic mercurials with the cellular sulfhydryl groups which are considered to be essential for the maintenance of the intact structure of the erythrocyte. The interaction of mercury and sulfhydryl groups is also the basis for many explanations of the antibacterial mechanism of action of the mercurials (24).

Complete hemolysis of human erythrocytes in solutions of phenylmercuric nitrate was not accomplished because of the aqueous solubility limitations of the chemical. It was shown, however, that hemolysis commenced in solutions of 0.004% concentration. Rabbit erythrocytes hemolyzed in lower concentrations of the mercurial; 0.001% induced trace hemolysis, and 0.003% caused total hemolysis.

Shukis and Tallman (25), working with a series of aliphatic mercurials, found better antibacterial action with the compounds having a partition coefficient in favor of the lipid phase. Hess and Speiser (26) reported phenylmercuric borate, with its higher solubility in lipids, to be much more active against microorganisms than thimerosal which is poorly lipid soluble. They further describe the action of mercurials as a two-stage process: (*a*) the adsorption of the mercurial onto the membrane, and (*b*) the penetration through the lipid barrier. Thimerosal has been found to enter the rabbit red blood cell after a subcutaneous injection (27).

Cationic Surfactants.—Benzalkonium and benzethonium chlorides exerted similar effects on both human and rabbit erythrocytes. In all experiments, hemolysis of red corpuscles was initiated in cationic concentrations of between 0.0010 and 0.0017%, with total hemolysis occurring in solutions of between 0.0022 and 0.0027% concentration (Table I).

TABLE III.—BACTERICIDAL AND HEMOLYTIC CONCENTRATIONS OF SOME ALCOHOLS

Alcohol	—Concn., %, of Alcohol Causing— Total Hemolysis of Erythrocytes[a] Human	Rabbit	Total Killing of *S. aureus*[b]
Benzyl	1.20	0.94	0.90
Phenylethyl	0.80	0.62	0.50
Chlorobutanol	0.50	0.32	0.28
p-Chloro-*β*-phenylethyl	0.18	0.14	0.10

[a] Data from Table I. [b] Data from Lachman, *et al.* (21).

The hemolysis of human erythrocytes by ionic detergents has been attributed to a lowering of the interfacial tension and the collapse of the membrane's cholesterol-phospholipid-lipoprotein complex, with the cholesterol monolayer being thought of as the main site of the attack (28). As shown in Table I, the erythrocytes showed a delicate sensitivity to the presence of the cationic surface-active agents. The cationic agents examined were about 300 times as effective in inducing trace hemolysis and about 200 times as active in causing total hemolysis as phenol. Reported phenol coefficients for benzalkonium chloride on *S. aureus* at 37° range from 293 to 407 (29).

Rabbit blood in 0.05% benzalkonium chloride became denatured into a voluminous brown sediment.

Parabens and Sodium Formaldehyde Sulfoxylate.—Solutions of the parabens were employed in concentrations up to their individual maximum solubilities, *i.e.*, 0.25% methylparaben, 0.17% ethylparaben, 0.05% propylparaben, and 0.02% butylparaben (30). No hemolysis was induced by the paraben solutions, except for a saturated butylparaben solution which lysed 12% of the rabbit and 6% of the human erythrocytes it contacted. Butylparaben has been reported (30) to be the most toxic of this relatively nontoxic group of preservatives.

Sodium formaldehyde sulfoxylate, in amounts normally used in parenterals (0.1%), had no hemolytic effects on human or rabbit red blood cells. In 1% concentrations the erythrocytes blackened but remained unhemolyzed; in 5% solutions, hemolysis was induced with the liberation of a red-black exudate.

General Considerations.—It appears that preservative agents which affect the permeability and/or membrane integrity of the erythrocyte may have a like mechanism of action when functioning as antibacterial agents. Antibacterial preservatives which are not hemolytic may act through the disruption of a specific biological process or on a site unknown to, and thereby not affecting, the erythrocyte.

Further studies on the hemolytic activities of these and other preservative agents are being conducted with emphasis on the relationship between chemical structure and hemolytic activity.

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