The Mode of Action of Some Antibiotics on Red Blood Cell Membranes

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Abstract. Data are presented on the interaction of gramicidin, primycin and valinomycin with red blood cell membranes and compared with those obtained for artificial lipid bilayer membranes. The channel forming antibiotics gramicidin and primycin show specific kinetic behaviour in living cell membranes. It could be shown that the penetration of these antibiotics into the red blood cell membrane is a cooperative process resulting in the occurrence of aggregates in the lipid lattice of the membrane.

Key words: Red blood cell membrane — Antibiotics-tracer exchange — Ion selectivity

Introduction

In recent decades membrane active antibiotics have gained special interest as models of transmembrane ion transporting systems. Two types of cation transport across membranes induced by membrane modifiers are now considered: transport through channels formed in membranes, and facilitated transport by mobile carrier mechanism. Gramicidin A, a linear pentadecapeptide of known primary chemical structure (Urry 1971) is considered as one of the simplest models of integral polypeptides. There is strong evidence obtained on model lipid bilayer membranes that this antibiotic induces ion transport by forming cation conducting channels. In organic solvents and lipid bilayers, dimers of head-to-head (formyl to formyl ends) linked gramicidin A molecules in β^6 (LD)-helical conformation, stabilized by intra- and intermolecular hydrogen bonds, form channel-like structures about 2.6 nm long with an effective inner pore diameter of about 0.4 nm (Urry et al. 1971; Bamberg and Janko 1977; Weinstein et al. 1979). An interwined antiparallel or parallel double helical conformation (Veatch et al. 1974) forming nearly similar pore structure is also considered possible in lipid bilayers. The antibiotic

primycin is another channel-forming compound (Vályi-Nagy et al. 1954). The amphiphilic molecule of this antibiotic consists of a lactone ring, a sugar and a guanidine group. The latter carries a positive charge at neutral pH (Aberhardt et al. 1970, 1974). Our recent investigations on artificial lipid bilayer membranes showed that primycin molecules penetrated into the hydrophobic core of the bilayers and, in a dimeric form, they created ion conducting channels (Blaskó et al. 1979, 1983).

The dodecadepsipeptide, valinomycin, and a series of macrotetralides are modifiers able to induce transmembrane cation transport through a mobile carrier mechanism. These ligand compounds form positively charged hydrophobic complexes with cations which can be translocated across lipid bilayers (Lev and Buzinsky 1967; Mueller and Rudin 1967; Markin and Chizmadzhev 1974; Ovchinnikov et al. 1974).

The mode of action of the above classes of modifiers has been studied in detail on model lipid bilayer membranes (Szabó et al. 1973, Markin and Chizmadzhev 1974; Ovchinnikov et al. 1974; Lev 1976; Urban et al. 1980; Läuger 1980; Sandblom et al. 1983; Anderson 1984).

Also, numerous studies have appeared on the action of these compounds on biological membranes (Henderson et al. 1969); Bielawski and Kvinto 1975; Caffier and Shvinka 1979, 1982; Shvinka and Caffier 1981; Shvinka et al. 1982; Cass and Dalmark 1979; Hunter 1977; Sobieski and Bielawski 1983; Horváth et al. 1979a, b).

Nevertheless, experimental evidence for a complete similarity of the action of modifiers on artificial and cell membranes is still lacking. The exact determination of the dependences of cell membrane cation permeability on modifier concentration is an example of problems to be solved.

In the present paper data are presented on interactions of gramicidin, primycin and valinomycin (at different concentrations) with membranes of living cells and compared with those obtained for artificial lipid bilayer membranes. For this purpose, the antibiotic-modified tracer exchange of alkali cations through red blood cell (RBC) membranes, considered as a model of plasma membranes was studied.

Materials and Methods

Human blood obtained from healthy volunteers was stabilized by citrate buffer and stored at 4 °C up to 3 days before the experiments. The composition of the media were chosen to prevent any net flux of cations across the red blood cell membranes. The flux of radioactive cations was balanced by an equal and opposite movement of non-radioactive cations as the cation concentrations outside and inside the cells were equal. Under these conditions, the addition of a radioactive isotope to one of the compartments (to the inside of the cell or to the bathing solution) allowed to measure isotope exchange through the cell membrane.

Determination of the efflux of alkali ions

The blood was equilibrated with radioactive isotopes (^{22}Na, ^{42}K, ^{86}Rb, ^{137}Cs) for 1.5 hours at 37 °C for tracer loading. Then, the erythrocytes were washed 3 times with a solution of the following composition (in mmol/l): KCl 130; NaCl 20; $CaCl_2$ 2.5; $MgCl_2$ 1; sucrose 27 (pH = 7) and resuspended in the same solution to obtain the haematocrit value required. The final suspension was kept in a shaking bath (temperature 20 °C) and samples of the suspension were taken at given intervals. The samples were centrifuged and the radioactivity of aliquots of the supernatants was measured by a γ scintillator counter (Gamma, Hungary). To assure completeness of mixing of the suspension, sampling was started 15—17 min after resuspension. The initial radioactivity was taken for the background value. At a given time, appropriate amounts of one of the antibiotics tested (gramicidin, primycin or valinomycin) were added (from ethanolic stock solutions) to obtain concentrations of the modifiers as required. The final concentration of ethanol in red blood cell suspensions did not exceed 1 % v/v. This ethanol concentration was shown to be without any effect on he ionic permeability of the erythrocyte membrane.

Determination of the influx of alkali ions

Erythrocytes were washed twice and resuspended in the solution as above. One of the antibiotics dissolved in ethanol was added into the suspension, and the suspension was then incubated at 35 °C for 40 minutes. The sample was then kept at this temperature, or cooled down to a lower temperature (20 °C or 3 °C), and radioactive isotopes were added to the system. Samples were taken in appropriate intervals, centrifuged, and the radioactivity of aliquots of supernatants was determined.

In the Figures, the amounts of radioactive ions (N_i) transported are expressed as percentages of the total radioactivity of RBC suspensions. The transport curves are presented in semilogarithmic scale: $-\ln\left(1-\frac{N_i}{N_\infty}\right)$ over time, where N_∞ is the percentage of radioactivity at isotopic equilibrium. Estimated lines are fitted to the experimental points. Gramicidin was obtained from Sigma, valinomycin from Calbiochem (USA) and primycin from Chinoin Pharmaceutical Works (Hungary). All other chemicals used were of analytical grade.

Results and Discussion

Results obtained in the present study confirmed our previous data (Blaskó et al. 1984) and showed that the kinetic curves of the radioactive cation efflux from gramicidin and primycin-treated red blood cells cannot be represented by a single exponent (Fig. 1, 2) as it might be expected for a two-compartment system consisting of one integral inner and one outer fixed volume. At least a sum of two exponents is required to fit the experimental kinetic data: one fast and one slow exponent. The slope of the slow exponent was found to be same for all the concentrations of gramicidin and primycin studied, and it also was equal to the transport rate established for unmodified red blood cells. This fact suggests the presence of two populations of RBC, one with membranes modified by the channel forming antibiotics, and another one free of the modifiers. However, a time-

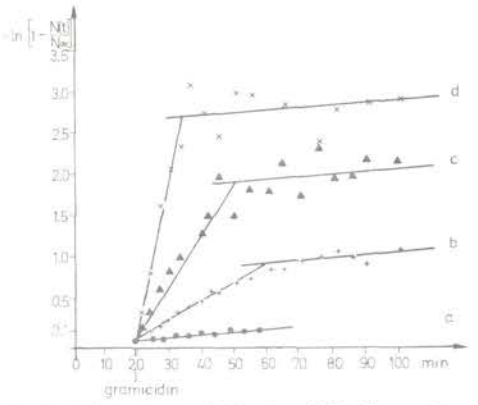

Fig. 1. Effect of gramicidin A on ^{42}K efflux under exchange conditions. Extracellular solution contained (in mmol/l): 130 KCl; 20 NaCl; 2.5 $CaCl_2$; 1 $MgCl_2$; 27 sucrose; pH: 7. H: 0.4. $t = 20$ °C. Gramicidin concentration: *a*) control; *b*) 3×10^{-10} mol/l; *c*) 3×10^{-9} mol/l; *d*) 6×10^{-9} mol/l.

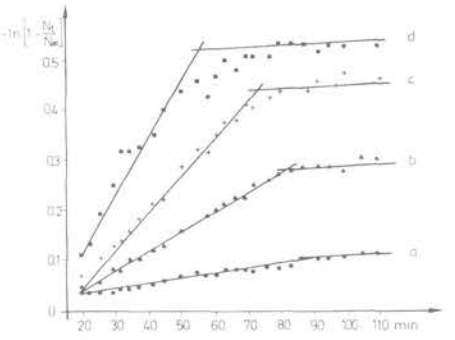

Fig. 2. Effect of primycin on ^{42}K efflux under exchange conditions. Primycin concentration: *a*) control, *b*) 3×10^{-6} mol/l, *c*) 6×10^{-6} mol/l, *d*) 1.2×10^{-5} mol/l (for other conditions see legend to Fig. 1.).

limited action of the modifier may also correspond to the kinetic data obtained. Studies of the modifier concentration dependence on the isotope exchange kinetics seemed to be useful for the understanding of the nature of the phenomenon.

The initial (fast) component of the kinetic curves may reflect a process of membrane modification (channel formation), or isotope exchange through antibiotic treated membranes, or both. The second (slow) component of the transport plots, being straight lines when represented in a semilogarithmic scale, was extrapolated to the ordinate, and an equilibrium value of isotope distribution between modified cells and the media (a) was found from the intercept (A) using the relationship

$$a = N(1 - e^{A}).$$

With the known equilibrium level, the portion of modified cells in the suspension, b, can be determined. The calculation of b from the isotope efflux data was made on the assumption that there was no significant potential difference across the cell membranes; this assumption seemed reasonable for our experimental conditions.

Than, at equilibrium

$$\frac{NX}{H} = N_{out} + C_{in} V_{in}$$

where N is the total amount of radioactive ions in the blood, N_{out} is the amount of radioactive ions in the extracellular solution, C_{in} is the intracellular concentration of radioactive ions, V_{in} is the volume of intracellular aqueous solution in modified

erythrocytes, X is the integral volume of modified erythrocytes, and H is the total volume of red blood cells.

Since

$$N_{out} = aN, \quad C_{in} = C_{out} = \frac{N_{out}}{1-H} \text{ and } V_{in} = 0.68\ X$$

(the concentration of haemoglobin is 32 % of the total intracellular volume). Thus,

$$b = \frac{X}{H} = \frac{a}{1 - \frac{0.68aH}{1-H}}$$

The calculated values of b were then plotted against gramicidin concentration (Fig. 3). In this Figure, the amounts of gramicidin molecules per one erythrocyte are indicated on the abscissa. To estimate the amounts of gramicidin molecules per one erythrocyte, the partition coefficient of gramicidin A between erythrocytes and a ueous salt solution was taken as 10^4 (Blaskó et al. 1983, 1984).

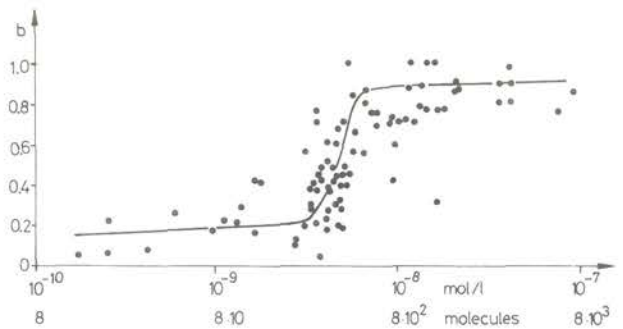

Fig. 3. Proportions of gramicidin modified erythrocytes relative to the total amount of erythrocytes (b) as a function of gramicidin concentration. Gramicidin concentrations are expressed in terms of mol/l of erythrocytes and as numbers of molecules per one erythrocte. For the latter calculation, a lipid : water partition coefficient of 10^4 was taken.

The S-shaped curve can be considered as suggesting cooperativity in the interaction of gramicidin A with red blood cell membranes. Up to a ratio of 300—600 gramicidin molecules per one erythrocyte only a small portion of erythrocytes was found to be modified, while above this ratio the modification was nearly complete.

The kinetic of accumulation of K^+ in the media obtained for ^{42}K loaded erythrocytes treated by primycin (Fig. 2) seemed to be similar to that obtained for gramicidin A modified cells. The dependence of b on the primycin concentration is shown in Fig. 4. The initial part of the plot resembles that for gramicidin A treated erythrocytes; however, "active" concentrations of primycin were found to be

shifted by about 2 orders of magnitude to the higher region, and the slope of the rise of b with the antibiotic concentration was not as steep. The saturation level of b for this modifier could not be obtained because of the pronounced haemolytic effect of the compound at concentrations higher than 6×10^{-5} mol/l.

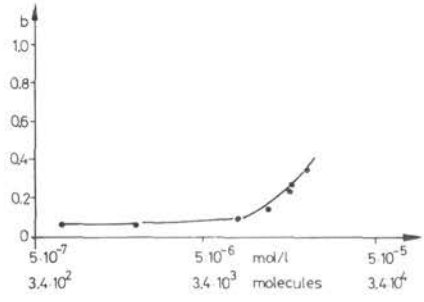

Fig. 4. Proportions of primycin modified erythrocytes relative to the total amount of erythroctes (b) as a function of primycin concentration. For the calculation of the numbers of primycin molecules per one erythrocyte, a lipid: water partition coefficient of 1 was taken.

One possible explanation for the specificity of the action of gramicidin A and primycin on RBC membranes, reported in our previous publication (Blaskó et al. 1984) was based on the assumption that the molecules of the antibiotic entering the membrane cause structural defects in the lipid lattice. The presence of defects facilitates the entering of further molecules of the antibiotic. This model considers the penetration of these antibiotics into the membrane as a cooperative process characterized by a critical antibiotic concentration. Our experimental results support this concept. However a weak point of this explanation was the very high critical concentration of antibiotics to make the membrane permeable for cations. It could be shown that only few channels were necessary to obtain fast equilibration of tracers between the erythrocytes and the media. According to our calculations, for equilibration in about 3 minutes not more than 10 channels with a conductance of about 17 pS (in 0.1 mol/l K^+ solution at 20 °C) were enough (Urban et al. 1980). It still is unclear why the critical number of channel formers required for the modification of RBC membranes is as high. We may speculate that not all the members of the antibiotic aggregate take part in the channel formation. The possibility of a quite different organization of cation transporting pores formed in RBC and in model lipid bilayer membranes cannot be ruled out either.

On the other hand, the questions arise whether the cooperativity mentioned reflects the formation of large aggregates (like those responsible for the haemolysis of RBC at high concentrations of primycin) and whether these aggregates destroy the structure of the membrane nonspecifically, thus forming unselective ionic leaks. One way to learn the nature of the channels formed in RBC membranes was to compare them with channels formed by the same substances in lipid bilayers, by studying the cation selectivity of ionic pathways.

A series of experiments were carried out in order to determine the exchange coefficients for different cations (^{22}Na, ^{86}Rb, ^{137}Cs, ^{42}K) on the basis of influx measurements of gramicidin treated red blood cells. As mentioned above, the conditions of our efflux experiments did not allow an exact determination of the rate coefficients of cation exchange, since the initial component of the kinetic curves possibly is complex, pertaining to channel former incorporation and tracer exchange processes.

To investigate the selectivity of cation transport, it was therefore reasonable to pretreat the cells with a membrane modifier. After a period of 40—50 minutes, required for the process of the antibiotic incorporation into the membranes, the tracer was added into the suspension and the radioactive isotope influx was measured as described before.

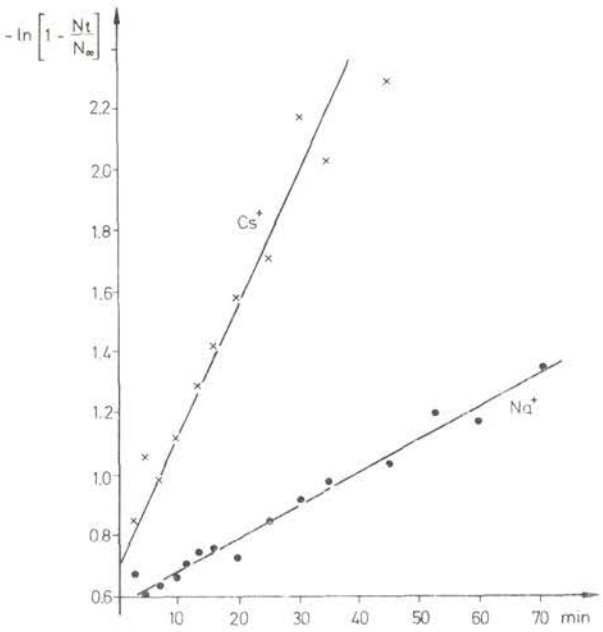

Fig. 5. Effect of gramicidin A on the influx of ^{22}Na and ^{137}Cs at 3 °C. For the composition of the extracellular solution see Fig. 1, pH: 7, H: 0.45. Gramicidin concentration: 1.2×10^{-8} mol/l of extracellular solution.

The results obtained showed that the entry of the isotope into the modified erythrocytes, when measured at 20 °C, was a very fast process. With the sampling protocol used, the precision of the determination of the influx rates was not high enough to provide reliable data on the selectivity of the cation transport. To slow the process and to obtain a better resolution, the cell suspension was cooled and kept at 3 °C. As it can be seen from Fig. 5, the slopes of the first components of the

kinetic curves of Na^+ and Cs^+ differed from each other. Similarly different slopes were got for K^+ and Rb^+. The following mean ratios of rate coefficients were found from 4 series of experiments:

$$k_{Rb} : k_{Cs} : k_K : k_{Na} = 4 : 3.4 : 2.4 : 1$$

The accuracy of the rate coefficient determinations was better than ± 15 %. This sequence of permeabilities was similar to that reported for single gramicidin A channel: $G_{Rb} : G_{Cs} : G_K : G_{Na} = 3.5 : 3.48 : 2.48 : 1$ in lipid bilayers (Anderson 1983). The numerical values of the ratio of rate coefficients found for gramicidin A treated RBC-membranes did not much differ from those established by single gramicidin channel or integral conductance measurements. The existence of a prominent selectivity of the cationic pathway for K^+ as compared with Na^+, induced by gramicidin A in the erythrocyte membranes is very important. It permitted us to exclude the above mentioned possibility concerning the formation of large, and thus nonselective, defects in RBC-membranes as a result of the formation of aggregates in the course of the cooperative action of the modifier.

If the channels formed by gramicidin A in erythrocyte membranes are of the same nature as those in model lipid membranes, the peculiarities of the antibiotic action on the living membranes mentioned above, look even more strange. Taking into account the limited number of detailed studies on the mode of interaction of membrane active antibiotics with the living cell membranes (this is even true for such a compounds as valinomycin widely used as a "chemical tool") we might suggest the existence of a general difference in the modifying action of antibiotics on the living membranes. To check this possibility, we investigated isotope exchange on erythrocytes treated by valinomycin under exactly the same conditions as those used in gramicidin experiments. The results of these experiments shown in Fig. 6—8 can be summarized as follows.

1. The kinetic curves of the efflux of $^{86}Rb^+$ at all the concentrations of valinomycin used were well approximated by a single exponent (Fig. 6) in accordance with the prediction for a two-compartment system.

2. The dependence of efflux rate coefficients on valinomycin concentration (Fig. 7) in the erythrocyte suspension showed a linear relation between membrane cation permeability and the concentration of the modifier in the system within a range of 10^{-7} to 4×10^{-6} mol/l.

3. Studies on cationic selectivity showed that the ratio of rate coefficients, $k_{Rb} : k_{Cs}$, was equal to 5 and was in good correspondence with the figure for permeability ratio ($p_{Rb} : p_{Cs}$) determined potentiometrically for valinomycin treated lipid bilayers (Lev and Buzinsky 1967; Mueller and Rudin 1967) and close to the ratio of conductances ($G_{Rb} : G_{Cs}$) (6) for bilayers prepared from ox brain lipids (Mueller and Rudin 1967) and that for dipalmitoyl phosphatidylcholine

bilayers (4.6) (Benz et al. 1973). The rate coefficient for Na^+ could not be determined as no $^{22}Na^+$ occurred during the 45 min period of observation, suggesting extremely high $p_{Rb} : p_{Na}$ and $p_{Cs} : p_{Na}$ ratios for the valinomycin induced transport.

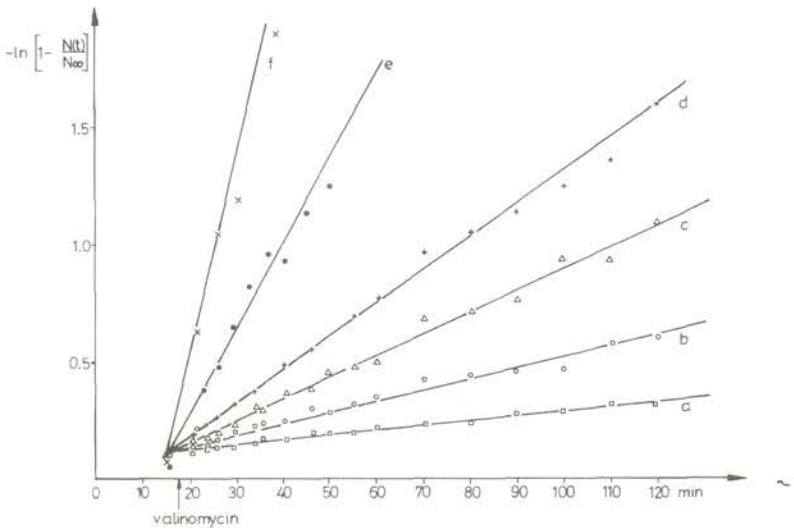

Fig. 6. Effect of valinomycin on ^{86}Rb efflux from red blood cell membranes under isotope exchange condition. Valinomycin concentration: *a)* 1×10^{-7} mol/l, *b)* 3×10^{-7} mol/l, *c)* 6×10^{-7} mol/l, *d)* 1×10^{-6} mol/l, *e)* 2×10^{-6} mol/l, *f)* 4×10^{-6} mol/l (for other conditions, see Fig. 1)

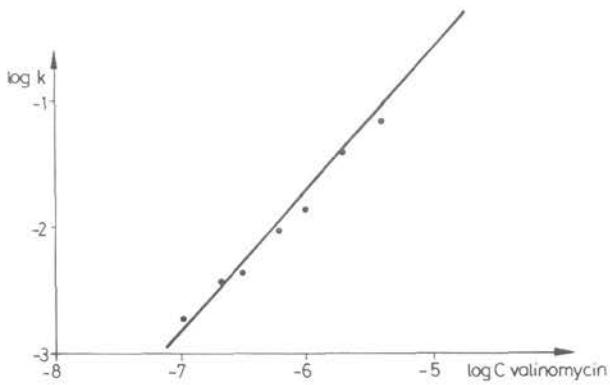

Fig. 7. Rate constants of ^{86}Rb efflux as a function of valinomycin concentration. For experimental conditions, see Fig. 8.

4. Contrary to the data on cation permeability of gramicidin A treated erythrocytes at 3 °C (see Fig. 5), the efflux of Rb^+ and Cs^+ (Fig. 8) was not detectable. For valinomycin in the suspension cooled to 3 °C these data were in full

agreement with the temperature dependence of valinomycin induced cation transport in lipid bilayers reported by Krasne et al. (1971). Our data were in agreement with those published by Hunter (1977) for erythrocytes under symmetrical electrolyte conditions ($c_i^{in} = c_i^{out}$); he found a linear dependence of erythrocyte permeability coefficients for K^+ on the valinomycin concentration in the media. The complex dependence of RBC permeability coefficients for cations on the valinomycin concentration found by Sobieski and Bielawski (1983) could not directly be compared with our data since the former were obtained under different (asymmetrical) electrolyte conditions.

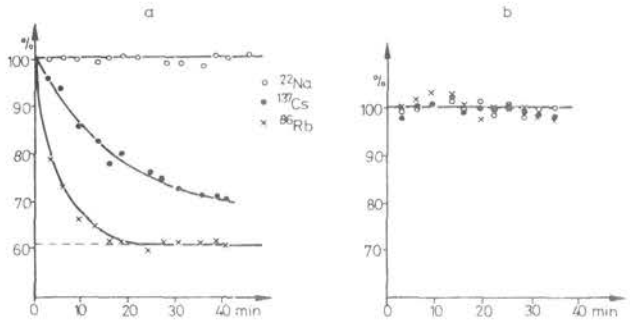

Fig. 8. Effect of valinomycin on the efflux of ^{22}Na, ^{86}Rb and ^{137}Cs under exchange diffusion at 35 °C (*a*) and at 3 °C (*b*). For the composition of the extracellular solution see Fig. 1, pH: 7, H: 0.47; valinomycin concentration: 2×10^{-6} mol/l of extracellular solution.

A comparison of data obtained for gramicidin A and primycin-treated erythrocytes, and those for valinomycin-modified erythrocytes showed that these channel forming antibiotics had a specific behaviour in living cell membranes as compared with model lipid bilayers. Special studies aimed at analysing these differences as well at investigating the kinetic peculiarities observed are now in progress.

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