Hemolysis of Erythrocytes by Antibacterial Preservatives IV

Hemolytic Activity of Chlorhexidine Diacetate

By HOWARD C. ANSEL

Chlorhexidine diacetate has been shown, *in vitro*, to be destructive to rabbit erythrocytes in a range of concentrations similar to that required for its antimicrobial effect, with total hemolysis and bacteriolysis occurring at similar dosage levels. It appears that the mode of action of chlorhexidine diacetate against microorganisms and erythrocytes is similar; that is, through the disruption of the structure and function of the cellular membrane. The hemolytic activity of chlorhexidine diacetate was reduced by the presence of polyethylene glycols of various molecular weights and by dimethylsulfoxide. This interference in the activity of chlorhexidine diacetate was considered from two possible modes: (*a*) chemical interaction between the chlorhexidine diacetate and the added substances, and (*b*) cellular effects of the added substances and chlorhexidine diacetate, making the hemolytic activity of the latter less pronounced. The latter mode of interference by the added substances is more strongly suspected.

CHLORHEXIDINE is a potent antibacterial compound, the properties of which were first described in 1954 (1). Since that time, investigations conducted mainly in Great Britain have been directed at determining the mode of action of chlorhexidine and its efficacy as a preservative agent particularly for ophthalmic preparations (2–10).

The present investigation represents a continuation of studies (11–14) to determine the hemolytic activity of antibacterial preservatives alone and in the presence of pharmaceutical adjuncts. Thus far, the work in the series has demonstrated a similarity between the drug concentrations required of certain preservatives to act effectively as antibacterial and hemolytic agents. Also, certain pharmaceutical adjuncts as the polyethylene glycols and dimethylsulfoxide (DMSO) have been shown to interfere with the hemolytic activity of certain preservatives. The information gathered in this series is expected to contribute both to the understanding of the cellular effects of preservative agents and to the effective preservation of pharmaceutical products, including those intended for intravenous administration. Chlorhexidine was selected for inclusion in the over-all study principally because its chemical structure, 1,6-di(4-chlorophenyldiguanido)hexane, is sufficiently diverse from those antibacterial agents already examined for hemolytic activity. Also, the bacteriological data for chlorhexidine, as reported in the current literature, provided a ready means for the comparison and possible correlation between the effects of the compound on microorganisms and on red blood cells.

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EXPERIMENTAL

Materials—The chlorhexidine diacetate employed in this investigation was pharmaceutical grade obtained through the courtesy of Ayerst Laboratories, Inc., New York, N. Y., and the dimethylsulfoxide was reagent grade obtained commercially.

Blood Samples—Rabbit blood, obtained by heart puncture and defibrinated in the manner previously described (15), was used throughout the study. Washed erythrocytes, prepared as reported in an earlier communication (14), were employed in certain experiments to reveal the influence of serum proteins on the hemolytic activity of chlorhexidine diacetate.

Each blood sample was collected just prior to its use and was verified for osmotic normalcy (11) during the course of each experiment.

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 (11). 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.

Experimental Solutions—Aqueous solutions were employed throughout the investigation. Chlorhexi-

dine diacetate test solutions of varying concentration were prepared in 0.6% sodium chloride. The sodium chloride afforded the erythrocytes protection against osmotic hemolysis, and therefore any hemolysis occurring in the test solutions could be attributed to the cytotoxic effects of the preservative agent (11). The data thus obtained established the minimum concentrations of chlorhexidine diacetate required to induce trace and total hemolysis of rabbit erythrocytes. The latter concentration, producing the optimum effect, was subsequently employed in experiments designed to determine the influence of polyethylene glycols of various molecular weights and of DMSO on the hemolytic activity of chlorhexidine diacetate.

Test solutions were prepared to contain the optimum hemolytic concentration of chlorhexidine diacetate in 0.6% sodium chloride and varying concentrations of polyethylene glycols 400, 1540, and 4000, or dimethylsulfoxide. The ability of the polyethylene glycols or of DMSO to interfere with the hemolytic activity of chlorhexidine diacetate would be reflected by decreased hemolytic response. The test solutions were equilibrated overnight at room temperature prior to conductance of hemolysis experiments at 37°. Appropriate control solutions were employed concurrently to affirm the effects attributed to the presence of the polyethylene glycols and DMSO.

The concentration of chlorhexidine diacetate in the various test solutions is expressed in terms of mcg./ml., in keeping with the precedent established by other investigators of the properties and activities of this preservative agent. In doing so, a direct comparison was facilitated between the data obtained in this study employing erythrocytes and that of previous studies employing microorganisms.

Kinetic Studies—The general experimental design of the research series is such that the hemolytic effects of the preservative agents and the influences of the pharmaceutical adjuncts are determined after a constant 45-min. incubation period. In the current project the hemolytic activities of certain representative test solutions were also determined at incubation intervals of from 5 to 20 min., during periods up to 120 min.

RESULTS AND DISCUSSION

Hemolytic Activity of Chlorhexidine Diacetate—Chlorhexidine diacetate at concentrations as low as 20 mcg./ml. induced trace hemolysis of both washed and unwashed rabbit erythrocytes (Fig. 1). As the concentrations of the test solutions were increased, hemolysis increased and at a concentration of 110 mcg./ml., total hemolysis first occurred. As can be seen in Fig. 1, the washed cells appeared slightly more sensitive to the lytic effects of chlorhexidine diacetate than did the unwashed cells. The absence of serum proteins and the mechanical effects of the repeated washings during the preparation of the washed cell samples could account for the increased cellular fragility and sensitivity to chlorhexidine diacetate.

It is interesting to note that in various studies (4, 5, 10) involving the application of chlorhexidine to the preservation of ophthalmic solutions, the successful concentrations (depending on such conditions of the experiment as: cell count, micro-

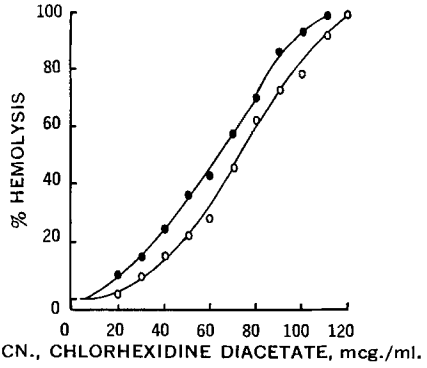

Fig. 1—Hemolytic activity of chlorhexidine diacetate in the presence of 0.6% NaCl. Key: ○, using unwashed erythrocytes; ●, using washed erythrocytes.

organism tested, temperature, and duration of incubation) ranged between 20 and 100 mcg./ml., a range which is nearly identical to that determined in the present investigation for trace to total destruction of erythrocytes. In practice, the most popular concentration of chlorhexidine employed as a preservative in ophthalmic solutions is reported to be 50 mcg./ml. (10).

Hugo and Longworth (2, 4) have investigated the mode of action of chlorhexidine diacetate against *Escherichia coli* and *Staphylococcus aureus*. They found chlorhexidine to be rapidly adsorbed by the cells. This adsorption was accompanied by cytological changes involving the permeability of the cells and their optical properties. The two microorganisms responded similarly to the effects of various dosage levels of chlorhexidine diacetate. At concentrations of 20 mcg./ml., the cells showed little damage to biochemical properties as well as an unchanged appearance as revealed by electron microscopy. At concentrations of 90 mcg./ml., chlorhexidine caused cytological damage resulting in widespread cellular leakage and in the formation of cellular ghosts devoid of cytoplasmic constituents. At concentrations of 200 and 500 mcg./ml., which greatly exceeded that required for cellular damage, the cells failed to exude their cytoplasmic constituents; however, compared to the controls, the cytoplasm appeared granular to electron microscopy and the cellular membranes appeared altered. They concluded from their studies that chlorhexidine exerts its bactericidal action by combining with the cell surface in a manner which lethally affects its structure and function thereby causing a disruption in its permeability barriers and over-all integrity. The release of cellular constituents by the bacterial cells was related to the level of drug adsorption. However, cellular release was not necessarily the sign of cellular death. This was demonstrated with highly bactericidal concentrations of chlorhexidine diacetate which as a consequence of their high concentration failed to induce bacteriolysis.

In the present study in which erythrocytes rather than microorganisms were exposed to various concentrations of chlorhexidine diacetate a similar pattern of cell response was noted (Fig. 1) with one exception. At high (500 mcg./ml.) chlorhexidine diacetate concentrations, which affected the bacterial cell membrane without inducing bacteriolysis, the

erythrocytes hemolyzed completely as they had at drug levels as low as 110 mcg./ml. In general, however, the red blood cells behaved similarly to the microorganisms, an observation which had been made before in this laboratory for other preservative agents (11–14). It is not unlikely that the mode of action suggested for chlorhexidine diacetate against microorganisms is similar against erythrocytes, that is, the disruption and disorganization of the structure and function of the cell membrane.

Kinetic Study of Chlorhexidine Diacetate—As depicted in Fig. 2, the hemolytic effects of chlorhexidine diacetate are manifest after a lag time apparently required for drug adsorption and consequent disruption of the permeability barriers of the cell. Gross cytolytic damage supported by cellular leakage proceeds rapidly, providing that the appropriate level of drug is present. In their observations on the effects of chlorhexidine diacetate on *E. coli*, Hugo and Longworth (4) concluded that of the two parmeters, time and concentration, the latter was the more important with respect to induced cytological damage.

Influence of Polyethylene Glycols and DMSO on the Hemolytic Activity of Chlorhexidine Diacetate—As noted in previous communications (12, 14), the presence of pharmaceutical adjuncts including the polyethylene glycols and dimethylsulfoxide interfere with the hemolytic activities of certain preservative agents suggesting like interference with preservative function.

The hemolytic activities of solutions containing 110 mcg./ml. of chlorhexidine diacetate, an amount sufficient to induce total hemolysis of erythrocytes (Fig. 1), in 0.6% sodium chloride and varying amounts of polyethylene glycols 400, 1540, and 4000 were determined along with the appropriate control solutions. As can be seen in Fig. 3, the presence of polyethylene glycols in increasing amounts and in increasing molecular weights was increasingly effective in preventing chlorhexidine-induced hemolysis. A similar influence of the polyethylene glycols in preventing hemolysis induced by antimicrobial preservatives was noted for phenolic compounds and was reported in an earlier communication (12). In that study a chemical interaction between the phenolic compound and the various polyethylene glycols was demonstrated titrimetrically and verified through the literature. In the current study, the possibility of a chemical interaction between the polyethylene glycols and the chlorhexidine diacetate, thereby rendering the preservative agent ineffective in its hemolytic capability, should be considered especially in light of the incompatible nature of both the polyethylene glycols (12) and chlorhexidine (5, 10) with other chemical materials. No visible signs of incompatibility were seen during the study. A second possibility for the protective action of the polyethylene glycols is a cellular effect of this chemical which interferes in some manner with the adsorption of chlorhexidine diacetate onto the erythrocytic membrane. This interference will be explored to a greater extent in a subsequent presentation.

The controversial chemical dimethylsulfoxide has been the subject of conflicting reports concerning its ability to act as a penetrant carrier of drugs through biologic membranes (16–20). Although DMSO freely penetrates the erythrocytic membrane

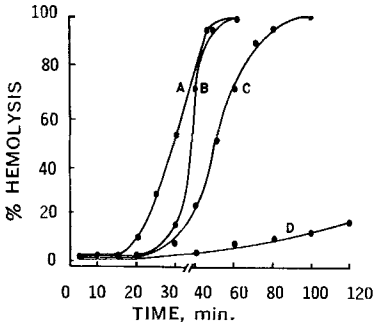

Fig. 2—Kinetic study of the hemolytic activity of various concentrations of chlorhexidine diacetate in the presence of 0.6% NaCl. Key: A, *110 mcg./ml.;* B, *90 mcg./ml.;* C, *70 mcg./ml.;* D, *20 mcg./ml.*

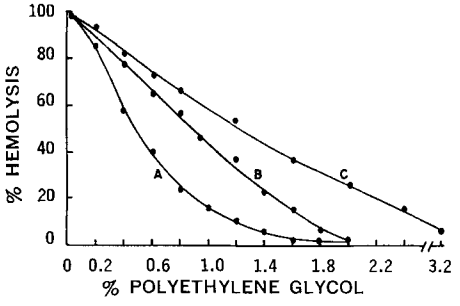

Fig. 3—Influence of PEG's on the hemolytic activity of 110 mcg./ml. of chlorhexidine diacetate in the presence of 0.6% NaCl. Key: A, *PEG 4000;* B, *PEG 1540;* C, *PEG 400.*

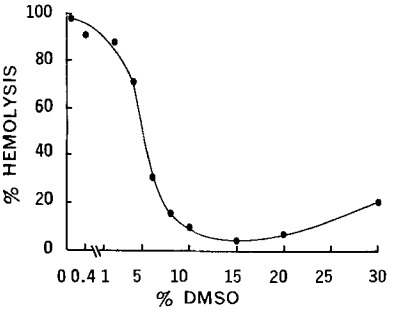

Fig. 4—Influence of DMSO on the hemolytic activity of 110 mcg./ml. of chlorhexidine diacetate in the presence of 0.6% NaCl.

and, in high concentrations, is destructive to red blood cells, it was shown (14) to greatly reduce, not enhance, the phenol-induced hemolysis of both washed and unwashed erythrocytes. It was suggested that this interference may be manifest through chemical interaction with the phenol or through a direct cellular effect. The present experiment involving the combination of DMSO and chlorhexidine diacetate was an outgrowth of the prior study. As can be seen in Fig. 4, the presence of DMSO reduced the hemolytic capability of an otherwise hemolytic concentration of chlorhexidine diacetate (110 mcg./ml.). All test solutions contain-

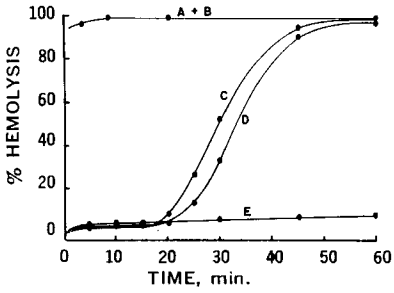

Fig. 5—Kinetic study of the hemolytic activity of various test solutions. Key: A, water; B, 15% DMSO; C, 110 mcg./ml. chlorhexidine diacetate in 0.6% NaCl; D, 110 mcg./ml. chlorhexidine diacetate, 0.6% NaCl, and 1% DMSO; E, 110 mcg./ml. chlorhexidine diacetate, 0.6% NaCl, and 15% DMSO.

ing concentrations of DMSO exceeding 35% caused the denaturation of blood, as noted in an earlier report (14).

As can be seen in Fig. 5, both distilled water and 15% DMSO induced total hemolysis within the first 5 min. of the study. Hemolysis occurring in the 15% DMSO test solution can be largely attributed to osmotic hemolysis resulting from the penetration of this hygroscopic material through the erythrocytic membrane (14). The addition of 0.6% sodium chloride to DMSO solutions of less than 35% concentration prevents osmotic hemolysis (14); hemolysis occurs in solutions containing greater amounts of DMSO due to the cytotoxic action of the more concentrated solutions. The addition of 1% DMSO to a solution containing 0.6% sodium chloride and 110 mcg./mg. of chlorhexidine diacetate slowed the rate of hemolysis induced by the latter and the addition of 15% DMSO all but prevented the chlorhexidine diacetate-induced hemolysis. The suspicion that DMSO exerts an independent cellular effect such as to prevent the preservative from exerting its own cellular activity has been bolstered by recent data obtained in this laboratory (21) showing a similar influence of DMSO on the hemolytic activities of such chemically diverse preservatives as phenylethyl alcohol, benzalkonium chloride, *p*-chlorophenol, benzyl alcohol, phenylmercuric acetate, and *m*-cresol. In each instance, the concentration of DMSO required to all but eliminate the hemolysis normally induced by the hemolytic concentrations of these preservatives was between 7 and 15%. Spectrophotometric analysis failed to reveal complexation between the DMSO and the chlorhexidine diacetate, further adding support to interference by DMSO by a cellular mechanism.

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Drug Standards

Determination of Phenylephrine in Combinations with Other Drugs

By JOSEPH LEVINE and THOMAS D. DOYLE

Phenylephrine combines with di-(2-ethylhexyl) phosphoric acid to form an ion-pair which can be extracted with immiscible solvents. Application of this means of extraction in conjunction with partition chromatography provides a method for the analysis of phenylephrine in its various combinations with other drugs.

THE ISOLATION and determination of phenylephrine by the usual methods for the analysis of alkoids[1] is not feasible because of the highly unfavorable extraction characteristics of the compound. Most of the published procedures for its analysis are not applicable in the presence of the drugs with which it is compounded in many formulations. Pratt (2), applying the acetylation procedure of Welsh (3), converted phenylephrine in aqueous solution to the diacetyl derivative, which is readily extracted with im-

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[1] Because alkaloids and many pharmaceutically important synthetic organic bases present identical analytical problems, and since the word "alkaloid" is reserved for the natural product, it has been proposed (1) that the word "alkoid" be used to encompass the entire group.