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Distribution of pore sizes in black lipid membranes treated with nystatin

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Resistance changes were measured on artificial membranes made of oxydized cholesterol and treated with nystatin. The experimental data were smoothed and then fitted to a mixture of Gaussian functions using the maximum likelihood method; the best fit was to a mixture of four gaussians centered approximately at 4, 6, 9 and 12 molecules per pore, leading to the conclusion that these are the most likely pore sizes.

In the early 70s research groups that were interested in the study of the ion passage through the lipid membranes treated with antibiotics polyenes agents, grasped the following experimental evidence [1–10]: (1) Polyenes modify the membrane permeability only when certain sterols are present, among them cholesterol. (2) Antibiotic–cholesterol, antibiotic–lipid and cholesterol–lipid interactions are of hydrophobic nature and compete among them, the first being the most favored. (3) The measured stoichiometric ratios antibiotic-cholesterol are found in the range 0.3–3.9. (4) In the case of the polyenes nystatin and amphotericin B the conductance dependence on the antibiotic concentration obeys a power law whose exponent varies between 4 and 12.

Those research groups proposed some models of pores that agree in their fundamental features [2,8,9], which are: (1) A small number of antibiotic and cholesterol molecules are needed to build up a pore; these molecules are aggregated on an interdigitated and closed antibiotic-cholesterol array in which the antibiotic OH groups are located in the inner part of the pore. (2) A complete pore is formed by two half-pores, one on each side of the membrane. (3) The pore radius ranges from 4 to 5 Å and the length is approx. 60 Å. The De Kruijff model [2] was one of the most relevant among those proposed at that time. On this model, eight amphotericin B and eight cholesterol molecules form a highly stable and closed array. In 1978 Van Hoogevest and De Kruijff [5] refined the model in order to include the observation that on thinner membranes half pores can communicate the two regions separated by the membranes. This observation was confirmed by another group [11].

Studies of the membrane conductance on bilayer lipid membranes (BLM) and biological membranes are reported in the literature. In the case of BLM, both wet [4,6] and dry [11] membranes have been used (Mueller's and Montal's techniques, respectively [12,13]). On these studies the measured molecularity of the pores, α, was found to vary between 4 and 12. There are ambiguities, however, on the reproducibility of α from one experiment to another [2,8,11].

The study reported here is an analysis of a set of experiments made on oxydized cholesterol

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BLM. These membranes were formed across a hole (approx. 1 mm^2) in a thinned sidewall of a teflon cup, using the Mueller brush technique [12] from a 1% oxydized cholesterol solution in decane. Each of the two compartments separated by the membrane contains approximately 5 ml of a 10 mM NaCl solution. Mixing was accomplished by magnetic fleas. Voltage across the membrane was generally kept at zero. The membrane's conductance was measured by means of a high impedance inverting amplifier on which the membrane acted as the input impedance. KCl agar bridges and Ag/AgCl electrodes were used to connect the solutions separated by the membrane to the electronics. Nystatin (from Squibb Mycostatin) was added to both sides of the membrane from a 1 mg/ml stock methanolic solution.

In our experiments the untreated BLM formed in a 1 mm^2 hole have a resistance between 10^9 and 10^{10} Ω. When the nystatin is added this value can decrease from 4 to 6 orders of magnitude, as shown on Fig. 1. There is a threshold of nystatin concentration, C_u, below which there is no resistance variation. Above the threshold, however, the resistance can reach values of the order of 10^4 Ω before the membrane's breakdown. From the threshold concentration the R_m has a power law dependence on the nystatin concentration C with an exponent α. According to the kinetic theory of reactions, α is interpreted as the pore molecular-

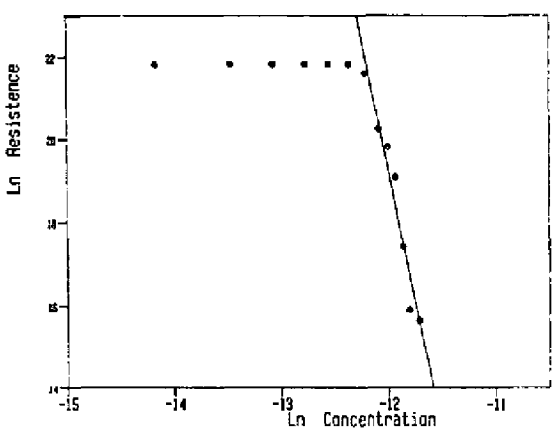

Fig. 1. Log resistance vs. log concentration plot for two-sided addition of nystatin to cholesterol-oxydized membrane. This graph illustrates the power law dependence of membrane resistance on nystatin concentration. The slope α, which is related to the pore molecularity, is in this case 12.7.

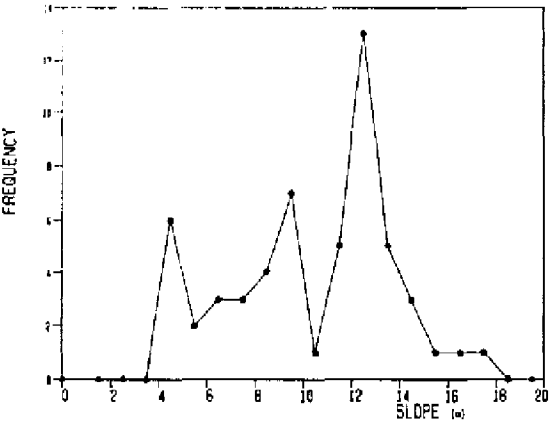

Fig. 2. Polygonal plot of experimental data. A set of 55 experimental data were classified by value in 14 classes one unit wide. The graph shows that the frequency of the α values peak around 4, 9 and 12 and the frequency values of the peaks are 6, 7 and 13, respectively.

ity. To get it from the experimental data a plot of log R_m vs. log C (Fig. 1) is required. The experimental parameters are: the initial resistance value, R_{m_i}, the nystatin threshold concentration value C_u and the slope of the descendant branch α. The values of the α slopes, obtained from the 55 experiments performed, vary in the range 3–17, which are in the range of those reported in the literature.

The results of our experiments were classified in 14 classes with a base of 1.0. A polygonal plot of frequency versus the class value is shown on Fig. 2. The graph shows that the frequency of the α values peak around 4, 9 and 12. The frequency values of the peaks are 6, 7 and 13, respectively.

Another representation of the data is by means of a smoothed curve that results from the convolution of the experimental results (α's) and a gaussian of certain width. To obtain such a curve each individual experimental result is represented by a Dirac's delta function (or a unit impulse response) centered on the corresponding α value; the width, σ, is chosen according to the degree of smoothing wanted (in this case σ was 0.5). The smoothed plot obtained by this procedure is shown on Fig. 3. From the inspection of the polygonal and the convolution plots it can be seen that: (i) the corresponding peaks of both representations match, as one would have expected, and (ii) a

distribution mixture is suggested by the convolution plot. Therefore, as a first approximation, the data were considered as a distribution mixture of three gaussians.

In order to treat the data as a distribution mixture several methods were tried [14–17] and finally the maximum likelihood method (MLM) was chosen [18]; the algorithm proposed by Agha and Ibrahim [19] was used. To fit the data the restrictions imposed by the MLM were made, i.e. the relation between the total number of data, the number of classes and the separation of the means.

The convoluted data curve in Fig. 3 shows clearly three peaks centered at 4.5, 9.2 and 12.3; there is also a shoulder between the two first that suggests the presence of a fourth gaussian. Taking this into account, fittings of the convoluted experimental data to mixtures of three and four gaussians were made, obtaining the best agreement with four gaussians. The results are also shown on Fig. 3 and the parameters of the fitting are reported on Table I. Then the use of this algorithm has allowed us to separate from a mixture of four gaussians, the single distributions for the chlorine ion.

These distributions have their maxima localized at 4.5, 6.4, 9.1 and 12.5, with a proportion in the mixture of 10%, 16%, 18% and 56%, respectively. If a single experiment is performed then the probability of finding an α value is given by the above

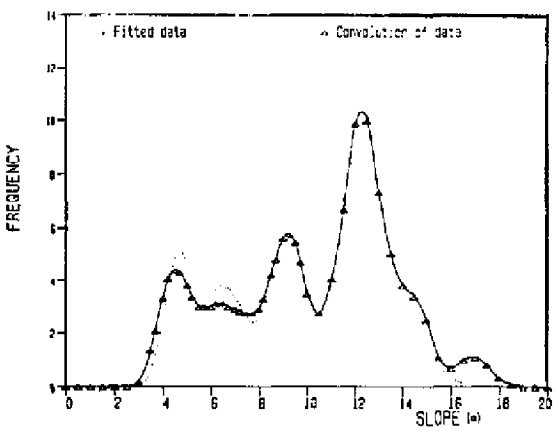

Fig. 3. The plot shows the convolution of experimental data and a gaussian with $\sigma = 0.5$. The graph also shows the result of the fitting of the convoluted data to a mixture of four gaussians. The parameters of the fitting are reported on Table I.

TABLE I

FITTING PARAMETERS OF THE CONVOLUTION OF DATA TO A MIXTURE OF FOUR GAUSSIANS WITH $\sigma = 0.5$

The proportions give the probability of finding an α value, for a single experiment, in a certain distribution.

Gaussian	Proportion	Mean	σ
1	0.10	4.5	0.53
2	0.16	6.4	1.00
3	0.18	9.1	0.69
4	0.56	12.5	1.29

percentages. However, in the membrane under study there is in general a population of pore sizes. We have then two types of distributions, one within the membrane an the other for the whole set of experimental data.

The conductance of a single membrane, G_m, must be governed by the general expression:

$$G_m = g_1[(N\text{-}C)_{\alpha_1}] + g_2[(N\text{-}C)_{\alpha_2}] + g_3[(N\text{-}C)_{\alpha_3}] + \ldots$$

or

$$G_m = \sum_i \gamma_i [N\text{-}C]_{\alpha_i}$$

where N-C is a nystatin-cholesterol complex, α_i is the molecularity of the i-pore and γ_i is a function of the reaction constants.

The experimental α must be a function of the γ_i's and α_i's, $\alpha = \alpha(\gamma_1, \alpha_1, \gamma_2, \alpha_2, \ldots)$. This α represents the molecularity of the resultant distribution of several pore sizes. It is expected that in the formation of pores a cooperative process is involved, consequently only a few sizes will appear in a single experiment. The sharpness of the distribution will depend on how many γ_i's are relevant.

Concerning the permeability characteristics of this kind of membranes one would expect differences depending on the pore size. Since 1970 A. Cass et al. raised this question emphasizing that the invariance of permeability with α made difficult to adopt the molecularity interpretation but they had not found any viable alternative to it.

Recent studies on pore permeability however, explore some fundamental physical quantities that must be involved in the permeability processes, as

the potential field in the neighborhood of the pore's mouth, its geometry, the dipole charges, hydrogen bonds, etc. All this factors might help to explain the surprising fact that a "conduction site made up of four nystatin molecules should have the same permeability as one consisting of 12 molecules" [9].

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References

1 McLaughlin, S. and Eisenberg, M. (1975) Annu. Rev. Biophys. Bioeng. 4, 335–364.
2 De Kruijff, B. and Demel, R.A. (1974) Biochim. Biophys. Acta 339, 57–70.
3 De Kruijff, B., Gerritsen, W.J., Oerlemans, A., Demel, R.A. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 30–43.
4 Andreoli, T.E. and Monahan, M. (1968) J. Gen. Physiol. 52, 300–325.
5 Van Hoogevest, P. and De Kruijff, B. (1978) Biochim. Biophys. Acta 511, 397–407.
6 Holz R. and Finkelstein, A. (1970) J. Gen. Physiol. 56, 125–145.
7 Holz, R.W. (1974) Ann. N.Y. Acad. Sci. 235, 469–479.
8 Andreoli, T.E. (1974) Ann N.Y. Acad. Sci. 235, 448–468.
9 Cass, A., Finkelstein, A. and Krespi, V. (1970) J. Gen. Physiol. 56, 100–124.
10 Flick, C. et al. (1976) Mol. Cryst. Liq. Cryst. 37, 71–80.
11 Kleinberg, M.E. and Finkelstein, A. (1984) J. Membr. Biol. 80, 257–269.
12 Mueller, P., Rudin, D.O., Tien, H.T. and Wescott, W.C. (1963) J. Phys. Chem. 67, 534–535.
13 Montal, M. (1974) in Methods in Enzymology, Vol. 33 (Fleischer, S. and L. Packer, eds.), pp. 545–554, Academic Press, New York.
14 Do, K. and G.J. McLachlan, G.J. (1984) J. R. Stat. Soc., C 33, 1134.
15 Marriot, F.H.C. (1975) Biometrics 31, 767–769.
16 O'Neill, T.J. (1978) J. Am. Stat. Ass. 73, 821–826.
17 Quandt, R.E. and Ramsey, J.B. (1978) J. Am. Stat. Ass. 73, 730–738.
18 Day, N.E. (1969) Biometrika 56, 463–475.
19 Agha, M. and Ibrahim, M.T. (1984) J. R. Stat. Soc. C 33, 327–332.