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III. A METHOD FOR DISTINGUISHING SPECIFIC FROM NONSPECIFIC HEMOLYSINS

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Abstract—1. A wide variety of surface-active and/or lipid-soluble substances (vitamin A alcohol, lysolecithin, lecithin, stearic acid, sodium taurocholate, lithocholic acid and other steroids, butanol and other alcohols, prochlorperazine ethane disulfonate, sodium lauryl sulfate, and Triton X-100) in low concentrations will protect or stabilize human erythrocytes against hypotonic hemolysis; high concentrations of most of these compounds cause direct hemolysis. None of these hemolysins, therefore, may be considered specific in eliciting this biphasic effect.

2. Specific hemolysins (digitonin, holothurin A, saponin, filipin), which may be considered as specific in the sense that they have a very high affinity for cholesterol and presumably hemolyze by binding to or extracting the membrane cholesterol, do not cause erythrocyte stabilization at any concentration.

3. The nonspecific stabilization has a long duration (3 hr) without any diminution in the degree of stabilization.

4. Two polyene antibiotics, amphotericin B and nystatin (whose cholesterol affinity is not so high as that of filipin), in low concentrations produce a stabilization lasting only a few minutes, the concentration–hemolysis curve soon approaching the monophasic pattern of the four specific hemolysins.

5. Two other polyene antibiotics, pimaricin and ascosin, also cause a transient stabilization. Ascosin has the longest period of transient stabilization (1 hr).

6. Whether a membrane lysin does or does not in low concentrations cause a prolonged and steady membrane stabilization may therefore be a simple possible criterion for distinguishing between specific and nonspecific lysins. On this basis tyrocidine B would be considered a specific hemolysin.

THE PURPOSE of this paper is (1) to suggest a simple method for determining whether a chemically pure hemolysin causes hemolysis by acting on some specific receptor or component of the erythrocyte or whether it acts as a nonspecific detergent; and (2) to demonstrate that a wide variety of lipid-soluble and surface-active compounds in very low concentration will protect and stabilize human erythrocytes from hypotonic hemolysis.

Although there is a voluminous literature describing the action of various hemolysins,[1] there does not appear to be any procedure that will distinguish between specific and nonspecific hemolysins. The words "specific" and "nonspecific" here refer to whether or not there is a "receptor" or a "component" on the erythrocyte membrane that strongly interacts or binds the hemolysin (see Ref. 2 for broader definitions).

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Until now most of the experiments determining the hemolytic potency of various compounds have been done with plasma or isotonic saline, 0·9% NaCl, as the suspending medium for the erythrocytes. In isotonic saline all lysins, whether they are specific or not, will at high concentrations cause hemolysis; in low concentrations there is no effect on hemoglobin release. This monophasic lytic effect for all lysins in 0·9% NaCl allows no qualitative and simple distinction between the specific and nonspecific hemolysins. It will be shown in this paper that the routine use of a hypotonic saline test medium will bring out a difference in the mode of hemolysis between these two groups of hemolysins. All the nonspecific hemolysins in very low concentrations stabilize the erythrocytes from hypotonic hemolysis; the specific hemolysins, on the other hand, cause no such stabilizing effect at any concentration.

In 1908 Traube[3] reported that low concentrations of amyl alcohol inhibited the hypotonic hemolysis of human erythrocytes but that high concentrations of the alcohol caused lysis. Since then a biphasic effect of membrane protection–lysis *in vitro* by a variety of surface-active agents has been reported for miscellaneous biological membranes. Hypotonic hemolysis is inhibited by low concentrations of anesthetics and soaps (see Jacobs and Parpart[4] for some references), Triton X-100[5, 6] (a polyoxyethylene ether), vitamin A,[7] phenothiazine tranquilizers,[8, 9] and steroids.[10] At higher concentrations all these surface-active compounds are hemolytic. It has also been reported that some steroids[11, 12] and certain detergents[13] will reduce the amount of hemolysis caused by prolonged blood storage, by sulfhydryl inhibitors, and by mechanical stress, although the effect of these compounds on hypotonic hemolysis was not tested. Biphasic effects of drugs on subcellular membrane-bounded organelles *in vitro* have also been noted in the literature (see Ref. 8 and 9 for bibliography).

Since such a wide variety of hemolysins causes membrane stabilization at low concentrations, a working hypothesis was formed that erythrocyte stabilization by drugs was nonspecific in the sense that virtually any surface-active or lipid-soluble compound would exert the effect. The studies described in this paper were undertaken, therefore, to check the hypothesis that the biphasic stabilization–lysis effect was a property of surface-active and lipid-soluble compounds acting on membranes *in vitro*. The results support this hypothesis with one important exception: hemolysins which are known to have a specific affinity for a membrane component will not cause stabilization.

MATERIALS AND METHODS

Digitonin (certified reagent) and 1-butanol were obtained from Fisher Scientific Co. Lithocholic acid (cholanic acid-3 α-ol) was from Steraloids, Inc., New York, N.Y. Sodium lauryl sulfate and sodium taurocholate (impure) were from Mann Research Laboratory. Tyrocidine B was purified and generously supplied by Dr. Michael Ruttenberg of the Rockefeller University. Crystalline vitamin A alcohol was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Triton X-100 came from Rohm and Haas, Ltd. Synthetic L-α-dipalmitoyl lecithin was from Calbiochem Corp., and lysolecithin from K & K Laboratories. Stearic acid was obtained from Eastman Organic Chemicals, Rochester, N.Y. Saponin ("pure white") was from Merck AG, Germany. Holothurin A (a saponin preparation purified from the sea cucumber, *Actinopyga agassizi*) was purified and graciously donated by Dr. J. D. Chanley, the

Mount Sinai Hospital, New York, N.Y. The following firms and laboratories kindly donated drug samples: Smith, Kline and French, Philadelphia: prochlorperazine ethane disulfonate (Compazine); The Upjohn Co., Kalamazoo, Mich.: filipin; Commercial Solvents Corp., Terre Haute, Ind.: ascosin; The Squibb Institute for Medical Research, New Brunswick, N.J.: amphotericin B type 1, and nystatin; American Cyanamid Co., Agricultural Div., Princeton, N.J.: pimaricin (Myprozine).

In order to calculate the molarities, the following molecular weights of the polyene antibiotics were used: pimaricin, 681; ascosin, 823-867 ($C_{43-33}H_{69}NO_{14-16}$; private communication from Dr. W. F. Phillips of Commercial Solvents Corp.); filipin, 571; nystatin, 932; amphotericin B, 960. According to the u.v. spectroscopic analysis performed by Commercial Solvents Corp., the purity of ascosin was given as approximately 77 per cent. The purity of the other four polyene antibiotics was assumed to be around 90 per cent, in keeping with the estimate made by Demel *et al.*[14] The molarities reported in this paper take into account these values for purity. The molecular weights used for the other compounds were as follows: saponin, 1500;[15] holothurin A, 1150;[15] digitonin, 1215; stearic acid, 284·5; lysolecithin (assumed that palmitic acid is the fatty acid moiety), 296; dipalmitoyl-α lecithin, 722; 1-butanol, 74·1 (with a density of 0·810 gram/ml); lithocholic acid, 376·6; prochlorperazine ethane disulfonate, 563·5; sodium lauryl sulfate, 288·4; sodium taurocholate, 573; vitamin A alcohol, 286·5; Triton X-100, average molecular weight 680.[16]

The experiments were carried out as follows. A stock suspension of human erythrocytes (from a freshly drawn blood sample of a fasting volunteer) of about 4×10^8 cells/ml in 154 mM NaCl, 10 mM sodium phosphate buffer, pH 7·0, was prepared as previously outlined.[8, 9] Aliquots of 0·1 ml were pipetted from this suspension into 1·5 ml or 1·55 ml of hypotonic test solution. The final concentration of erythrocytes was between 2×10^7 and $2{\cdot}5 \times 10^7$ cells/ml. The tube containing the drug and the erythrocytes was immediately mixed twice, thoroughly but briefly (4 sec), with a Vortex mixer.

The hypotonic test solution consisted of between 66 and 69 mM NaCl in 10 mM sodium phosphate buffer, pH 7·0, containing a drug in concentrations ranging between 10^{-10} and 10^{-2} M; the molarity of NaCl was chosen such that approximately 50–60 per cent of the erythrocytes were hemolyzed. After remaining at room temperature (21° to 24°) for a certain time (indicated in Results) the unhemolyzed erythrocytes were centrifuged down in a swinging-bucket centrifuge at 1500 *g* for 1 min. The hemoglobin concentration of the clear supernatant was measured by recording the optical density at 543 mμ in a Beckman DU spectrophotometer. All experiments were done in duplicate or triplicate.

Some of the drugs were dissolved into and diluted directly with the aqueous hypotonic test solution; this group included holothurin A, saponin, tyrocidine B, vitamin A alcohol, Triton X-100, sodium lauryl sulfate, sodium taurocholate, prochlorperazine ethane disulfonate, and 1-butanol. The five polyene antibiotics were prepared as concentrated stock solutions in dimethylsulfoxide (commercial grade, unpurified) The other compounds of low aqueous solubility, stearic acid, lecithin, lysolecithin, lithocholic acid, and digitonin, were prepared as concentrated stock solutions in ethanol. The drugs in dimethyl sulfoxide and in ethanol were added as 0·05-ml aliquots to the 1·5-ml hypotonic test solution about 20 sec before adding the erythrocytes. Separate experiments indicated that ethanol and dimethylsulfoxide did not affect

the erythrocytes at these low concentrations; for example, identical results were obtained with butanol prepared in either aqueous or ethanolic solution.

RESULTS

A. Nonspecific hemolysins. The results shown in the bottom half of Fig. 1 and in Fig. 2 demonstrate that wide varieties of different chemical compounds in low concentration stabilize erythrocytes against hypotonic hemolysis; at higher concentrations these compounds cause complete hemolysis or precipitate the erythrocyte protein. The ordinate in Figs. 1 and 2 is that of relative hemolysis; this was obtained by dividing

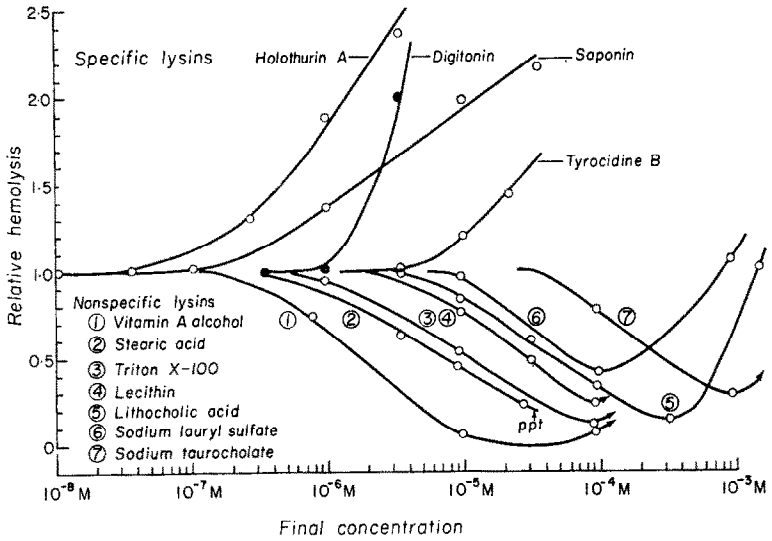

FIG. 1. A heterogeneous group of compounds, cationic, anionic, and nonionic (1–7, above), in low concentrations protect or stabilize human erythrocytes against hypotonic hemolysis; high concentrations of these compounds lead to complete hemolysis (arrowheads, above). The steroid glycoside hemolysins, digitonin, holothurin A, and saponin do not in low concentrations cause stabilization against hypotonic hemolysis. All compounds above were dissolved in hypotonic NaCl (66–69 mM, pH 7) except lithocholic acid, stearic acid, and lecithin, which were added from concentrated ethanolic solutions; ppt indicates that stearic acid precipitated before the cells were added. A relative hemolysis of 1·0 indicates an absolute hemolysis of about 45 per cent. The cell density was 2·4 × 10^7 cells/ml.

the amount of hemoglobin released (during 5-min hypotonic hemolysis) in the presence of the drug by the amount of hemoglobin released in the absence of the drug (but with ethanol or dimethylsulfoxide added in those cases where these solvents were employed).

Erythrocyte stabilization occurred at low concentrations of nonionic hemolysins (Triton X-100, 1-butanol, vitamin A alcohol), anionic hemolysins (sodium taurocholate, sodium lauryl sulfate, stearic acid, and lithocholic acid), and cationic hemolysins (prochlorperazine ethane disulfonate, lecithin, and lysolecithin). All these compounds will be referred to as nonspecific stabilizers, since the phenomenon of stabilization is not specific for any special group of hemolysins.

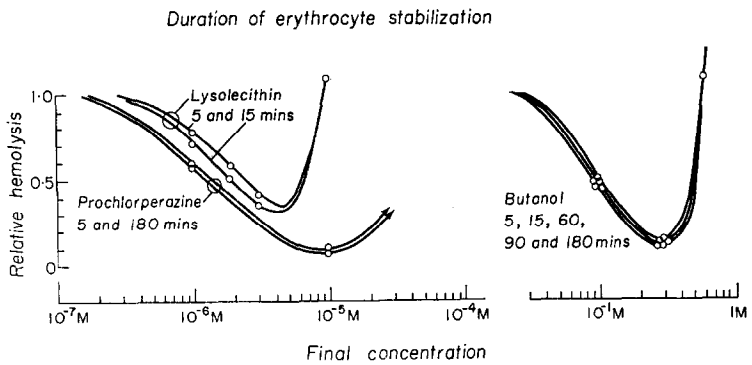

FIG. 2. Erythrocyte stabilization by low concentrations of nonspecific compounds, a cationic detergent (prochlorperazine ethane disulfonate), an amphoteric detergent (lysolecithin), and a non-ionic detergent (1-butanol), lasts for many minutes or hours without diminution in the degree of stabilization. Lysolecithin was added from concentrated ethanolic solutions. A relative hemolysis of 1·0 represents a hemolysis of about 50 per cent.

The results of Fig. 2 indicate that the duration of erythrocyte stabilization by these nonspecific compounds was quite long and extended to at least 3 hr without any significant loss in the degree of stabilization.

B. Specific hemolysins. Compounds such as digitonin, saponin, and holothurin A are known for their extremely high affinity for cholesterol (see Discussion), and this is presumably the basis for their mode of hemolytic action. When these three steroid glycosides were tested for their effect on hypotonic hemolysis, it was observed that stabilization did not occur at low drug concentrations; these three saponins either caused no effect or caused hemolysis, depending on the concentration, and the results are shown in the top part of Fig. 1.

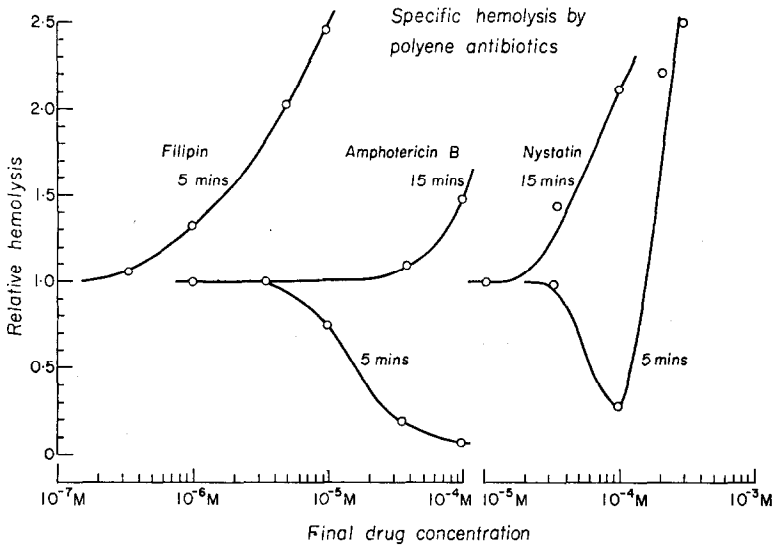

FIG. 3. Low concentrations of polyene antibiotics did not stabilize (filipin) or only transiently stabilized (amphotericin B and nystatin) human erythrocytes against hypotonic hemolysis. A relative hemolysis of 1·0 corresponds to an absolute hemolysis of about 40 per cent.

A monophasic lytic effect was also observed with the cyclic decapeptide antibiotic tyrocidine B (Fig. 1).

The polyene antibiotics are also known to have a very high affinity for cholesterol, and this affinity has been offered as the explanation for their hemolytic mechanism (see Discussion). The effects of five polyene antibiotics on hypotonic hemolysis are depicted in Figs. 3 and 4. Filipin causes effects very similar qualitatively and quantitatively to digitonin, namely, a monophasic lytic effect without any stabilization at low

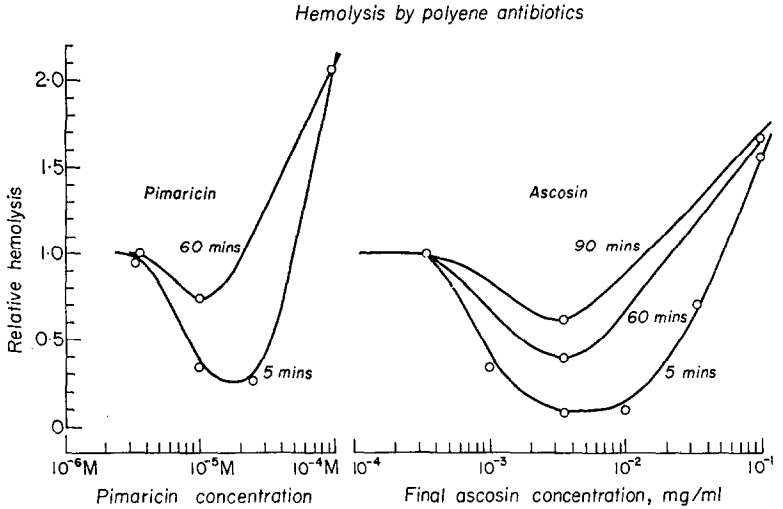

FIG. 4. Low concentrations of two polyene antibiotics, pimaricin and ascosin, cause a transient stabilization of human erythrocytes against hypotonic hemolysis; high concentrations elicit rapid hemolysis. A relative hemolysis of 1·0 corresponds to an absolute hemolysis of about 40 per cent.

concentrations. The other four polyene antibiotics, amphotericin B, nystatin, pimaricin, and ascosin, cause stabilization at low concentrations. This stabilization is only transient, however, diminishing steadily after the onset of action. It is impossible to say whether this transient stabilization is a property of the polyene compounds themselves or whether it is caused by some lipid impurity. The purity of these compounds has been estimated at 90 per cent with the exception of ascosin which is 77 per cent.

DISCUSSION

The results indicate that the use of hypotonic saline appears to be of some value in distinguishing specific from nonspecific hemolysins. A qualitative difference between these two classes of hemolysins is that the nonspecific hemolysins can cause stabilization against hypotonic hemolysis while the specific ones do not (or only transiently do so). A second and only quantitative difference is that the specific hemolysins hemolyze at concentrations which are 10 to 100 times lower than the nonspecific compounds.

Since the phenomenon of erythrocyte stabilization is seen with many chemically different and heterogeneous agents it may be said that the effect of stabilization is not specific for any one group of hemolysins. All these nonspecific compounds cause

the biphasic effect of stabilization and lysis. The only factor that these agents seem to have in common is that of surface activity and/or high lipid solubility. While probably all membrane stabilizers are surface-active and/or lipid-soluble, not all surface-active compounds will cause membrane stabilization. Saponin, holothurin A, and tyrocidine B are surface-active but do not cause stabilization.

On the basis of studies of cholesterol monolayer penetration by saponin, a very high specific affinity between cholesterol and saponin is known to exist.[17, 18] The recent work of Demel *et al.*[14] shows that filipin penetrates monolayers of cholesterol and ergosterol in the face of high initial film pressures.

While a wide variety of hemolysins caused the biphasic effect of stabilization and lysis, only those hemolysins which are known to have a very high specific affinity for a certain membrane component (e.g. cholesterol) caused a monophasic effect. According to this criterion the monophasic lytic effect caused by tyrocidine B suggests that it is of the "specific" type, there being presumably some receptor or component on the membrane to which the antibiotic combines tenaciously.

Although vitamin A is a polyene compound, it does not reveal a mode of hemolytic action similar to that of the polyene antibiotics, since the vitamin causes long-term stabilization at low concentrations. This finding is consistent with the work of Bangham *et al.*,[19] who found that the vitamin interacted more strongly with lecithin than with cholesterol.

Lysolecithin, normally comprising about 1 per cent of the total erythrocyte lipids or about 2 or 3 per cent of the phospholipids was found to cause 50 per cent erythrocyte stabilization at around 10^{-6} M. This stabilizing effect of lysolecithin is a confirmation of the effect first described by Collier.[20]

The transient stabilization caused by amphotericin B, Nystatin, pimaricin, and ascosin is presumably a part explanation of Kinsky's observation[21] that nystatin and pimaricin required much longer incubation periods for erythrocyte hemolysis (in isotonic solution) and much higher concentrations than those required with filipin.

Other studies indicate that the nonspecific stabilization may be associated with an increase in the surface area/volume ratio of the erythrocytes; this area/volume ratio may increase by an expansion of the cell membrane. The work of Rand and Burton[22] and of Castle and Daland[23] has shown that cells having high area/volume ratio hemolyze in solutions of lower osmotic pressure. The membrane expansion created by the nonspecific stabilizers will therefore be associated with an increase in the "critical hemolytic volume" of the erythrocyte. Such an effect has been noted by Barac-Nieto *et al.*,[5] who found that Triton X-100 (2 mg/100 ml) raised the critical hemolytic volume by 11 per cent.

The saponins and the polyene antibiotics were selected in this study as examples of specific hemolysins because only for these two types of compounds is there adequate information on binding specificity. It will be necessary to test other categories of hemolysins with hypotonic saline. It is hoped that those working with agglutinating and hemolytic antibodies or with bacterial hemolysins, and also those working with subcellular organelles, will be encouraged to try the simple procedure suggested in this paper.

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