Action of Polymyxin B on Bacterial Membranes, II

Formation of Lipophilic Complexes with Phosphatidic Acid and Phosphatidylglycerol

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Salmonella typhimurium, phospholipids, cell membrane, polymyxin B, antibiotic

The bactericidal mechanism of action of polymyxin B against susceptible bacteria has been suggested to result from electrostatic interactions of the basic polypeptide antibiotic with acidic cell envelope phospholipids [1,2]. This process causes the breakdown of the selective permeability barrier of the cytoplasmic membrane, and finally cell death. The identities of the polymyxin receptor molecules in sensitive bacteria, however, have never been determined with certainty. The problem is further complicated by the fact that polymyxin exhibits a strikingly selective activity against Gram-negative bacteria [3] which, in addition to the plasma membrane, possess a second membrane structure, the outer membrane or plastic layer composed of proteins, lipopolysaccharide and phospholipids [4]. A possible strategy to identify the polymyxin receptors consists in examining isolated, individual components and substructures of the cell envelope (= outer membrane, murein and inner membrane) for their ability to combine with polymyxin B *in vitro* under physiological conditions. We have previously reported [2] that extraction of isolated cell envelopes from *Salmonella typhimurium* with chloroform-methanol reduced their binding capacity for radioactive polymyxin B by 70%. In the present communication, it is shown that phosphatidic acid and phosphatidyl glycerol, but not phosphatidylethanolamine, phosphatidylserine or cardiolipin (diphosphatidylglycerol), form stable complexes with polymyxin B which can be isolated by paperchromatography or extraction into chloroform. This is the first instance for a specific preference of polymyxin B for defined acidic phospholipids.

The phospholipids occurring in *S. typhimurium* [5], phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylethanolamine (PE) and cardiolipin (CL), were purchased from Supelco Inc. (Bellefonte, Pa., USA). PE and PG were also purified from nutrient broth grown *S. typhimurium* G30 according to G. F. Ames [5].

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Mono-*N*-acetyl-^{14}C-derivatives of polymyxin B (PX) were prepared as previously reported [6]. The specific activity was 14.7 μCi/μmole. Polymyxin B sulfate (sterile powder) was a gift of Pfizer GmbH (Karlsruhe, Germany). Radioactivity was determined in a toluene based scintillation liquid with a Beckman LS100 spectrometer.

Since it is generally assumed that phospholipids are arranged in biological membranes in bilayers forming the hydrophobic permeability barrier [7], it was of interest to search for a polymyxin-phospholipid complex which would be stable in a hydrophobic environment. Because the polymyxin molecule is very polar at neutral pH (5 free aminogroups) it does not migrate away from the origin when chromatographed with chloroform-methanol-water on Whatman No. 1 paper. However, when mixed with PA or PG, part of the radioactive anti-

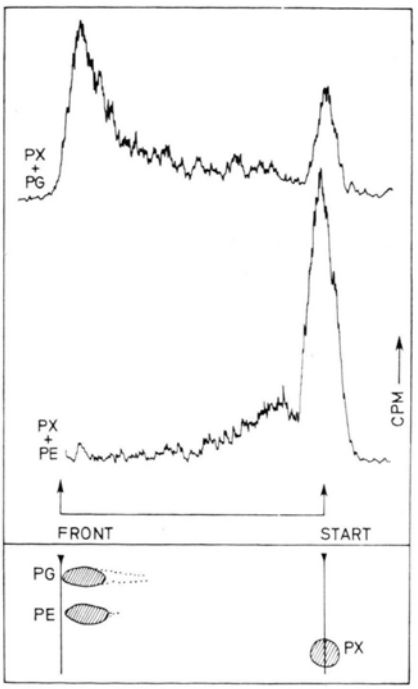

Fig. 1. Paperchromatographic evidence for lipophilic complexes of polymyxin B (PX) with phosphatidylglycerol. 25 μg of PE and 30 μg of PG both dissolved in chloroform were spotted onto separate start positions on Whatman No. 1 paper. The dried spots were each carefully wetted with 10 μl of 0.14 M NaCl containing 2 nmoles mono-*N*-acetyl-^{14}C-polymyxin B. The dried paper was developed with the solvent chloroform–methanol–water (95 : :50 : 5, v : v : v) until the solvent front had moved 9 cm above the origin. Phospholipids were detected with the molybdate-sulfuric acid spray [5], radioactive PX with a chromatogramm scanner. All phospholipids with or without PX moved with the front. PX, free or mixed with PS, CL or PE stayed at the origin (lower tracing). PX mixed with PA or PG moved with the front (upper tracing).

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biotic cochromatographed with these phospholipids near the solvent front (Fig. 1). Mixtures of polymyxin with PE, CL and PS did not exhibit this behaviour. PE and PG isolated from *S. typhimurium* showed identical properties like the commercial phospholipids. These results were checked by extraction experiments with the phospholipids dissolved in chloroform and polymyxin B dissolved in aqueous 0.14 M sodium chloride pH 6.9. Again, only PA and PG were able to transfer the radioactive PX derivatives from the aqueous into the organic phase (see Table). The chloroform layer containing PX and PG was optically clear. The use of fluorescent mono-*N*-dimethylaminonaphthalenesulfonyl polymyxin B [8] confirmed that the PX-PG complex was not deposited at the glass walls of the reaction tube but was evenly distributed in the organic phase.

Table. Phospholipid-dependent extractability of mono-*N*-acetyl-^{14}C-polymyxin B (14.7 μCi/μmole) from the aqueous into the organic phase. 0.1 ml of chloroform containing 25 μg (phosphatidic acid, phosphatidylserine, phosphatidylethanolamine) or 30 μg (phosphatidylglycerol, cardiolopin) of phospholipids were overlaid with 0.12 ml of 0.14 M sodium chloride pH 6.9 containing 4 nmoles (about 5 μg) of radioactive polymyxin B derivatives. The two phases were mixed for 15 sec at 20 °C with rapid stirring and separated by 15 min centrifugation at 2500 rpm. 10 μl Portions of the clear phases were counted for their content of radioactive polymyxin B.

Phospholipid in chloroform layer	^{14}C-Polymyxin B derivative [cpm] in 10 μl of	
	aqueous phase	organic phase
none	4 900	130
phosphatidic acid	1 270	2 760
phosphatidylglycerol	395	3 800
phosphatidylserine	4 720	400
cardiolipin	5 560	490
phosphatidylethanolamine	5 070	350

These properties make the system amenable to spectroscopic investigations, *e. g.* nuclear magnetic resonance, infrared and fluorescence spectroscopy. A detailed quantitative analysis of the physicochemical properties of the polymyxin-phosphatidylglycerol complex is in progress in our laboratory.

Whereas phosphatidic acid is only a trace component of the membrane phospholipids in *S. typhimurium* [5], phosphatidylglycerol makes up 17% of the phospholipids in the outer membrane and 33% in the plasma membrane [9]. Since polymyxin B has to penetrate the outer membrane before it gains access to the plasma membrane [10], this could occur *via* the phosphatidylglycerol-polymyxin B complex and/or the complex with the lipid A region of lipopolysaccharide as described in an accompanying paper [11]. The presence of these compounds in the outer membrane of Gram-negative bacteria would therefore be a reasonable explanation for the specific action of polymyxin against these organisms.

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6,8-Dihydroxypurine in the Hen's Egg Yolk

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6,8-Dihydroxypurine, egg yolk, purine metabolism

During an investigation on nucleic acid catabolism in chicken eggs, we were able to identify several purine and pyrimidine derivatives in white and yolk [1]. Isolation of these products from yolk (Leghorn) was achieved by dialysis against ammonia solutions (pH 9.0 – 9.2). Further separation and purification was done as described for the products isolated from white [1]. One of the peaks obtained after chromatography on Dowex 2 × 8 (acetate) and gel filtration on Sephadex G-10 shows an UV spectrum resembling that of hypoxanthine. Paper chromatography in different solvent systems revealed the presence of at least two products, one of them being hypoxanthine (evidence for the presence of hypoxanthine was obtained by means of UV spectroscopy and paper chromatography). The purification of the other product was achieved by paper chromatography with isopropanol/H_2O/NH_3 (28%) : 85/15/1.3 [2]. The elution volume on Sephadex G-10 sug-

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gested a purine derivative. A spectrophotometric titration carried out in the range pH 2 to pH 12 reveals two pK values: 7.9 and 10.5. The isosbestic points are rather sharp, indicating only a low contamination by other UV absorbing substances. The obtained spectra were identical with those of 6,8-dihydroxypurine [3]. The product was then compared with an authentic sample of 6,8-dihydroxypurine (Aldrich).

A spectrophotometric titration of this product gave the same pK values, isosbestic points and other UV characteristics as those obtained for the product isolated from yolk. Further evidence for the identity of both samples comes from paper chromatography in six different solvent system and from two dimensional thin layer chromatography on cellulose [4]. Paper electrophoresis carried out at pH 1.9 and at pH 10 confirms the identity. The observed spectral changes after UV irradiation at pH 4.6 (0.01 M acetate) and pH 9.2 (0.01 M carbonate) are identical for both samples. UV spectra and R_F values do not change after treatment with 1 N HCl for 1 hour at 100 °C, neither for 6,8-dihydroxypurine nor for the product isolated from yolk.

The role of 6,8-dihydroxypurine in the yolk of chicken eggs is not elucidated. Catabolizing enzymes that may account for it as a degradation product of other purines were not yet found. Bergmann and Dikstein [3] considered it as a possible intermediate in the oxidation of hypoxanthine to uric acid, although they did not prove this. They also showed that 6,8-dihydroxypurine is a substrate for the xanthine oxidase from milk. On the other hand, Wyngaarden and Dunn [5] found that 8-hydroxyadenine is the major intermediate in the enzymic oxydation of adenine to 2,8-dihydroxyadenine.

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