Part A 26, 1813.
Lichter, R. L., and Roberts, J. D. (1970b), *J. Org. Chem. 35*, 2806.
Lichter, R. L., and Roberts, J. D. (1971), *J. Amer. Chem. Soc. 93*, 3200.
Lichter, R. L., and Roberts, J. D. (1972), *J. Amer. Chem. Soc. 94*, 2495.
Pachler, K. G. R. (1963), *Spectrochim. Acta 19*, 2085.
Pachler, K. G. R. (1964), *Spectrochim. Acta 20*, 581.
Pregosin, P. S., Randall, E. W., and White, A. I. (1971), *Chem. Commun.*, 1602.
Ramachandran, G. N., Chandrasekaran, R., and Kopple, K. D. (1971), *Biopolymers 10*, 2113.
Randall, E. W., and Gillies, D. G. (1971), *Progr. Nucl. Magn. Resonance Spectrosc. 6*, 119.
Sunners, B., Piette, L. H., and Schneider, W. G. (1960), *Can. J. Chem. 38*, 681.
Webb, L. E., and Lin, C. F. (1971), *J. Amer. Chem. Soc. 93*, 3818.
Ziauddin and Kopple, K. D. (1970), *J. Org. Chem. 35*, 253.

The Mechanism of Polymyxin B Action and Selectivity toward Biologic Membranes†

Chuen-Chin HsuChen and David S. Feingold*

ABSTRACT: The selectivity of action of the polymyxin antibiotics was studied using lipid spherules in aqueous suspension (liposomes) as a model system; the release of trapped glucose marker was the index of membrane damage. The polymyxin B induced glucose release was observed to be a specific process which differentiates among phospholipids with various "polar head" structures. Liposomes prepared from phosphatidylethanolamine were extremely sensitive to polymyxin B while none of those prepared from the *N*-methyl-substituted analogs (*N*-methylphosphatidylethanolamine, *N*,*N*-dimethylphosphatidylethanolamine, phosphatidylcholine) were sensitive to the antibiotic. The N-methylated analogs of phosphatidylethanolamine protected phosphatidylethanolamine-containing liposomes from polymyxin B. The efficiency of this protection was a function of the molar per cent of the analog in the membrane model and the antibiotic concentration. The pH optimum of the polymyxin B induced glucose leakage in liposomes with phosphatidylethanolamine as the major component was between 7.0 and 8.5 with a marked decrease in activity at extremes of pH. We conclude that the polymyxin susceptibility of biologic membranes requires both the presence of target molecules such as phosphatidylethanolamine, and a threshold density of these molecules on the membrane surface. On the basis of these data with the model membranes a mechanism to explain the selective toxicity of the polymyxin antibiotics is proposed. According to this mechanism proton transfer between the antibiotic and the membrane may be the initial step leading to the irreversible damage in membranes containing phosphatidylethanolamine as the dominant phospholipid.

The polymyxin family of peptide antibiotics has potent bactericidal activity against most Gram-negative bacilli. These antibiotics share several structural features including: (1) a cyclic heptapeptide; (2) a preponderance of the basic amino acid, α,γ-diaminobutyric acid, and (3) a side chain which terminates with a short-chain fatty acid such as methyloctanoic acid. Our present knowledge on the mode of action of the polymyxins began with the pioneer studies of Newton in the early 1950's (reviewed in Newton, 1956). He showed that the antibiotics interacted with cell surface components, probably phospholipids, and demonstrated the irreversible breakdown of the permeability barriers associated with the bactericidal action of polymyxin. Few (1955) demonstrated the interaction of polymyxin with monolayers of phospholipids and lipids of bacterial origin. The strict structural requirements for biological activity of the polymyxin molecule were recognized during the chemical synthesis of this compound (Vogler and Studer, 1966); the structural requirements of the phospholipid molecule determining antibiotic susceptibility have not been systematically studied. This is of special interest since the polymyxins are among the very few membrane-active antibiotics which show sufficient selective membrane toxicity to be of use in treating infections in man.

Previous work suggest that one can sharply differentiate two types of resistance to the polymyxins. *Proteus mirabilis*, and probably other bacteria, are resistant because the cell wall of the organism blocks access of the antibiotic to the susceptible cytoplasmic membrane (Teuber, 1969; Sud and Feingold, 1970; HsuChen and Feingold, 1972). Mammalian cells are also resistant but they lack cell wall and the membranes must be inherently resistant to destruction by the polymyxins. The studies reported herein were designed: (1) to define the nature of the inherent cytoplasmic membrane resistance to the antibiotic and (2) to demonstrate that phospholipids with different "polar head" structures respond to polymyxin differently. The model membrane system used was similar to that described by Haxby *et al.* (1968).

Experimental Section

Preparation of Lipids and Liposomes for Study. Lipids were extracted from *Escherichia coli 200P* (a K12 strain) as described by Kanfer and Kennedy (1963) followed by pre-

† From the Infectious Disease Unit, Beth Israel Hospital-Children's Hospital Medical Center and the Departments of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215. *Received June 12, 1972.* Supported in part by U. S. Public Health Service Grants AI-06313 and TO1-A1-00350 from the National Institute of Allergy and Infectious Diseases.

cipitation with cold acetone. Lipids were isolated from human erythrocyte membrane as previously described (Blostein, 1968; Ways and Hanahan, 1964). Phosphatidylethanolamine (isolated from bacteria) and *N*-methyl-L-phosphatidylethanolamine (dipalmitoyl, synthetic) were purchased from Calbiochem; L-3-phosphatidyl-*N*,*N*-dimethylethanolamine was obtained from Sigma, and egg lecithin was from General Biochemicals. Cholesterol and dicetyl phosphate were also purchased from commercial sources. All of the phospholipids were chromatographically homogenous on silica gel thin-layer plates developed with chloroform–methanol–water (65:25:4, v/v). The stock preparations of phospholipid, containing 1–2 μmol of phosphorus/ml of benzene, were stored at $-20°$ under a nitrogen atmosphere. Total phosphorus was determined by the method of Bartlett (1959).

In general liposomes were prepared by dispersing 0.5 μmol of phospholipid phosphorus into 0.5 ml of aqueous glucose (0.3 M) as described by Kinsky *et al.* (1969). When more than one phospholipid was present, the molar ratio of phospholipid–cholesterol was 1:0.5; when dicetyl phosphate was present, the molar ratio of phospholipid–cholesterol–dicetyl phosphate was 1:0.5:0.1, respectively. Glucose not associated with the liposomes was removed by passing the liposome suspension through a Sephadex G-75 column packed in 0.15 M NaCl (saline). In general 0.5 g of Sephadex was packed in columns of 1.2-cm diameter. The final concentration of liposome was 0.2 μmol of phosphorus/ml; 0.1 ml of this suspension was used per assay.

Testing for Polymyxin B Sensitivity. To test for polymyxin B susceptibility, freshly drawn human erythrocytes were washed in saline until the supernatant was clear (4°, 3000*g*, 10 min), diluted into ten volumes of saline, and 0.1 ml of this suspension was added to tubes containing polymyxin B at various concentrations in saline. The final volume of each sample was adjusted to 1.5 ml with saline, and the sets of tubes incubated at 37°. The final erythrocyte concentration was about 3×10^7 cells/ml. After 1 hr, the samples were centrifuged and the extent of hemolysis was determined by the absorbancy of the supernatant at 540 nm. The experimental data were corrected for the release of hemoglobin observed in the absence of polymyxin B. The 100% hemolysis value was obtained by diluting 0.1 ml of cell-suspension into 3 ml of distilled water; 100% hemolysis yielded an absorption of 0.844 at 540 nm.

The effect of polymyxin B on liposomes was determined by comparing the release of glucose from liposomes in the presence and absence of the antibiotic. The assay system was essentially the one developed by Kinsky *et al.* (1969) with the following modifications. (1) The final concentrations of $MgCl_2$ and $CaCl_2$ in the assay mixture were 10^{-4} and 5×10^{-6} M, respectively. (2) Saline (0.15 M NaCl) was used instead of Veronal-buffered saline; the assay system was still buffered with 50 mM Tris-HCl at pH 7.5. (3) The enzymatic assay reaction for glucose was started by the addition of TPN instead of liposomes. To control for the light-scattering changes due to interaction of polymyxin with the liposomes and to account for variations in turbidity of liposome preparations of different lipid compositions, the reaction mixtures were preincubated for 30 min at room temperature (about 20°) prior to the addition of TPN. The difference in absorbancy at 340 nm just before and 30 min after the addition of TPN were used for calculation of glucose release. Preliminary experiments showed that over 90% of the polymyxin-dependent glucose release was complete within 30 min and that there was no appreciable further absorbancy change between the 30-min reading and readings taken 2 hr later. (4) The amount of total glucose enclosed in liposomes was determined by preincubating 0.1 ml of liposomes with 0.1 ml of 10% Triton X-100, heating in a boiling water bath for a few seconds, and cooling to room temperature prior to glucose assay; these values are referred to as the Triton control. For liposomes prepared in the absence of dicetyl phosphate, the total amount of glucose trapped was 90 nmol of glucose $\pm 30\%$ per 0.1 ml of liposome suspension. The presence of dicetyl phosphate did not change the amount of trapped glucose in liposomes prepared from *E. coli* phospholipid or human red blood cell lipid, but increased the amount of trapped glucose in liposomes prepared from all the methylated phosphatidylethanolamines by approximately twofold. The final volume in the assay tubes was 1.1 ml; hence, where the antibiotic concentration is recorded in micrograms per assay the same numerical value can be considered as micrograms per milliliter for practical purposes.

To examine the effect of pH on polymyxin-dependent release of glucose from liposomes, the above assay procedures were slightly modified since the enzymatic system for quantitating glucose did not work at extremes of pH. Thus 0.2 ml of liposome suspension (at 0.5 μmol of phosphorus/ml) was added to tubes containing 10 μg of polymyxin, 50 mM NaCl, and 10 mM buffers at various pH's in a final volume of 1.0 ml. For each sample, there was a parallel blank control with buffers at the same pH but without antibiotic. After incubating at 20° for 1 hr, the reaction mixtures were centrifuged at 10,000*g* for 30 min and 0.2 ml of the clear supernatant was analyzed for glucose released by the enzymatic system described. The reagents for the enzymatic glucose assay (hexokinase, glucose-6-phosphate dehydrogenase, NaTPN, Na_2-ATP were obtained from commercial sources and polymyxin B sulfate (Aerosporin) from Burroughs-Wellcome, Inc. Melittin was purchased from the Sigma Chemical Co.

Preliminary experiments revealed that the incorporation of cholesterol into the liposomes had no effect on the action of the polymyxin B, but did result in more stable liposomes as evidenced by lower control values for glucose marker release. Hence, except with erythrocyte lipids, cholesterol was incorporated into all lipid suspensions at a ratio of 0.5 mol of cholesterol to 1.0 mol of phospholipid (to 0.1 mol of dicetyl phosphate when this was included).

Results

The Specificity of Membrane Phospholipid Composition in Conferring Polymyxin B Sensitivity. The sensitivity of fresh washed human erythrocytes to polymyxin B in 0.15 M NaCl was examined by measuring the hemolgobin released to the supernatant (Table I); the cells were very resistant to hemolysis even on exposure to 500 μg/ml of polymyxin B. This was reflected in the resistance of the liposomes prepared from the lipids extracted from the erythrocytes; the data in Table I also show that only small amounts of glucose were released by the antibiotic. Lipid analyses of human erythrocyte membrane regularly show the phospholipids phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and phosphatidylserine. The former 2 phospholipids with methylated polar head groups comprise about 60% of the total phospholipids whereas phosphatidylethanolamine approximates 20% of the total (reviewed by Rouser *et al.*, 1968). Cholesterol makes up about 25% of the total lipid.

In contrast to red blood cells, *E. coli* cells and liposomes prepared from their extracted lipids were exquisitely susceptible to membrane damage caused by polymyxin B. The ex-

TABLE I: Effect of Polymyxin B on Human Erythrocytes and Liposomes Prepared from Extracted Erythrocyte Lipids.[a]

Polymyxin Concn (μg/ml)	Polymyxin B Effect (% of Control)			
	On Erythrocytes		On Eyrthrocyte Liposomes	
	Expt 1	Expt 2	Expt 1	Expt 2
5	0.47	0	16.1	11.9
50	5.30	3.14	28.6	26.2
500	8.70	6.53		

[a] Polymyxin B action on erythrocytes was measured from the amount of hemoglobin released as described in the text; polymyxin B action on erythrocyte lipid-derived liposomes was measured from the amount of marker glucose released as described in the text.

tracted lipids from these organisms contained 79% phosphatidylethanolamine and 18% phosphatidylglycerol (by thin-layer chromatography, extraction and phosphorus analysis). From the data shown in Figure 1A it can be seen that at 1 μg of antibiotic/assay or approximately 1 mol of polymyxin B/25 mol of phospholipid, liposomes prepared from the *E. coli* lipids released about 30% of their trapped glucose; at a fivefold increase in the antibiotic concentration (1 mol of antibiotic/5 mol of phospholipid) over 80% of the glucose was released.

It appeared possible that polymyxin, on its interaction with phospholipid, was able to discriminate on the basis of structural variations of the polar heads of the phospholipid. The qualitative and quantitative aspects of the lipid–antibiotic interactions were examined by testing the sensitivity of liposomes prepared from the N-methylated analogs of phosphatidylethanolamine and mixtures of the analogs with phosphatidylethanolamine. A methodologic problem arose immediately in that pure phosphatidylethanolamine did not form effectively sealed liposomes (Papahadjopoulos and Miller, 1967). We used two approaches to circumvent this difficulty. Dicetyl phosphate was incorporated into liposomes at one-tenth the molar concentration of phospholipid; these liposomes effectively trap aqueous glucose solution. Figure 1B shows the sensitivity of phosphatidylethanolamine–cholesterol–dicetyl phosphate (1.0:0.5:0.1) liposomes to polymyxin B. The results were similar to those seen with liposomes from *E. coli* lipid. As a second approach, *E. coli* lipid was used instead of 100% phosphatidylethanolamine. Included in Figure 1 are the data on the susceptibility of liposomes prepared from the N-methylated analogs of phosphatidylethanolamine without dicetyl phosphate (1A) and with it (1B). Although liposomes prepared from the methylated analogs trapped more glucose marker in the presence of dicetly phosphate, as noted in the previous section, none of these liposomes released more than 10% of the marker on incubation with the antibiotic. Melittin (Sessa *et al.*, 1969; Williams and Bell, 1972), another membrane-active polypeptide, also served as control and was as effective as Triton in disrupting all the liposomes studied.

The striking lack of polymyxin B sensitivity with all three N-methylated phosphatidylethanolamines was unexpected. The next series of experiments was designed to determine the consequence of mixtures of phosphatidylethanolamine and

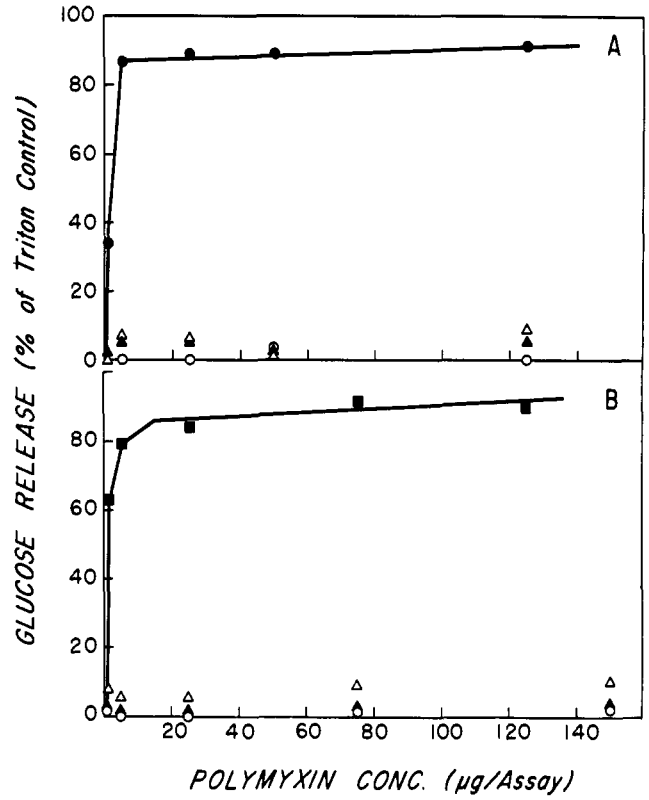

FIGURE 1: Effect of polymyxin B on liposomes prepared from various phospholipids. In part A liposomes were prepared from mixtures containing phospholipid and cholesterol (1:0.5, molar ratio); in part B liposomes were prepared from mixtures containing phospholipid, cholesterol and dicetyl phosphate (1:0.5:0.1, molar ratios). Liposomes were used at a concentration of 0.02 μmol of phosphorus/assay. Glucose released was determined spectrophotometrically as described in the text. The reactions were run in 50 mM Tris-HCl at pH 7.5. The various phospholipids used are designated as follows: (●) phospholipids extracted from *E. coli*, (○) *N*-methylphosphatidylethanolamine, (▲) *N,N*-dimethylphosphatidylethanolamine, (△) phosphatidylcholine, and (■) phosphatidylethanolamine.

the insensitive N-methylated phospholipids in model membranes.

The Protective Effect of N-Methylated Phosphatidylethanolamines on Susceptibility of Model Membranes to Polymyxin B. Phosphatidylcholine was added to *E. coli* phospholipids at various molar percentages of the total phospholipid (since one could not prepare 100% phosphatidylethanolamine controls, *E. coli* phospholipids were used throughout these phospholipid mixing experiments). No detectable N-methylated phosphatidylethanolamine was present in the *E. coli* lipid batches used. Release of trapped glucose was then examined at two polymyxin B concentrations. Polymyxin B concentrations tested were chosen to reflect a blood concentration which can be achieved when the antibiotic is used to treat infections in man (5 μg/ml) and a feasible renal or urine concentration achieved in the same situation (50 μg/ml) since the drug is concentrated in the kidney. These data, graphed in Figure 2, indicate that the incorporation of lecithin into the liposomes produced a "protective effect" against polymyxin B action and that such protection could be reversed partially by increasing the antibiotic concentration. Similar protection was obtained when *N,N*-dimethylphosphatidylethanolamine (Figure 3) and *N*-methylphosphatidylethanolamine (Figure 4) were used in place of lecithin. It is important to emphasize that in this series of studies, the polar head portions of phos-

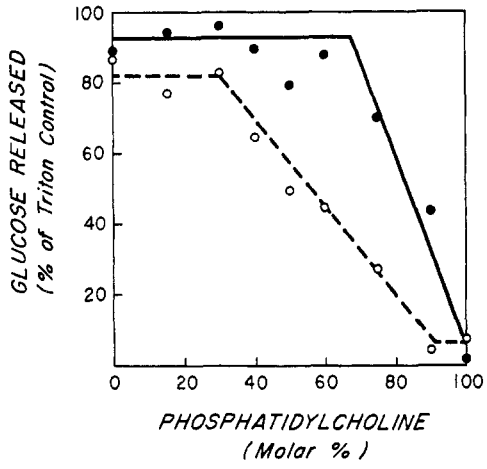

FIGURE 2: Polymyxin B sensitivity of liposomes as a function of phosphatidylcholine content: (○) polymyxin B at 5 μg/assay; (●) polymyxin B at 50 μg/assay. Liposomes were prepared from various combinations of *E. coli* phospholipid and phosphatidylcholine, with cholesterol added to give a final composition of total phospholipid–cholesterol (1:0.5, molar ratio). The molar per cent of phosphatidylcholine was calculated from: 100× (moles of phosphatidylcholine/moles of total phospholipid). Cholesterol was not included in the calculation. Liposomes were used at a concentration of 0.02 μmol of phosphorus/assay.

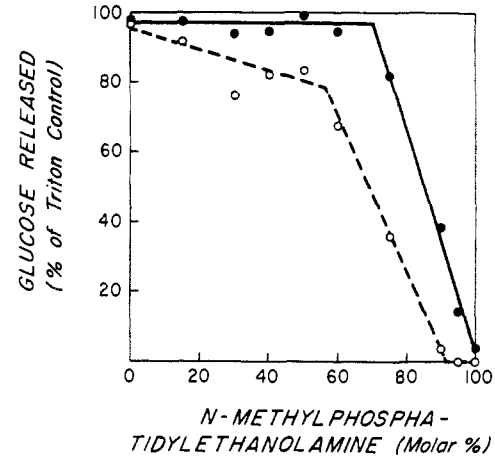

FIGURE 4: Polymyxin B sensitivity of liposomes as a function of *N*-methylphosphatidylethanolamine content. For experimental conditions, refer to legend for Figure 2.

pholipids were manipulated; the possible role of fatty acid composition of the phospholipids was not examined. It should be noted that, although qualitatively similar, the shape of the "protection" dose–response curve for the dimethyl phospholipid (Figure 3) was different from those observed with the mono- and trimethylated compounds. The reason for this is not clear although it may reflect the different hydrocarbon chains in the lipids used.

The Polymyxin Susceptibility of Model Membranes Derived from E. coli Phospholipids as a Function of pH. The polymyxin effect on sensitive (*E. coli* lipid) liposomes as a function of pH is presented in Figure 5. Controls without antibiotic indicated that liposomes prepared from *E. coli* phospholipid were stable with regard to glucose release over this wide pH range in the absence of polymyxin. The antimicrobial activity of polymyxin after incubation for 1 hr at 20° at the extremes of pH employed (pH 1.0 and 10.5) was also examined. After neutralizing the antibiotic solution, bioassay on an *E. coli* of known sensitivity revealed that greater than 95% of the original potency was retained.

Maximum efficacy of the antibiotic is achieved at neutral pH values. From pH 8.5 to 10.5 the polymyxin activity decreases by approximately 58%. This corresponds with what might be expected if a protonated amino group on the antibiotic polymyxin is required for the antimembrane activity. The gradual decrease of more than 85% in activity on altering the pH from neutral to 2.0 is most likely also an indication of involvement of ionizable groups on the polymyxin molecule. There are five free amino groups in polymyxin B, three located in the ring moiety. The p*K* values of these closely associated amino groups theoretically may deviate considerably from those normally encountered, by dint of their interaction; the p*K*'s of the amino groups in a molecule such as polymyxin B may be scattered over a wide pH range (Edsall and Wyman, 1958).

The efficacy of melittin as a function of pH was also studied As shown in Figure 5, this antibiotic at 5 μg/ml was equally

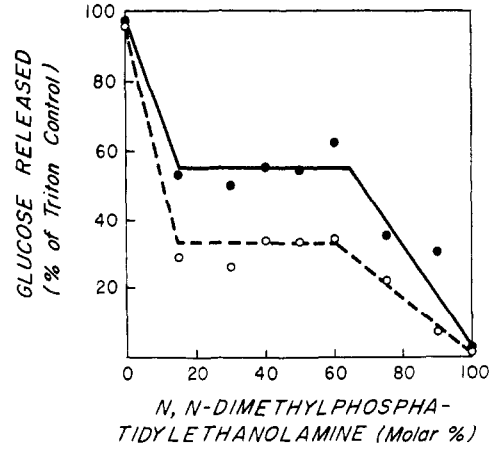

FIGURE 3: Polymyxin B sensitivity of liposomes as a function of *N*,*N*-dimethylphosphatidylethanolamine content. For experimental conditions, refer to legend for Figure 2.

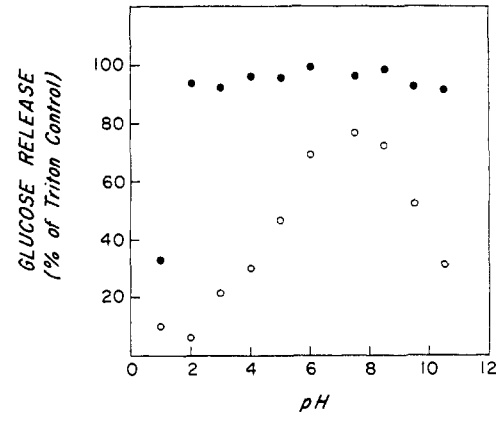

FIGURE 5: Effect of pH on the polymyxin susceptibility of liposomes prepared from *E. coli* phospholipid. The polymyxin susceptibility was measured by assay of marker glucose released as described in the text. The buffers (10 mM) used were NaCl–HCl buffer from pH 1.0 to 2.0, acetate buffer from pH 3.0 to 6.0, Tris-HCl buffer from pH 7.5 to 8.5, glycine–NaOH buffer from pH 9.5 to 10.5. (○) Polymyxin B used at 10 μg/ml; (●) melittin used at 5 μg/ml.

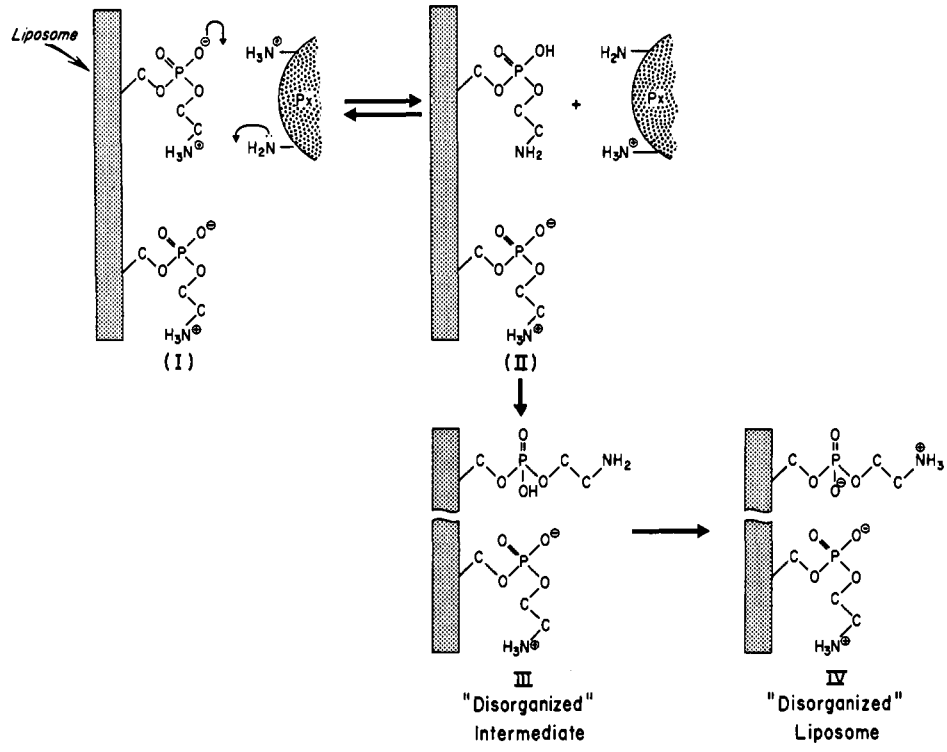

FIGURE 6: A diagrammatic illustration of a suggested mechanism of polymyxin action with phosphatidylethanolamine as the "target" molecule. In this multisite-type mechanism, the ammonium (NH_3^+) and amino (NH_2) groups on polymyxin B ring moiety interact, respectively, with the phosphate (PO_4^-) and ammonium (NH_3^+) groups on the phosphatidylethanolamine polar head through proton transfer. At the first stage, the interaction is initiated when a NH_3^+ group on the polymyxin ring moiety approaches the PO_4^- group on phosphatidylethanolamine (I); electrostatic interaction brings all four participating groups into proper alignment. At the second stage, simultaneous proton transfer leads to neutralization of charges on the polar head (II). The stabilizing forces derived from hydrophilic and electrostatic interactions are, thus, temporarily interrupted and may lead to disorganization of the bilayer structure (III) which then may yield a permanantly "disorganized" liposome (IV). Although the figure shows interaction of the two functional groups on the polymyxin molecule with the opposite charges on a single phosphatidylethanolamine molecule, a similar reaction could occur with the PO_4^- and the NH_3^+ of neighboring molecules.

effective throughout the pH range 2–10.5 and approximated the Triton activity. Of interest was the markedly diminished efficacy of melittin at pH 1.0; we cannot explain this based on the proposed mechanism of action (see Discussion) although the p*K* of the ionized phosphate group on phosphatidylethanolamine is between 1.0 and 2.0.

Discussion

From these experiments we conclude that the susceptibility of biologic membranes to the polymyxins depends on the presence of target sites in the membrane, on the number or possibly density of these sites and on the antibiotic concentration. It is clear from Figures 2 through 4 that the effect of polymyxin B on liposomes increases in proportion to the molar percent of *E. coli* phospholipid present. Since this phospholipid is primarily phosphatidylethanolamine and since phosphatidylethanolamine is the only major phospholipid class in most bacteria sensitive to the polymyxins (Ikawa, 1967), we suggest that phosphatidylethanolamine represents one such target molecule on membranes. Other phospholipids beside phosphatidylethanolamine may act as similar sensitive targets for the polymyxins; in these experiments we have only examined phosphatidylethanolamine and its methylated analogs.

The integrity of liposomes relies on several factors, among them: (1) hydrophobic interactions which are facilitated by orienting the fatty acid chains toward the interior of the particles; (2) hydrophilic interaction associated with the close packing of the polar groups on the interface between the phospholipids and the bulk aqueous phase; and (3) electrostatic interactions. One would expect the mechanism of polymyxin B action to involve steps which lead to the disruption of one or more of these noncovalent interactions as a first step. In Figure 6 such a mechanism is presented diagramatically; apposition of functional groups on the antibiotic and phosphatidylethanolamine result in proton transfer initiating disruption of stabilizing electrostatic forces. The data in support of this mechanism and the assumptions made are discussed. (1) The active functional groups on polymyxin B are the γ-amino groups (Teuber, 1970) of the cyclic peptide moiety (Nakajima, 1967). At least two polymyxin γ-amino groups are involved. The relative position of these groups is defined by the conformation of the entire ring. These functional groups, for electrostatic reasons (Edsall and Wyman, 1958), are not likely to be either simultaneously protonated or uncharged near neutral pH. The experiments with glucose marker release from liposomes as a function of pH are consistent with a proton transfer hypothesis since under conditions where one may expect all the amino groups of the antibiotic to be protonated (low pH) and under conditions where the amino groups may be minimally protonated (high pH) the glucose release is at a minimum. (2) Chapman and his coworkers based on physical evidence have determined that there is an interaction between the phosphate group in lecithin and amino group on polymyxin B (Pache *et al.*, 1972). They had previously shown that the electrostatic environment of the phosphorus atom in phosphatidylethanolamine and lecithin

is the same (Chapman and Morrison, 1966). Thus, although we have not directly demonstrated an "interaction" of the phosphate group of the lipid and an amino group of the antibiotic, its occurrence seems well established. Since lecithin liposomes are not disrupted by polymyxin B, this interaction is likely essential but insufficient for membrane disruption. Since the phosphorus atoms of the four structural analogs studied, likely have the same electrostatic environment, they may compete with each other for the polymyxin B ring-NH_3^+ groups when presented in the same system. (3) The fatty acid portion on the polymyxin B side chain can penetrate into the hydrophobic region of the liposome bilayer (Pache *et al.*, 1972). This helps to bring about, but is not necessary for, the suitable alignment among the several, probably four, participating functional groups. Nakajima (1967) showed directly that the cyclic peptide moiety cleaved from the antibiotic had bactericidal activity; on a weight basis it was about one-third as active as the complete antibiotic. (4) The amino group on polar head of phosphatidylethanolamine is practically indistinguishable from the amino group on the polymyxin ring moiety; proton transfer among these two identical primary amino groups should be possible if they are in apposition. As depicted in Figure 6 such proton-transfer reactions would transiently disturb the electrostatic forces which help stabilize the liposomes. Proton transfer is ruled out for lecithin, since there is no exchangeable proton available. In case of other *N*-methyl-substituted analogs, proton transfer may be less likely for at least two reasons. The presence of methyl groups may prevent the proper alignment of the methylamino group and the amino group on polymyxin B (steric effect); the methyl group also increases the basicity of the attached nitrogen atom (induction effect) making proton transfer to a less basic amino group (primary amino group on the polymyxin B ring) unfavored. (5) With liposomes from *E. coli* lipids (or phosphatidylethanolamine), on interaction of the protonated amino group of polymyxin B with the phosphate group of the phospholipid, the suggested apposition of amino groups with consequent proton transfer may be a frequent event. Molecular models of one possible conformation in polymyxin B show that the distance between 2 ring γ-amino groups approximates that between phosphate and amino groups of phosphatidylethanolamine (C.-C. HsuChen and D. S. Feingold, unpublished observations). As one increases the percentage of methylated phosphatidylethanolamines in mixed phospholipid liposomes, it becomes less likely that interaction of a polymyxin molecule with a phospholipid phosphate group will result in the apposition of an appropriate amino group for proton transfer. Increasing the polymyxin concentration should increase the activity in mixed liposomes; this is what we found.

Several reservations must be stated concerning the proposed mechanism. (1) The role of water is not discussed, mainly due to lack of information. (2) Thus far, no information is available concerning the titration behavior of the amino groups on polymyxin and the three-dimensional structure of polymyxin is not yet defined. Until such data are available, the possible role of other functional groups in the action of polymyxin B such as the hydroxy group on threonine in the cyclic peptide moiety should not be overlooked. (3) We interpret the pH profile (figure 5) based on the assumption that the ionization state of certain groups may produce a decisive effect on polymyxin action, such as the amino groups of the polymyxin molecule. However, the gradual change in the ionization state of the polymyxin (and/or phopholipid) may also influence its conformation in such a way that the extent of interaction between the two molecules changes with pH. More experimental facts are needed to confirm the suggested mechanism or to define the mechanism of polymyxin action in further detail.

Biologic membranes throughout nature are generally similar and, hence, one would not expect clinically useful antimicrobics to work by attacking this organelle. Sessa *et al.* (1969) showed that the polypeptide melittin released marker from liposomes prepared from phosphatidylcholine with or without cholesterol and with net positive or negative charge. Recently Williams and Bell (1972) demonstrated by electron spin resonance spectroscopy that melittin disrupted membrane matrix with membranes of various phospholipid composition; this agent disrupted all the liposomes we examined and functioned effectively over the pH range 2.0–10.5. The antibiotic chlorothricin acts on phospholipids of *Bacillus subtilis* as well as on lecithin–water dispersions (Pache and Chapman, 1972). It has been suggested that both melittin and chlorothricin work by dint of nonpolar association with hydrocarbon chains of the phospholipids regardless of the polar head group. The macrocyclic, ionophorous antibiotics also effect membranes with little or no selectivity; they include valinomycin, the depsipeptide enniatins, the macrotetralide actins and the polypeptides gramicidin S and alamethicin (Hauser *et al.*, 1970) (subject reviewed by Kinsky, 1970).

It should be kept in mind that polymyxin action on membranes may be much more subtle than glucose release from liposomes or a lethal action on bacteria. For example, Schwartz and his coworkers (1972) have recently shown that the antibiotic which has no fungicidal action on *Candida albicans* can nevertheless foster the entry of tetracycline into the yeast. Hasselbarth (1972) observed that polymyxin B has no overt action on monkey kidney cells in culture, but augments the release of polio virus from these cells. Polymyxin B toxicity in man is primarily neurologic and renal. It is of interest that nervous tissue contains among the highest phosphatidylethanolamine levels in lipids from mammalian sources (Rouser *et al.*, 1968). Renal tubular cell damage may be expected as a toxic effect since the antibiotic is concentrated severalfold in the urine and it is clear from our data that the "protective effect" of methylated phospholipids to phosphatidylethanolamine-containing membranes can be partially reversed by increasing the polymyxin B concentration.

One can identify few major differences among membranes which seem to be foci for antibiotic selective toxicity and, hence, usefulness as therapeutic agents. Many bacterial membranes differ from mammalian membranes in the large percentage of phospholipid which is phosphatidylethanolamine and the absence of the methylated derivatives such as lecithin. We suggest that the selective toxicity of the polymyxins may depend on this later difference in the comparative biochemistry of membranes.

References

Bangham, A. D. (1968), *Progr. Biophys. Mol. Biol. 18*, 29.

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.

Blostein, R. (1968), *J. Biol. Chem. 243*, 1957.

Chapman, D., and Morrison, A. (1966), *J. Biol. Chem. 241*, 5044.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. 1, New York, N. Y., Academic Press, pp 457, 540.

Elwood, C. M., Lucas, G. D., and Muehrcke, R. C. (1966), *Arch. Intern. Med. 118*, 326.

Few, A. V. (1955), *Biochim. Biophys. Acta 16*, 137.

Hasselbarth, A. (1972), *Med. Microbiol. Immunol. 157*, 239.
Hauser, H., Finer, E. G., and Chapman, D. (1970), *J. Mol. Biol. 53*, 419.
Haxby, J. A., Kinsky, C. B., and Kinsky, S. C. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 300.
HusChen, C. C., and Feingold, D. S. (1972), *Antimicrob. Ag. Chemother. 2*, 331.
Ikawa, M. (1967), *Bacteriol. Rev. 31*, 54.
Kanfer, J., and Kennedy, E. P. (1963), *J. Biol. Chem. 238*, 2919.
Kinsky, S. C. (1970), *Annu. Rev. Pharmacol. 10*, 119.
Kinsky, S. C., Haxby, J. A., Zopf, D. A., Alvin, C. R., and Kinsky, C. B. (1969), *Biochemistry 8*, 4149.
Koike, M., and Iida, K. (1971), *J. Bacteriol. 108*, 1402.
Nakajima, K. (1967), *Chem. Pharm. Bull. 15*, 1219.
Newton, B. A. (1953), *J. Gen. Microbiol. 9*, 54.
Newton, B. A. (1956), *Bacteriol. Rev. 20*, 14.
Pache, W., and Chapman, D. (1972), *Biochim. Biophys. Acta 255*, 348.
Pache, W., Chapman, D., and Hillaby, R. (1972), *Biochim. Biophys. Acta 255*, 358.
Papahadjopoulos, K., and Miller, N. (1967), *Biochim. Biophys. Acta 135*, 624.
Rouser, G., Nelson, G. J., and Fleischer, S. (1968), *in* Biological Membranes, Chapman, D., Ed., New York, N. Y., Academic Press, pp 52, 55.
Schwartz, S. N., Medoff, G., Kobayashi, G. S., Kwan, C. N., and Schlessinger, D. (1972), *Antimicrob. Ag. Chemother. 2*, 36.
Sessa, G., Freer, J. H., Colacicco, G., and Weissmann, G. (1969), *J. Biol. Chem. 244*, 3575.
Sud, I. J., and Feingold, D. S. (1970), *J. Bacteriol. 104*, 289.
Teuber, M. (1969), *J. Bacteriol. 98*, 347.
Teuber, M. (1970), *Z. Naturforsch. 25B*, 117.
Vogler, K., and Studer, R. O. (1966), *Experientia 22*, 345.
Ways, P., and Hanahan, D. J. (1964), *J. Lipid Res. 5*, 318.
Williams, J. C., and Bell, R. M. (1972), *Biochim. Biophys. Acta 288*, 255.

Isolation and Partial Chemical Characterization of Cell-Surface Glycopeptides from AS-30D Rat Hepatoma Which Possess Binding Sites for Wheat Germ Agglutinin and Concanavalin A†

David F. Smith,‡ Giovanni Neri,§ and Earl F. Walborg, Jr.*

ABSTRACT: AS-30D rat ascites hepatoma cells were agglutinable by wheat germ agglutinin (WGA), but only weakly agglutinable by high concentrations of concanavalin A (Con A). Treatment of the intact cells with papain rendered them highly agglutinable by both lectins and released a glycopeptide fraction from the cell surface. The cell-surface glycopeptide fraction inhibited the cytoagglutination of AS-30D cells by WGA, indicating that this fraction contained components that possessed the minimum requirements of cell-surface binding sites for WGA. The cell-surface glycopeptide fraction exhibited both WGA- and Con A-hemagglutination inhibitory activity. Partial resolution of the glycopeptides by gel filtration and ion-exchange chromatography resulted in the isolation of a sialoglycopeptide fraction, which represented 12% of the weight of the papain-labile, cell-surface glycopeptides and possessed the major portion of the WGA-binding activity. This glycopeptide fraction exhibited specific hemagglutination inhibitory activities which reflected the cytoagglutination properties of the intact AS-30D cell. Purification of Con A-binding activity from the papain-labile, cell-surface glycopeptides resulted in the isolation of a sialoglycopeptide fraction which possessed only Con A-hemagglutination inhibitory activity.

Chemical alterations at the cell periphery have been implicated in many of the manifestations of the cancer cell which are responsible for its altered social behavior (Abercrombie and Ambrose, 1962; Wallach, 1968). The phytoagglutinins, Con A[1] and WGA, have become valuable tools in the detection of altered oligosaccharide moieties present at the tumor cell periphery (Burger and Goldberg, 1967; Inbar and Sachs, 1969a). Transformed cells, in contrast to their normal counterparts, express binding sites at their cell periphery which are responsible for their cytoagglutination by Con A and WGA (Aub *et al.*, 1965; Inbar and Sachs, 1969a). With the exception of embryonic (Moscona, 1971; Noonan and Burger, 1971) and mitotic cells (Burger, 1973), nontransformed cells become agglutinable only after treatment with proteases (Burger, 1969; Inbar and Sachs, 1969a).

† From the University of Texas at Houston Graduate School of Biomedical Sciences and Biochemistry Department, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025. *Received October 2, 1972.* Supported by research grants from the National Cancer Institute (No. CA 11710) and the Paul and Mary Haas Foundation.

‡ Recipient of a Rosalie B. Hite Predoctoral Fellowship in Cancer Research. This research was presented to the Faculty of The University of Texas at Houston Graduate School of Biomedical Sciences in partial fulfillment of the requirements for the Ph.D. degree, June 1972. Present address: The American National Red Cross Blood Research Laboratory, Bethesda, Md. 20014.

§ Present address: Università degli Studi di Perugia, Istituto di Patologia Generale, Policlinico Monteluce, Perugia, Italy.

[1] Abbreviations used are: WGA, wheat germ agglutinin; Con A, concanavalin A.