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HAEMOLYTIC ACTIVITY OF AROMATIC HEPTAENES

A GROUP OF POLYENE MACROLIDE ANTIFUNGAL ANTIBIOTICS

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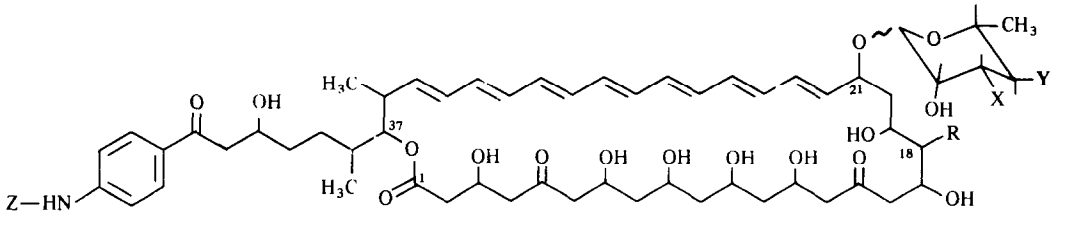
Abstract—The aromatic heptaene vacidin A induces ion selective channels in human red blood cells. The ion flux induced leads to a secondary effect—colloid osmotic haemolysis. Molecular variations at ionizable polar groups of the antibiotic modify the properties of the permeability pathway concerning intercationic selectivity and the symmetry of ion flux.

Aromatic heptaenes are the subgroup of large ring polyene macrolide antibiotics. They are characterized by a biological activity on eukaryotic micro-organisms that is two orders of magnitude higher than the representatives of other groups of large macrolide ring polyenes like amphotericin B or nystatin, most commonly used in therapy [1–3].

The pharmacological application of polyene macrolide antibiotics in antifungal therapy relies upon their high activity against ergosterol-containing pathogenic micro-organisms and their lower activity against cholesterol-containing animal cells. Some of the aromatic heptaenes, being very active on yeast, exhibited low haemolytic activity, pointing to different structural relations in both effects. Red blood cells have been often used as a convenient model in the studies of the mechanism of action of polyene macrolide antibiotics on cholesterol-containing cells and on the toxic effects of these antibiotics [2, 4, 5].

Haemolysis is the final result of complex changes of membrane permeability and membrane structure. In the case of aromatic heptaenes it has been shown previously that their biological activity is due to a rather specific increase of permeability of the membrane to monovalent cations through channel formation [6–11]. Therefore the osmotic imbalance generated by uncontrolled ion distribution is responsible for their haemolytic effect. It might be expected that the haemolytic efficiency of a given aromatic heptaene is related not only to the ability of channel formation but also to the permeability characteristic of the pathway induced.

We have reported earlier that ionizable polar groups (carboxyl at C-18, amino group of amino sugar at C-21 and amino group of aromatic moiety) are essential for the permeability pattern induced by these antibiotics [10, 11]. In the present work an attempt was made to analyse the relation between the haemolytic activity of aromatic heptaenes, the ability of the channel formation and the ionic selectivity of the induced pathway. The studies were done with the aromatic heptaene vacidin A and a number



	R	X	Y	Z
Vacidin A	COOH	NH_2	OH	H
Gedamycin	COOH	NH_2	OH	CH_3
Perimycin	CH_3	OH	NH_2	CH_3
Vacidin A methyl ester/VME/	$COOCH_3$	NH_2	OH	H
Gedamycin methyl ester/GME/	$COOCH_3$	NH_2	OH	CH_3
N,N'-diacetyl vacidin A/NAV/	COCH	$NHCOCH_3$	OH	$COCH_3$
N'-acetyl gedamycin/NAG/	COCH	$NHCOCH_3$	OH	CH_3
N'-succinyl perimycin/NSP/	CH_3	OH	$NHCO/CH_2/_2COOH$	CH_3

Fig. 1. Structure of vacidin A and its natural and semisynthetic analogues.

of its natural and semisynthetic analogues with modified amino groups and carboxyl group. The structures of the examined compounds are shown in Fig. 1.

MATERIALS AND METHODS

Antibiotics and their derivatives. Vacidin A and gedamycin were isolated from crude aureofacin by counter-current distribution in chloroform–methanol–borate buffer, pH 8.3(2:2:1) [12]. Crude aureofacin originated from Pharmaceutical Works POLFA (Tarchomin, Poland). Perimycin A was isolated from crude perimycin [13], and supplied by Lundbeck Co. (Copenhagen).

The methyl esters of vacidin A and gedamycin (syn. mepartricin B and mepartricin A, respectively) [14] were prepared following the method of Bruzzesse *et al.* [15] with some modifications. *N,N'*-Diacetyl vacidin A, *N'*-acetyl gedamycin, and *N'*-succinyl perimycin A were obtained following the procedure described by Schaffner and Borowski for the acylation of heptaenes [16].

The compounds to be tested were dissolved in dimethyl formamide at a concentration of 1 mg/ml and were applied to the red cell suspension in microlitre amounts. The concentration of dimethyl formamide did not exceed 2% and this amount did not affect the red blood cells. Solutions of antibiotics were prepared immediately before use. The concentrations were expressed as μg/ml, calculated for pure compounds.

Media. Isotonic media of pH 7.4 were composed of 150 mM KCl, NaCl or choline chloride. They were buffered with 5 mM Tris–Cl.

Erythrocytes. Human blood, citrate anticoagulated, was kept at 4°. No differences in the sensitivity of cells to polyene macrolide antibiotics were observed when 14-day-old blood was used for cell preparation. Just before use, erythrocytes were separated from plasma and buffy coat by centrifugation for 15 min at 2000 *g*, and were then washed three times by suspending in 4 volumes of choline medium, followed by centrifugation. Packed cells were resuspended in ten volumes of choline medium (stock cell suspension). For antibiotic treatment the stock cell suspension was diluted 25-fold with tested medium. The final cell concentration was *ca* 2×10^7 cells/ml. After complete lysis and centrifugation the supernatant of this suspension had an absorbance of 0.7 at 540 nm.

Determination of haemolytic activity (H_{50}). Portions (2.5 ml) of cell suspension in 310 mOSM buffered potassium chloride, pH 7.4, were incubated for 15 min at 37° in a shaking water bath, then increasing amounts of the compounds to be tested were added. After 1 hr at 37°, the samples were spun and the absorbance of the supernatant was determined at 540 nm in a spectral colorimeter (Carl Zeiss, Jena). The dosage which led to 50% haemolysis (H_{50}) was extrapolated graphically from the haemolysis concentration curves obtained from three separate determinations. The value for 100% haemolysis was obtained by hypotonic haemolysis in water. The error of the haemolysis determination was ±5%.

Osmotic behaviour of the red cells. Washed human erythrocytes were suspended in 250 volumes of isotonic solutions of KCl, NaCl or choline chloride. Volume changes were monitored by changes in the turbidity at 690 nm as a function of time.

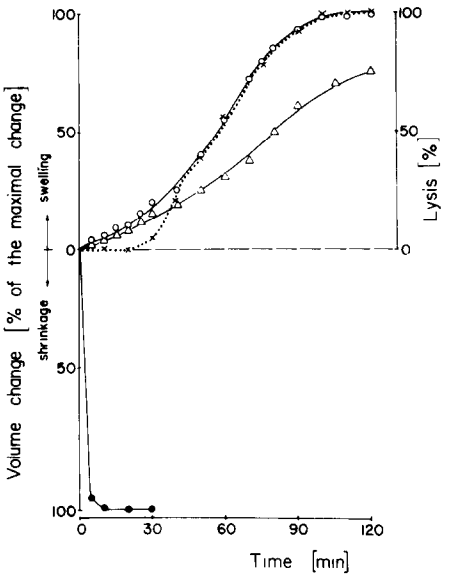


Fig. 2. Changes in erythrocyte volume upon treatment with vacidin A. 2×10^7 cells per ml of isotonic potassium chloride (○—○), sodium chloride (△—△), or choline chloride (●—●) were incubated at 37° with vacidin A at a concentration of 0.2 μg/ml and changes in transmittance were monitored at the indicated time intervals; they are expressed as % of maximal change, i.e. decrease in transmittance for untreated cells suspended in a hypertonic (1 M) solution of sodium chloride (shrinkage) or increase in transmitance for a haemolysed suspension (swelling). ×---×, Haemoglobin released to the medium.

RESULTS

Determination of haemolytic activity

The experiments with the basic antibiotic vacidin A, shown in Fig. 2, illustrate the osmotic behaviour of the red cells in iso-osmotic solutions of sodium chloride, potassium chloride and choline chloride. Subsequent changes in cell volume were monitored by light scattering measurements. The volume changes of the cells reflect the evolution of salt concentration inside the cells. This change is proportional to the difference between the influx and outflux of ions through the antibiotic-formed channels. In choline chloride cell shrinkage was observed, stable in time at the indicated concentration of vacidin A. This was due to the rapid efflux of intracellular potassium not compensated by any influx of choline. In potassium chloride swelling and haemoglobin release were observed due to the influx of K^+ through the channel, according to the concentration gradient. Swelling was followed by lysis. At this concentration of antibiotic swelling as well as shrinkage was suppressed by sucrose. These events indicate that the lysis is of a colloid osmotic type. In physiological sodium chloride solution swelling was also observed but was slower than in potassium chloride.

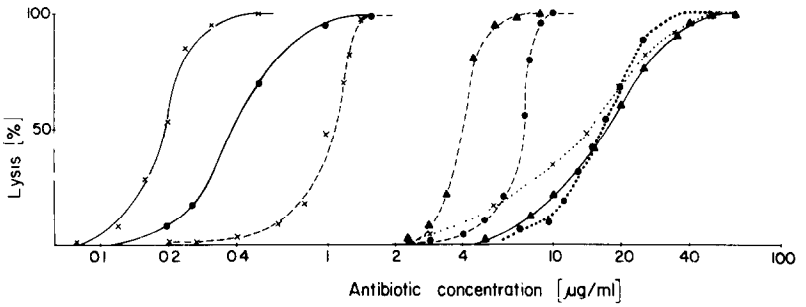


Fig. 3. Haemolysis as a function of antibiotic concentration. The degree of haemolysis of 2×10^7 cells per ml in isotonic potassium chloride after incubation with increasing concentrations of antibiotics for 1 hr at 37° was evaluated from the concentration of haemoglobin in the supernatant after low speed centrifugation. ×—×, Vacidin A; ×---×, NAV; × · · · ×, VME; ●—●, gedamycin; ●---●, NAG; ●···●, GME; ▲—▲, perimycin A; ▲--▲, NSP.

Here the sodium influx is partially compensated by the potassium efflux and the rate of haemolysis reflects the relative permeability of the channels for these two cations. It is obvious from the figure that for a given antibiotic haemolytic activity depends not only on the ability of channel formation but also on its selectivity.

A comparative study of the haemolytic activity of vacidin A and its natural and semisynthetic analogues was done in iso-osmotic potassium chloride. In this simple situation the rate and extent of haemolysis depend on two parameters only: the number of conducting channels and their permeability to K^+. In the bi-ionic situation, when cells are suspended in saline, the selectivity of the channel to cations is the additional parameter influencing the rate and extent of haemolysis.

As the decrease in turbidity to follow haemolysis was complicated by the decrease in turbidity due to prelytic swelling of the cells, the degree of haemolysis was evaluated from the concentration of haemoglobin released to the medium during incubation with the given concentration of antibiotic for the time arbitrarily chosen.

The lytic properties of vacidin A and its derivatives are compared in Fig. 3. For all the compounds examined the extent of haemolysis depended sigmoidally on the applied concentration. However, a broad range of haemolytic activity towards human erythrocytes was observed. The haemolytic concentration range of perimycin A was 100-fold higher than that of vacidin A. Besides, two types of haemolysis dose–response patterns could be distinguished: for compounds with a free carboxyl groups (vacidin A, gedamycin, NAV, NAG, NSP), regardless of its position and molecular variations at the amino groups, steep dose–response curves were observed. Lysis did not start to occur until a critical antibiotic concentration had been reached, but thereafter it was completed in the 2- to 4-fold concentration range of the antibiotic. Compounds without a free carboxyl group (perimycin A, VME, GME) were characterized by rather extended dose–response curves. For them lysis reached a maximal value in the 10-fold concentration range.

The strongest haemolytic activity on a weight basis was exhibited by vacidin A, which has all three ionizable polar groups unsubstituted (Fig. 1). The introduction of a methyl group to an aromatic amine (gedamycin) caused only a slight decrease in this activity. On the other hand, esterification of the carboxyl group (VME, GME) or replacement of the carboxyl by a methyl group (perimycin A) drastically diminished haemolytic properties. The close similarity of the lytic effects caused by the three latter compounds having no free carboxyl group indicates that the position of the amino group in the amino sugar moiety (4′ in perimycin A and 3 in VME and GME) is not essential for a haemolytic activity.

N′-Acylation decreased or increased haemolytic activity depending on the antibiotic used. NAG was less haemolytic than the parent antibiotic. The introduction of a second acetyl group to the aromatic amine (NAV) diminished this effect. On the other hand, NSP was more active on red cells than perimycin itself.

Effect of pore selectivity on haemolysis

Figure 4 shows the dose–response curves for vacidin A, perimycin A, gedamycin and NAG in isotonic solutions of potassium chloride, sodium chloride and choline chloride. As already shown in Fig. 2, haemolysis depends upon the relative permeability of the channel for potassium and sodium, or potassium and choline. It can be seen that, for vacidin A (Fig. 4A), in a Na^+-containing medium *ca* 30% more antibiotic is required to obtain the same haemolysis as that present in a K^+-containing medium. This means that vacidin A forms channels which are slightly more permeable for K^+ than for Na^+. The haemolytic efficiency of vacidin A in choline chloride is more than one order of magnitude lower, reflecting the very low permeability of the vacidin A channel for choline. At low concentrations of vacidin A, only stable shrinkage was observed (Fig. 2). Similar analyses carried out for other compounds (Fig. 4, Table 1) indicate that the analogues of vacidin A formed channels exhibiting a different selectivity for these three cations. Compared to the selectivity of the pathway induced by vacidin A, *N′*-acylation of

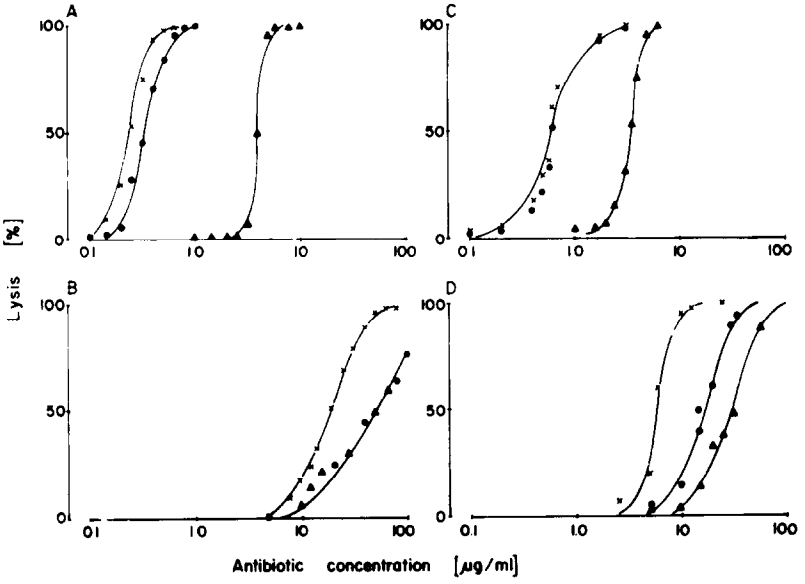


Fig. 4. Effect of medium composition on haemolytic activity. Determination of the degree of haemolysis as in Fig. 3. Vacidin A (A), perimycin A (B), gedamycin (C) and NAG (D) in KCl (×—×), NaCl (●—●) and choline chloride media (▲—▲).

the amino sugar (NAV, NAG) increased the ability of the channel to distinguish between K^+ and Na^+ but at the same time decreased the difference between the alkali cations and choline. The decrease in channel selectivity was observed when the carboxyl group was esterified (VME) or replaced by a methyl group (perimycin A). *N*-Methylation of the aromatic amino group (gedamycin) seemed to have a smaller effect, as the permeability pattern for this antibiotic was similar to that for vacidin A.

Some studies on intercationic selectivity of the permeability pathway formed by aromatic heptaenes for five alkaline monovalent cations have been published elsewhere [10, 11].

VME and perimycin A induced haemolysis only at high concentrations in all three media examined. Moreover, vacidin A, gedamycin, NAV and NAG exhibited haemolytic properties in choline chloride also at relatively high concentrations. In both cases it might be suspected that the haemolysis observed at higher antibiotic concentrations was not due to osmotic imbalance but rather to the detergent-like effect. The analysis of the time course of volume changes of the red cells upon addition of an antibiotic showed that even at high antibiotic concentrations the haemolysis was always the result of osmotic imbalance. As an example, the evolution of cell volume in choline chloride under the effect of increasing concentrations of vacidin A (Fig. 5A) and perimycin A (Fig. 5B) is shown. In both cases swelling and haemolysis of the red cells were always preceded by the shrinkage phase, due to rapid potassium efflux. This means that the membrane was not immediately disrupted by these antibiotics as would be observed in the case of the detergent-like action.

Moreover, there is some indication that the low haemolytic activity of perimycin, as compared to vacidin A, is not due to its low ability of pore formation but rather to the properties of the pore. In Fig. 6 are shown the time courses of cell volume changes in choline chloride and potassium chloride

Table 1. Effect of medium composition on haemolytic activity of aromatic heptaenes and their derivatives

Compound	Ionic state (pH 7.4)	H_{50} (μg/ml) KCl	NaCl	Choline chloride
Vacidin A	Zwitterion	0.2	0.3	4
Gedamycin	Zwitterion	0.4	0.4	3
N,N'-Diacetyl vacidin A (NAV)	Anion	1.5	3.0	8
N'-Acetyl gedamycin (NAG)	Anion	7	18	32
Vacidin A methyl ester (VME)	Cation	12	20	30
Perimycin	Cation	20	40	50

H_{50} is the concentration of compound causing 50% haemolysis of 2×10^7 cells/ml in isoosmotic buffered solutions of potassium chloride, sodium chloride and choline chloride after 1 hr incubation at 37°.

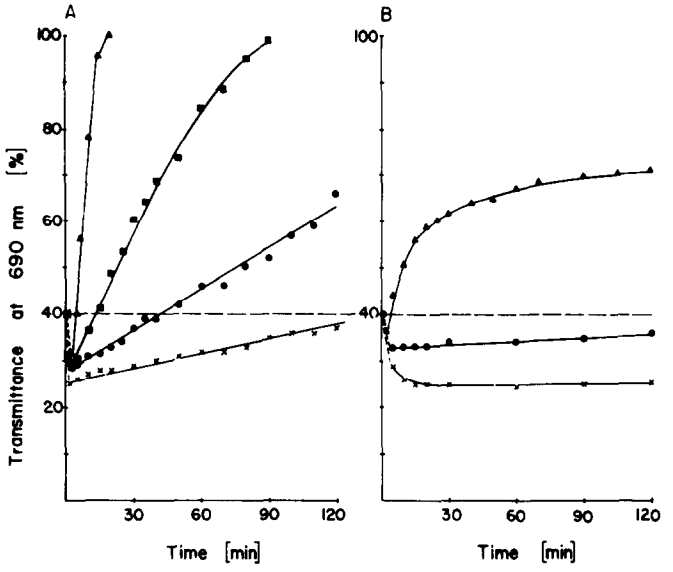


Fig. 5. Osmotic behaviour of erythrocytes under the effect of vacidin A and perimycin A. 2×10^7 cells per ml of isotonic choline chloride were incubated at 37° with various concentrations of vacidin A or perimycin A. Changes in transmittance at 690 nm were monitored at the indicated time intervals. (A) Vacidin A at a concentration of: ×—×, 1 μg/ml; ●—●, 3 μg/ml; ■—■, 4 μg/ml; and ▲—▲, 6 μg/ml. (B) Perimycin A at a concentration of: ×—×, 0.5 μg/ml; ●—●, 2.5 μg/ml; and ▲—▲, 25 μg/ml.

media induced by the same concentration of vacidin A and perimycin A. In choline chloride solution the rate of shrinkage was similar for both antibiotics. This means that the potassium efflux through the vacidin A- and perimycin A-induced channels is approximately the same. On the other hand, in potassium chloride medium the swelling of the cells which is due to the K^+ influx through the same channels was quite different. The permeability of the vacidin A and of the perimycin A channels seemed to be different depending on the direction of the K^+ flux. Although the K^+ outflux through the channels formed by the two antibiotics appeared to be similar, the K^+ influx was much lower for perimycin A than for vacidin A.

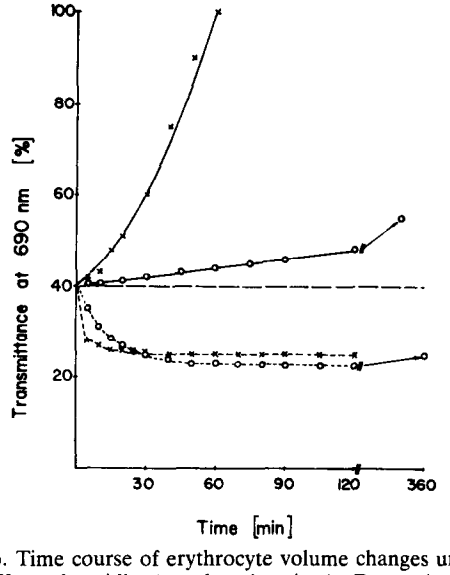


Fig. 6. Time course of erythrocyte volume changes under the effect of vacidin A and perimycin A. Determination as in the legend to Fig. 5. ———, KCl; ——— choline chloride; ×, vacidin A, 0.1 μg/ml; ○, perimycin A, 0.12 μg/ml.

DISCUSSION

Vacidin A and its natural and semisynthetic analogues appear to form pathways specific for cations rather than create a general permeabilization of the cell membrane through a detergent-like effect. This is consistent with the classification of polyene macrolide antibiotics proposed by Brajtburg *et al.* [4], according to which large ring polyene macrolides induce specific permeability, and small macrolide ring polyenes exert a detergent-like effect, although some exceptions were observed [17].

The conclusion which may be drawn after examination of the results (Fig. 3 and Table 1) is that haemolytic activity depends on the structure of the 'polar head' of the molecule. The 'polar head' consists of the carboxyl group at C-18 and the amino sugar at C-21. The Carboxyl and amino groups or the amino sugar confer the ionic character of the antibiotic. According to the ionic state of these two groups at physiological pH, the compounds studied can be divided into three classes: (a) zwitterionic—with free carboxyl and amino groups (vacidin A, gedamycin); (b) negatively charged—with free carboxyl and substituted amino groups (NAV, NAG) or with carboxyl introduced by succinylation (NSP); and (c) positively charged—having a free amino group and esterified carboxyl (VME, GME) or possessing no carboxyl (perimycin A). The highest haemolytic activity was exhibited by vacidin A and gedamycin—zwitterionic antibiotics, the lowest by positively charged perimycin A, VME and GME. The latter were also characterized by a more

extended concentration dependence as compared to the sharp sigmoidal curve of compounds with a free carboxyl group. The activity of the negatively charged compounds was intermediate between these two but greatly varied from one antibiotic to the other depending on the position of the amino group and/or the presence or absence of a carboxyl group.

A difference of two orders of magnitude in haemolytic activity between, for instance, vacidin A and perimycin A could be ascribed either to large differences in the ability to make the permeability pathway or to the permeability characteristic of the pathway when it was formed, i.e. mainly its selectivity. A comparison of the results obtained in potassium chloride, sodium chloride and choline chloride media demonstrates the importance of this last factor (Fig. 4). Here again the ionic character of the polar head controls the selectivity pattern: zwitterionic vacidin A and gedamycin seem to form narrow channels through which alkali cations easily penetrate but permeation of choline is very slow. Anionic compounds (NAV, NAG, NSP) discriminate between K^+ and Na^+, but the difference between the permeability of alkali cations and choline is much smaller than that observed for zwitterionic compounds. At the same time, positively charged VME and perimycin A seem to form channels of low selectivity.

These results point to the fact that caution must be taken in drawing conclusions about cell toxicity of polyene macrolides from haemolytic activity data. There is no correlation between the two events when antibiotics create a specific pathway for cations.

Haemolytic activity is a secondary result of imbalanced influx of cations into the cells. The permeability pathway might be present in the membrane but if it is selective a massive influx of ions will not always be promoted (Fig. 5). The haemolytic effect will be dependent upon the composition of the external medium.

More precise information can be drawn from the evolution of the cell volume in media different only in respect to cations (Fig. 6). In Fig. 6 the rates of volume change of red blood cells at a low concentration of vacidin A, which is highly active according to H_{50} values, and perimycin A, poorly active according to this test, are compared. It is clear that in choline chloride both antibiotics induce cell shrinkage at approximately the same concentration. Thus this test indicates that they are equally active in inducing potassium efflux, i.e. both are similarly cytotoxic, as the cytotoxicity is mainly ascribed to the intracellular K^+ leakage. This conclusion is quite different from that drawn on the basis of the H_{50} values.

This observation raises the following question: why are vacidin A and perimycin A equally active in promoting a K^+ leakage but very different in promoting the K^+ influx which is responsible for haemolysis in potassium chloride medium? One explanation can be found from the half-pore model proposed by van Hoogevest and de Kruijff for amphotericin B [18]. In this model the antibiotic molecules are organized together with cholesterol molecules in a circular manner in such a way that the polar heads of the antibiotic molecules are located on the water–membrane external interface while the macrolide rings form a porous structure perpendicular to the plane of the membrane. Thus the pore structure is asymmetrical and the ion flux depends on the state of ionization of the polar head. The reality of pore asymmetry was demonstrated on black films for amphotericin B and some aromatic heptaenes [8, 19].

Much work has to be done to check this preliminary observation and to obtain more information on the various problems of the mechanism of pore formation in biological membranes. Whatever the mechanism is, it appears from this study that although rapid and convenient the haemolytic test is informative about cytotoxicity only when is positive. When negative, a direct measurement of the ion efflux should be carried out in order to reach a firm conclusion about the toxicity of the antibiotic.

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