Distribution of alamethicin in lipid membranes and water

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Summary The concentration of alamethicin in aqueous solutions was quantitated using measurements of the spot area on thin-layer chromatograms. These data were utilized to measure a partition coefficient of 17 for alamethicin in a phospholipid membrane–water system under equilibrium dialysis conditions.

Supplementary key words thin-layer chromatography · cyclopeptide antibiotic · phospholipid membranes · equilibrium dialysis · partition coefficient

Alamethicin is a macrocyclic antibiotic which has been used to induce voltage-dependent changes in ionic conductances of bimolecular phospholipid membranes (1, 2). More recently, the antibiotic was employed to enhance the permeability of lipid membranes to $^{22}Na^+$ by diffusion (3). In evaluating its role as an Na^+ carrier, experimental data were required on the relative distribution of alamethicin in the membrane phase and water. The method adopted for this purpose employed phospholipid spheres rather than the usual liquid–liquid system to measure the partition coefficient of a solute.

Materials and methods. A chloroform–methanol 2:1 (v/v) extract of phospholipids from fresh beef brain was prepared as previously described (4). The solvent was

Abbreviations: TLC, thin-layer chromatography.

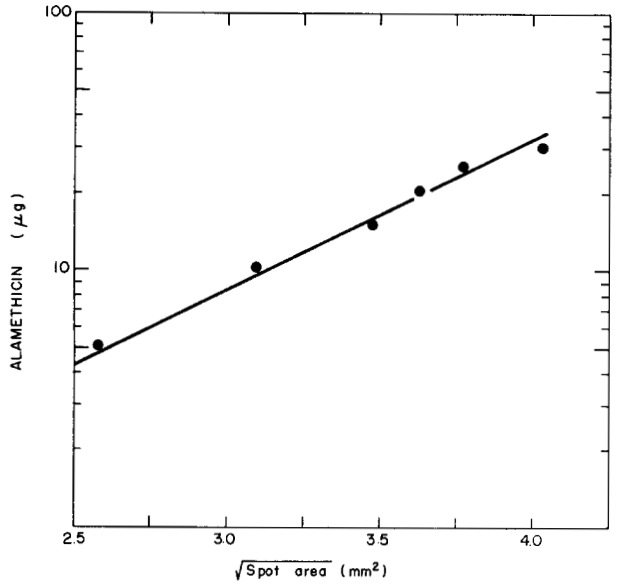

FIG. 1. Relation of amount of alamethicin in the chromatogram to spot area according to the equation, log amount $\propto \sqrt{\text{area}}$.

TABLE 1. Determination of the partition coefficient of alamethicin for spherical phospholipid membranes and water by TLC

Item	Description	Spot Number 1	2	3	4
1	Volume of alamethicin in ethanol per spot (μl)	3	4	5	6
2	Mean area per spot (mm²)	7.85	8.89	10.0	11.4
3	Amount of alamethicin per spot (μg)[a]	7.0	8.8	11	14
4	Concentration of alamethicin in ethanol (μg/μl)	2.3	2.2	2.2	2.3
	Mean concentration ± SD	2.25 ± 0.05			
5	Concentration of alamethicin in membrane (c_m)	770 ± 17 μg/g			
6	Concentration of alamethicin in water (c)	45 ± 1.0 μg/g			
7	Partition coefficient K where $K = c_m/c$	17 ± 0.5			

[a] Calculated from regression line of Fig. 1.

evaporated under a stream of filtered nitrogen. Spherical membranes were prepared under aseptic conditions by sonicating 3 g of the lipids in 30 ml of distilled water in an ultrasonic bath for 1 hr at 4°C. There was no evidence of lipid peroxides in the sonicate as measured by absorption spectrophotometry at 234 nm. The sonicate was centrifuged at 105,000 *g* for 30 min, and 20 ml of supernate (lipid content 13 mg/ml as measured by the difference in dry weight of 20 ml of distilled water and 20 ml of supernate) was transferred to an autoclaved dialysis bag. The dialysis tubing was high purity cellulose (Canadian Laboratory Supplies Limited, series D1615) with an average pore diameter of 4.8 nm. The pore diameter was too small to allow a measurable number of the lipid vesicles to pass through. Electron microscopy of freeze-etched lipid samples has shown the vesicles to be predominantly spherical, 20–25 nm in diameter, while phosphotungstic acid staining demonstrated that the vesicles were bounded by a single membrane (5). The contents of the dialysis bag were dialyzed against 20 ml of sterilized distilled water acidified to pH 4 with 0.1 N HCl to solubilize the alamethicin added to a concentration of 100 μg/ml. The alamethicin[1] was chromatographically pure, and preliminary experiments established that it equilibrated across the dialysis membrane. Thus, when a total of 2 mg of alamethicin was added to the 20 ml of distilled water outside the dialysis bag and dialyzed at ambient temperature against 20 ml of distilled water inside the dialysis bag, the amount of alamethicin inside and outside the dialysis bag after 4 days was 1.04 mg in each case. This also indicates that no measurable amount of alamethicin was lost due to its possible adsorption to the dialysis membrane or to the glass wall of the containing vessel. The dialysis with lipid supernate in the dialysis bag was likewise continued for 4 days with constant agitation on a mechanical shaker.

The dialysate was flash evaporated to dryness at 40°C and dissolved in 400 μl of ethanol for chromatography. Different amounts of the solution (Table 1) were spotted in 1-μl aliquots on layers of silica gel G (E. Merck, Darmstadt, Germany), 250 μm thick, on 20 × 20 cm air-dried TLC plates. The plate was developed in the upper phase of *n*-butanol–glacial acetic acid–water 100:10:30 solvent for a distance of 10 cm. The chromatogram was visualized by the chlorine–tolidine reaction of Brenner, Niederwieser, and Pataki (6). The amount of alamethicin in each spot was quantitatively estimated by measuring the spot area five times in each case and then relating the mean values graphically to a calibration curve (Fig. 1) constructed according to Gänshirt (7).

The slope of the regression line ($y = 9.79x - 22.2$; correlation coefficient = 0.99) through the data points varied slightly from one TLC plate to another and was attributed to variations in thickness of the layer of silica gel G. This dependence was minimized by spotting the alamethicin solutions of both known and unknown concentration on the same plate in varying amounts such that the spot areas of the unknown samples (Table 1) fell within the range of spot areas of samples where the concentration of alamethicin was accurately known (Fig. 1).

[1] Alamethicin, a cyclopeptide antibiotic derived from *Trichoderma viride*, was kindly made available by Dr. E. L. Masson of the Upjohn Company of Canada.

Results and discussion. The results in Fig. 1 indicate that an aqueous solution of alamethicin may be reliably estimated by the spot area method. Lipids in the solution interfered with the reliability of the method and necessitated the use of the dialysis technique. Since the volumes of the aqueous phase inside and outside the dialysis bag were equal at 20 ml, the concentration of alamethicin in the dialysate at equilibrium should be diluted one-half, from 100 to 50 μg/ml. Any further reduction in the concentration was taken as evidence of uptake of alamethicin by the spherical lipid membranes whose bimolecular nature has been previously described (5, 8).

In Table 1, the amount of alamethicin per spot (item 3) is estimated using the data on spot area (item 2) and the calibration line of Fig. 1. The concentration of alamethicin in ethanol was then calculated (item 4) for each spot by dividing the amount of alamethicin per spot (item 3) by the volume of solution per spot (item 1). The mean value of the four determinations was 2.25 μg/μl with a standard deviation of $\pm$ 0.05 (2.2%). Taking into account the fact that the alamethicin in 20 ml of dialysate was dissolved in 0.40 ml of ethanol, further calculations indicate that the concentration of alamethicin in the dialysate (c) at equilibrium was decreased from 100 to 45 μg/ml. Because equilibrium across the dialysis membrane was previously demonstrated and no undesirable adsorption to the dialysis bag or container walls was identified, this decrease in alamethicin in the dialysate is then due to the uptake of 200 μg of alamethicin by the membrane phase. Since the amount of phospholipid membrane material was initially 13 mg/ml in 20 ml, it is estimated that the concentration of alamethicin in the membrane phase (c_m) at equilibrium was 200 μg/(13 mg/ml $\times$ 20 ml) = 770 μg/g. The partition coefficient K as given by the ratio c_m/c is then 17 $\pm$ 0.5 (Table 1).

From the average size and shape of the spherical lipid vesicles and the thickness of the lipid membrane it is readily calculated that the volume of water enclosed in the lipid spheres is infinitesimal ($\approx 0.28 \times 10^{-3}$ cm^3 for every milliliter of sonicate). Thus, any effect of this small volume of enclosed water on the partition coefficient must be considered as insignificant.

Measurement of the partition coefficient for alamethicin in a biphasic system consisting of water–*n*-tetradecane (Eastman Kodak P2221, mp 2–4°C) yielded a value less than unity. The failure to obtain a value greater than one is not surprising because surface forces influence the partitioning process and the water–*n*-tetradecane interface in this respect is not a suitable approximation of the parallel array of dipoles that characterizes the bimolecular phospholipid membrane (9).

The value of 17 for the partition coefficient is more reliable than the figure of 40 quoted earlier (3) because the latter was obtained by a TLC technique that did not utilize the spot area method. The decrease is significant because it reduces the molecular ratio of alamethicin to lipid, at which the Na^+ permeability by diffusion was increased by a factor of 12, from ca. 2×10^{-5} to 8.5×10^{-6}. This reduction in the molecular ratio strengthens the argument against alamethicin acting as a mobile ion carrier of sodium (3) in a model phospholipid membrane. In addition, the method by which the value was obtained uses small quantities of a limited supply of alamethicin and would be of benefit in physical studies of alamethicin–lipid membrane interactions (10–13) where data on the molecular ratio(s) appear to be of importance in relating structure with function.

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