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THE INFLUENCE OF AMPHOTERICIN B ON THE PERMEABILITY OF MAMMALIAN ERYTHROCYTES TO NONELECTROLYTES, ANIONS AND CATIONS

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SUMMARY

The influence of the polyene antibiotic, amphotericin B, on the permeability of porcine and bovine erythrocytes was studied by measuring net and tracer movements of nonelectrolytes, anions and cations in these cells.

1. Amphotericin B (0.5–20 μM) enhances the rates of transfer of hydrophilic nonelectrolytes (glycerol, erythritol), anions (phosphate, lactate, glycollate, Cl^-, SCN^-) and cations (Na^+, K^+). Different concentrations of the antibiotic are required for equal effects on the different transfer processes. Bovine erythrocytes respond much less to amphotericin than porcine cells.

2. Nystatin enhances the transfer of all the permeants to a much lesser extent; gramicidin D, although producing a large increase of cation permeability, leaves unaltered anion and nonelectrolyte transfer.

3. The amphotericin-induced enhancement of erythrocyte permeability (ΔP) increases with time. It has a concentration dependence of the type $\Delta P = \alpha \cdot C^n{}_A{}^*$ ($n = 1.5$–2.5) and becomes more pronounced at low temperatures.

4. Partial depletion of membrane cholesterol, which in itself does not alter nonelectrolyte and anion permeability, reduces the effectivity of amphotericin B, indicating that in the erythrocyte membrane, too, a sterol acts as receptor for polyene antibiotics.

5. The selectivity of the amphotericin-induced pathway of transfer in the erythrocyte membrane is lower than that of the normal pathways of nonelectrolyte and anion transfer in this membrane.

The results support the view that amphotericin produces the same type of molecular reorganisation of lipid constituents in biological and artificial membranes. On the other hand, the polyene-induced pathway in the erythrocyte membrane seems to differ functionally from the normal transfer pathways in this membrane.

INTRODUCTION

Macrocyclic polyene antibiotics, notably amphotericin B (Fig. 1) and nystatin damage microorganisms as well as cells of higher organisms by making their membranes leaky towards metabolites, ions and other cell constituents[2–12]. In case of

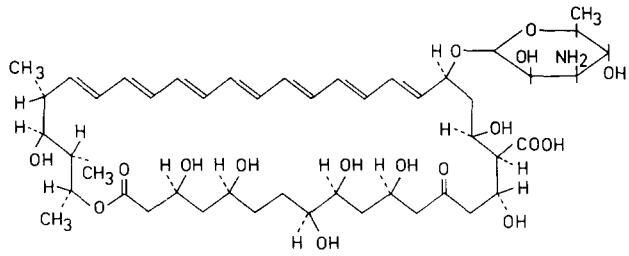

Fig. 1. Proposed structure of amphotericin B (ref. 1).

the erythrocyte, increases of permeability to K^+ and glucose have been described[8–12]. The polyene-mediated enhancement of permeability requires the presence of unesterified cholesterol or certain other sterols in the membranes[13,14]. Sterol-free membranes such as those of bacteria are virtually insensitive to polyene antibiotics[15].

The understanding of the mode of action of the polyenes has recently been greatly promoted by the analysis of their effects on the permeability of artificial lipid membranes[16–23]. These studies provided evidence in favour of the assumption, that the interaction of polyene antibiotics with membrane cholesterol induces a rearrangement of membrane lipids and thus produces pathways of transfer having the properties of aqueous channels or "pores"[19,20,22]. Measurements of permeabilities, hydraulic conductivities and reflection coefficients in artificial lipid membranes treated with amphotericin B or nystatin suggested "equivalent radii" of these pores of 4.3–4.6 Å[22,24]. Since these values strikingly resemble the "equivalent pore radii" calculated for the normal human erythrocyte membrane[25] it was proposed[24] that the pathways induced by polyene antibiotics in lipid bilayers might be a model of the hypothetical pores in the erythrocyte membrane.

In the light of these results and assumptions two questions arise concerning the effect of polyene antibiotics on biomembranes in general and the erythrocyte membrane in particular: (1) To what extent are polyene-induced pathways in biological membranes comparable to those created by these antibiotics in artificial membranes? (2) Are there similarities between the transfer pathways induced in the erythrocyte membrane by polyene antibiotics and the transfer pathways in the normal erythrocyte membrane?

These questions cannot be answered from the data available at present. We have therefore studied the influence of amphotericin B on the transfer of nonelectrolytes, anions and cations across porcine and bovine erythrocyte membranes. For reasons of comparison the effects of two other membrane-active antibiotics, nystatin and gramicidin D, and of digitonin were also investigated.

METHODS

The influence of antibiotics on the membrane permeability of erythrocytes was studied by measuring their effects on the tracer fluxes of anions (Cl^-, SCN^-, phosphate, lactate, glycollate) and nonelectrolytes (erythritol, glycerol) and on the net movements of cations (Na^+, K^+).

Tracer efflux of anions and nonelectrolytes

Loading of the cells. Porcine or bovine erythrocytes obtained at the local slaughter house were washed 3 times in 154 mM NaCl and suspended at a haematocrit of 5% in saline media containing in addition to the permeant to be tested: glucose (5mM), phosphate buffer (Na_2HPO_4–NaH_2PO_4, 10 mM) as well as NaCl and glycylglycine buffer in varying concentrations. The latter nonpenetrating compound was added in order to prevent colloid-osmotic hemolysis of the erythrocytes in the presence of the antibiotics[26]. The cells were incubated in these media at pH 7.35 and 37 °C for 60–180 min in order to attain equilibrium for all penetrating solutes. Subsequently, the suspensions were centrifuged ($6000 \times g$, 15 min) and the supernatant removed by aspiration until the volume left equalled the cell volume. The fluid removed (="preincubation medium") was kept at 0 °C and served as incubation medium during the tracer efflux period.

The 50% (v/v) cell suspensions thus obtained were incubated in the presence of $^{14}C(^{32}P)$-labelled permeant (0.1 μCi/ml of suspension) for 60–90 min. The isotope-loaded cells were separated from their media, washed once in an excess of cold "preincubation medium" and resuspended at a haematocrit of 5% in another portion of the isotope-free "preincubation medium" adjusted to the desired temperature. Special protocols followed in experiments on Cl^- and SCN^- efflux are described in the legend to Fig. 3.

Isotope efflux. Isotope efflux from the erythrocytes under control conditions and in the presence of antibiotics (added at the beginning of the efflux period) was followed by measuring the increase of radioactivity in the incubation medium. 1.5-ml aliquots of the suspensions were sampled at suitable intervals, the medium separated from the cells by rapid centrifugation (14000 rev./min, 15 s, Eppendorf centrifuge 3200) and traces of protein removed by precipitation with 60% $HClO_4$ (0.01 ml per ml of medium). 0.5-ml samples of the deproteinized medium were mixed with 10 ml scintillation fluid (5.5 g PPO, 0.15 g POPOP and counted in a liquid-scintillation spectrometer).

Calculations. The increase of extracellular radioactivity followed a simple exponential in the control experiments. The rate constant k of tracer efflux could therefore be determined by calculating the slope of the linear regression of a plot of $N = \ln[(cpm_\infty - cpm_t)_{ex}/(cpm_\infty - cpm_0)_{ex}]$ *versus* time (cpm = counts/min per unit volume). Subscripts $0, t$, and ∞ refer to the values at the beginning of the efflux period, after different times of incubation and after attainment of isotope equilibrium, respectively. In experiments on phosphate permeability the increase of ^{32}P radioactivity in the medium had to serve as a measure of transfer.

Net movements of cations

The leakage of Na^+ and K^+ from porcine and bovine erythrocytes into media free of these cations was studied by washing and subsequently incubating cells in isotonic (154 mM) solutions of choline chloride (pH 7.35) at an initial haematocrit of 30% with and without addition of antibiotic. After suitable intervals aliquots of the incubation media were analyzed for Na^+ and K^+ by flame photometry (Eppendorf) using in general the procedures of Funder and Wieth[27]. For all dilutions and the preparation of standards choline chloride solutions were used. The extracellular concentration changes of Na^+ and K^+ found under these conditions were not cor-

rected for the simultaneous increase of extracellular water space resulting from net loss of water from the cells.

MATERIALS

Incubation media and washing solutions were prepared from analytical grade or high purity reagents (Merck, Darmstadt; Fluka, Buchs). Labelled compounds ($H^{36}Cl$; $K^{14}CNS$; DL-[1-^{14}C]lactic acid sodium salt; [1-^{14}C]glycollic acid sodium salt; [U-^{14}C]erythritol, [1-^{14}C]glycerol, and [^{32}P]orthophosphate) were purchased from Amersham Buchler, Braunschweig.

Amphotericin B was used in form of the pharmaceutical preparation, *i.e.* in combination with sodium deoxycholate (4 mg per 5 mg amphotericin). A stock solution in water (5 mg amphotericin B per ml) was freshly prepared every week. Nystatin and gramicidin D were dissolved in dimethylformamide (5 mg/ml). All three antibiotics were kindly donated by von Heyden-Squibb, Munich.

RESULTS AND DISCUSSION

The specificity of the effect of amphotericin B

Amphotericin B. According to Fig. 2, low concentrations of amphotericin B greatly affect various transfer processes in porcine erythrocytes. The antibiotic not

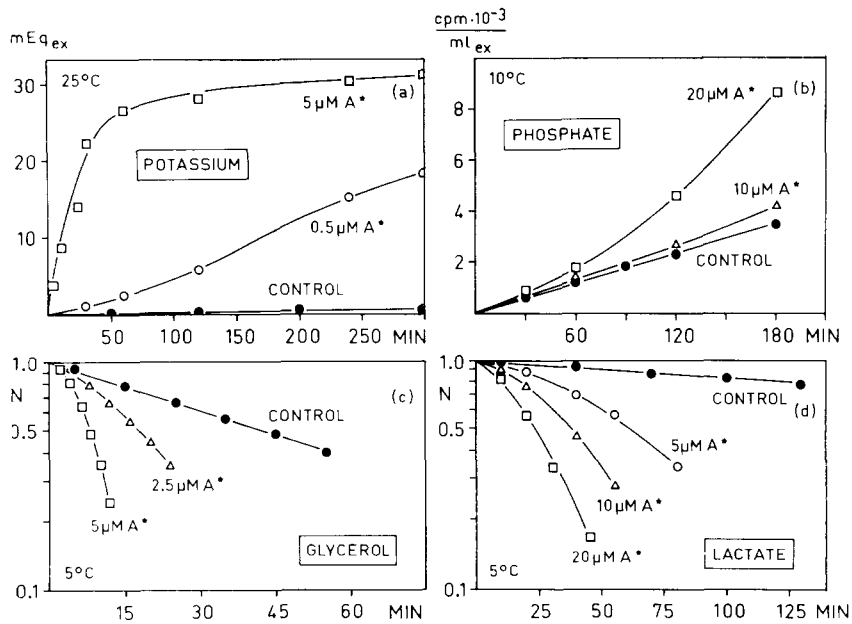

Fig. 2. Influence of amphotericin B (A*) on the rates of transfer of various solutes in porcine erythrocytes. a. Net leakage of K^+ into isoosmolar choline chloride solution. Ordinate scale: extracellular K^+ concentration. b. Efflux of [^{32}P]orthophosphate. Incubation medium (mM): NaCl, 90; Na_2HPO_4/NaH_2PO_4,12; glycylglycine, 50; pH 7.35. Cells preequilibrated with medium for 180 min at 37 °C. Ordinate: extracellular radioactivity. c. [^{14}C]Glycerol efflux. Incubation medium (mM): glycerol, 15; NaCl, 80; Na_2HPO_4/NaH_2PO_4, 12; glycylglycine, 55; pH 7.35. Cells preequilibrated with medium for 120 min at 37 °C. d. DL-[^{14}C]lactate efflux. Incubation medium (mM): sodium DL-lactate, 5.5; NaCl, 80; Na_2HPO_4/NaH_2PO_4,12; glycylglycine, 60. N values on the ordinate: *cf.* Methods.

only enhances the net leakage of K^+ as would be expected from similar results obtained by other investigators[8–12], but also greatly increases the transfer rates of glycerol and of anions such as phosphate and lactate. Similar effects were obtained for Na^+, erythritol and glycollate, which all penetrate much more rapidly in the presence of amphotericin B. Furthermore, an enhancement of the fluxes of Cl^- and SCN^- could be demonstrated under special conditions, namely in the presence of high concentrations of salicylate (Fig. 3). Salicylate reduces the extremely high halide permeability of the erythrocyte membrane by almost 3 orders of magnitude[28], thus making detectable amphotericin-induced changes of permeability which might be difficult to demonstrate in the range of the normal halide permeability. All these phenomena are caused by amphotericin B and not due to the deoxycholate added simultaneously. The latter compound was completely ineffective when added alone.

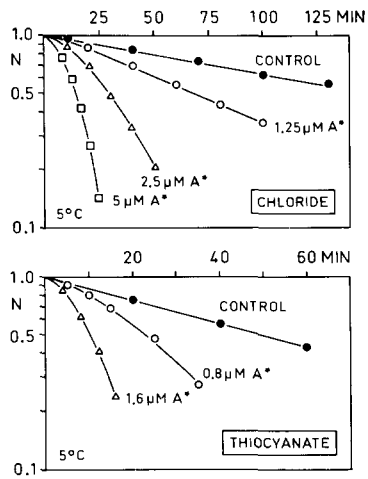

Fig. 3. Enhancement by amphotericin B (A*) of Cl^- and SCN^- efflux from pig erythrocytes into salicylate media. Cells preloaded with $^{36}Cl^-$ and $S^{14}CN^-$ in 0.154 M NaCl or NaSCN (15 min, pH 7.35, Hct. 50%). Efflux medium (mM): sodium salicylate, 85; glycylglycine, 60; NačHPO$_4$/NaHčPO$_4$, 5 (pH 7.65 at 5 °C).

A more detailed analysis of the effect of amphotericin B revealed two peculiarities: Firstly, the enhancement of transfer rates increases with time even during prolonged periods of incubation (Figs 2b, 2d and 3). Whether a new state of constant permeability is finally reached in the presence of the antibiotic could not be clarified in our investigations since amphotericin B was only present during the efflux period which is limited by the attainment of isotope equilibrium. A time dependence of the action of polyene antibiotics was also observed in microbial[4], epithelial[29] and artificial[18,21] membranes. Obviously, either the penetration of the antibiotic into the membrane or the subsequent formation of new transfer sites only occurs slowly.

As a second remarkable feature the magnitude of the antibiotic-induced enhancement of permeability depends on the species of the permeant. In order to accelerate lactate and phosphate transfer, higher concentrations of amphotericin are required than for an enhancement of cation leakage or the fluxes of glycerol and the halides.

Moreover, the transfer of SCN^- is enhanced by the antibiotic more effectively than the transfer of Cl^-. The pathway induced by amphotericin B, as characterized by the difference between the rate constants of transfer in normal and amphotericin-treated cells, thus seems to be more permeable to SCN^- than to the smaller Cl^-. A similar peculiarity was recently described[30] for the "anion-conductance pathway" [30,31] of the normal erythrocyte membrane, where as the rapid, electrically silent "anion-exchange pathway"[30–32], which mediates more than 99.8% of the normal Cl^- transfer, has a higher affinity for Cl^- than for SCN^-.

Gramicidin D. The results obtained with amphotericin B did not exclude the possibility that the transfer rates of anions and even nonelectrolytes might only become enhanced in the presence of the antibiotic as a consequence of net movements of cations, changes of membrane potential or a shifting of water between the cells and their incubation media. In an attempt to settle this problem indirectly, the rates of efflux of a nonelectrolyte and an anion were determined in the presence of gramicidin D[33], which has been shown to enhance considerably the cation permeability of erythrocytes and other membranes[34–36] but is assumed to have no influence on anion and nonelectrolyte permeability[36].

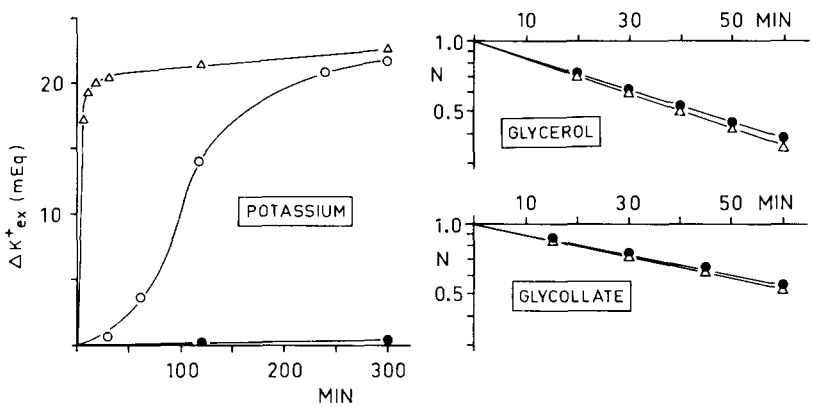

Fig. 4. Influence of gramicidin D on the transfer of K^+, glycerol and glycollate across the pig erythrocyte membrane. Experimental conditions as described in Fig. 2, glycollate efflux measured under the same conditions as that of lactate. ●, controls; ○, gramicidin D, 0.5 μg/ml; △, gramicidin D, 2 μg/ml.

According to the results in Fig. 4, concentrations of gramicidin D which induce rapid net movements of cations do not influence the efflux of glycerol nor that of glycollate. From this finding it may be concluded, that amphotericin B in fact enhances the permeability of the erythrocyte membrane towards anions and nonelectrolytes. Moreover, the results clearly support the view[36] that the transmembrane "channels" presumably induced by gramicidin D[37] are not accessible to glycollate and glycerol.

Nystatin. Other polyene antibiotics, in particular nystatin, also enhance permeability and electrical conductance of natural and artificial membranes[2–4,16,18–23]. Nystatin differs from amphotericin B, a heptaene, (*cf.* Fig. 1) mainly in one respect, namely the lack of one double bond, which makes the molecule a conjugated tre-

traene. As is evident from Fig. 5, this structural difference goes along with a marked difference in activity, nystatin being only slightly effective at concentrations at which amphotericin B greatly enhances membrane permeability. The same difference was also observed in studies on artificial membranes[18,21] and in connection with the haemolytic[8] and antifungal[38] effect of the two polyene antibiotics. It may be explained by the finding[39] that nystatin is bound by the erythrocyte membrane to a lesser extent than amphotericin. Obviously, the rigidity rendered to the large lactone ring of amphotericin B by a series of conjugated double bonds is of great importance for its effect on membranes (*cf.* ref. 21).

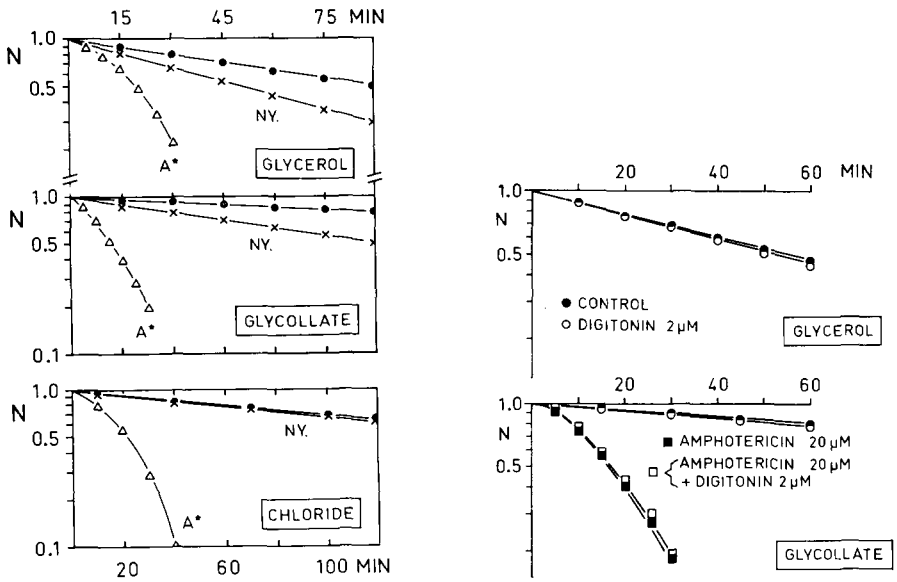

Fig. 5. Different effectivities of equal concentrations (20 μM) of amphotericin B (A*) and nystatin (NY). Pig erythrocytes, experimental conditions of efflux measurements as described in Figs 2–4. ●, controls.

Fig. 6. Lack of influence of digitonin on the permeability and on the amphotericin-induced increase of permeability in pig erythrocytes. Experimental conditions as described in Figs 2 and 4, amphotericin B and digitonin were added simultaneously.

Digitonin. Besides amphotericin B and nystatin, other lytic compounds, namely digitonin and other saponines are also assumed to interact with membrane cholesterol (*cf.* ref. 40). The morphological changes of membrane substructure induced by saponines, however, differ from those brought about by polyene antibiotics[40]. Moreover, amphotericin B—in contrast to saponines—induces haemolysis of the colloid-osmotic type which can be prevented by extracellular non-penetrating solutes [8,26]. On the other hand, similarities in the action of the saponines and the polyene antibiotics, as well as a competition between both for sterols in the membrane of *Neurospora* have been reported[26]. According to the data in Fig. 6, digitonin at the highest subhaemolytic concentration did neither enhance the permeability of porcine erythrocytes nor modify the action of amphotericin B. This finding supports the

view, that amphotericin B and digitonin induce different types of membrane disturbance.

The characteristics of the effect of amphotericin B

Species differences. Erythrocytes from different mammalian species differ considerably in the composition as well as the functional properties of their membranes[41,42]. These dissimilarities are also reflected by their sensitivity to amphotericin B: Bovine erythrocytes are influenced only slightly by concentrations of amphotericin B which are highly effective in porcine erythrocytes. They have to be exposed to 10–20 times higher concentrations of amphotericin B before showing a comparable increase of permeability (Fig. 7).

The membrane contents of free cholesterol, which considerably influences amphotericin B sensitivity (see section on *Cholesterol dependence*), are very similar in porcine and bovine erythrocytes[41] and therefore cannot account for these differences. There are some indications, however, that the external surface of the bovine erythrocyte membrane may be covered with a glycoprotein layer more bulky than that of the porcine erythrocyte membrane[43–45]. This glycoprotein layer could impede the access of the polyene molecules to their sites of action. The assumption of such a barrier function of membrane glycoproteins is corroborated by the finding[7] that the permeability of the serosal membrane of the turtle large intestine epithelium only responds to amphotericin B after treatment with proteolytic enzymes.

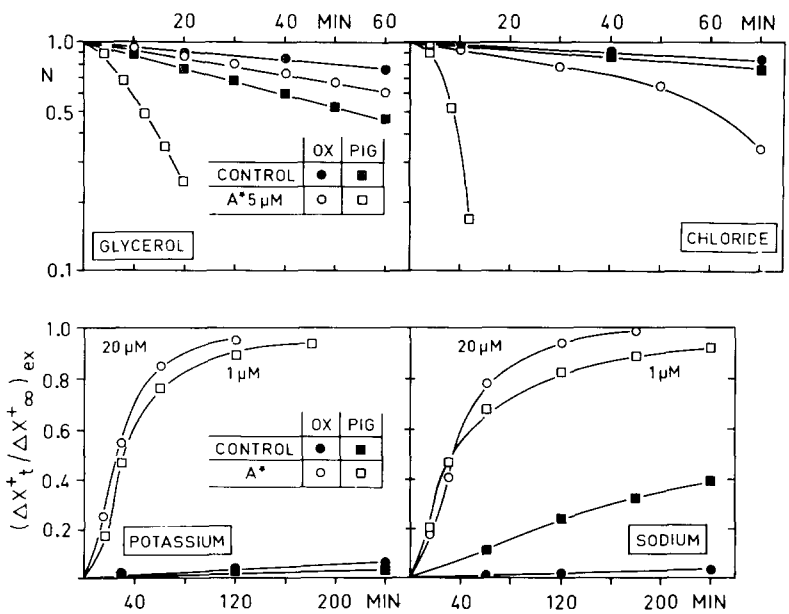

Fig. 7. Differences in the sensitivity of porcine and bovine erythrocyte membranes to amphotericin B (A*). [^{14}C]Glycerol and $^{36}Cl^-$ efflux and Na^+ or K^+ leakage were determined under the experimental conditions described in Figs 2 and 3. Cation concentrations (X^+) in the extracellular medium given in arbitrary units relative to the extracellular concentration after attainment of equilibrium (X^+_∞).

Cholesterol dependence. In microorganisms as well as in artificial membranes the effect of polyene antibiotics depends on the presence of cholesterol or certain other sterols[2,4,16–18], which act as receptors for the polyenes. The same role of cholesterol in the erythrocyte membrane could now be demonstrated by experiments, in which the influence of amphotericin B was studied in normal pig erythrocytes and in cells partly depleted of their membrane cholesterol[46] by the technique of Murphy[47]. According to the data compiled in Table I, a reduction of the membrane cholesterol content by about 30% does not increase the basic permeability of porcine erythrocytes to glycollate and to erythritol, in contrast to findings in other membrane systems (for references see ref. 46). The 5–6-fold enhancement of permeability induced by amphotericin B in the normal cells, however, diminishes to a mere 2-fold increase after the partial removal of cholesterol. This finding may have particular significance for the elucidation of the mode of action of amphotericin B and of the organisation of the erythrocyte membrane in general: On the basis of morphological and physicochemical data it has been proposed that the formation of complexes between polyene antibiotics and membrane cholesterol goes along with a reorientation of membrane lipids[39,40], which enhances solute permeability probably by creating pores for penetrating hydrophilic solutes. On the other hand, it is in general tacitly assumed that in natural membranes, as in artificial lipid membranes, the cholesterol which interacts with amphotericin B was originally part of a phospholipid–cholesterol bilayer, stabilized by the sterol and acting as a barrier for polar permeants. The obvious lack of influence of a substantial cholesterol depletion on the normal permeability of porcine erythrocytes clearly indicates that at least a fraction of membrane cholesterol is not part of the barriers limitingthe transfer of anions and nonelectrolytes, since otherwise depletion should lead to an increase of permeability. Nevertheless, this fraction can serve as a constituent of new transfer pathways which in the case of "pore" formation would have to extend through the permeability barrier of the erythrocyte membrane. This finding may be taken as evidence that the amphotericin-induced pathway runs parallel to and spatially distant from the normal pathways of anion and nonelectrolyte transfer in the erythrocyte membrane.

TABLE I

EFFECT OF AMPHOTERICIN B ON THE RATE CONSTANTS ($k \cdot 10^3$ (min^{-1})) FOR THE EFFLUX OF GLYCOLLATE AND ERYTHRITOL FROM NORMAL AND CHOLESTEROL-DEPLETED PIG ERYTHROCYTES

	Glycollate efflux (5 °C)		*Erythritol efflux* (30 °C)	
	Control	*Amphotericin B* (20 μM)	*Control*	*Amphotericin B* (10 μM)
Normal cholesterol (702 nmoles/μmole haemoglobin)	9.3	56.6	13.6	64.0
Cholesterol-depleted (508 nmoles/μmole haemoglobin)	9.6	18.6	14.6	31.5

Concentration dependence. Present ideas about the interaction of polyene antibiotics with their receptors in membranes are based in particular on the concentration dependence for the enhancement of permeability observed in artificial membrane systems[18,20,21]. Permeability increases as a large power of the antibiotic concentration according to a relationship of the type:

$$\Delta P = \alpha \cdot C_{A^*}^{n}, \quad \text{or} \quad \log \Delta P = \log \alpha + n \cdot \log C_{A^*} \tag{1}$$

ΔP is the increase of permeability; C_A*, concentration of antibiotic; α, n are constants. This relationship has been taken to indicate[18,21] that n molecules of antibiotic are involved in the creation of one new transfer site. The values of n observed in artificial membranes range from 3.5–10, depending on the type of the membrane and on the permeant. In order to clarify whether in the erythrocyte membrane too amphotericin acts in this way, the rates of tranfer of various solutes were determined at increasing concentrations of the antibiotic.

Due to the time-dependent rise of the rate constant k for the efflux in the presence of amphotericin B (*cf.* Figs 2 and 3) values for k in a strict sence were not available. Instead, the linear slope of the efflux kinetics between the 10th and the 20th min of the efflux period was taken as a relative measure of k. By subtracting the rate constants of the control fluxes from these values the transfer only due to amphotericin B (Δk_A*, $\Delta (dC_{ex}/dt)_A$*) was calculated. As is evident from Fig. 8, the logarithms

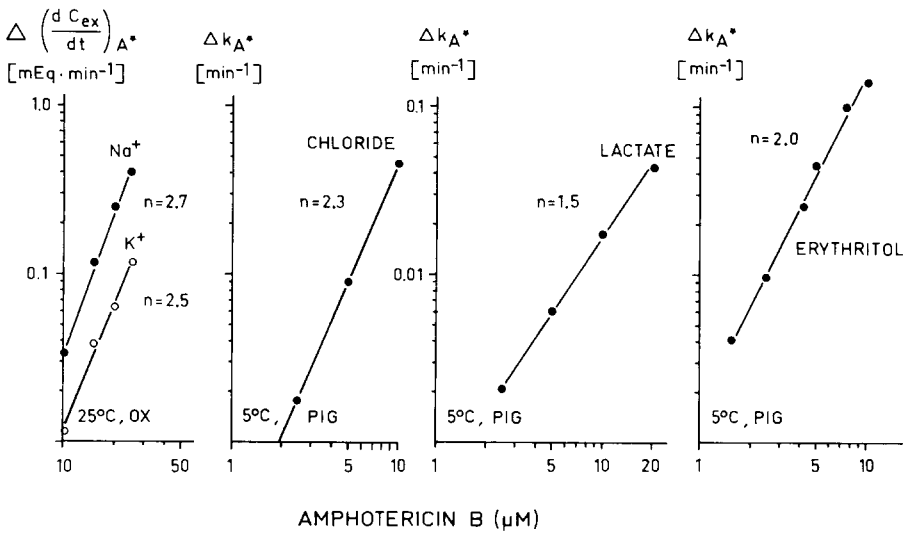

Fig. 8. Exponential increase of the amphotericin-induced permeability with the concentration of antibiotic. Data obtained under the experimental conditions described in Figs 2 and 3. Erythritol efflux measured under the same conditions as glycerol efflux. $\Delta (dC_{ex}/dt)_A$* and Δk_A*: Rates of cation leakage and rate constants of tracer efflux in the presence of amphotericin *minus* the respective values in absence of the antibiotic. n values refer to Eqn 1 in the text.

of these values increase linearly with log C_A*. The slopes vary in a range between 1.5 and 2.7 for the different permeants studied, but are essentially independent of the time interval chosen for the determination of k_A* (Table II).

TABLE II

TIME INDEPENDENCE OF THE EXPONENT n (Eqn 1) IN THE DOSE-RESPONSE RELATIONSHIP FOR AMPHOTERICIN B

n_{10-20}; n_{20-30}: Values determined from slopes of the efflux kinetics linearized between 10 and 20 or 20 and 30 min after beginning of the efflux period.

Species	*Permeant (at 5 °C)*	n_{10-20}	n_{20-30}
Ox	Glycerol	1.72	1.95
Pig	Glycollate	1.68	1.47
Pig	Erythritol	2.26	2.23

In terms of the model outlined above, this finding indicates that in the erythrocyte membrane, too, more than one molecule of amphotericin B is required for creating a transfer site. The values of n, however, are lower here than in artificial membranes. An interpretation of this difference in terms of numbers of interacting molecules of polyene and cholesterol will have to await the further elucidation of the physicochemistry of the complex formation[39,48,49].

Temperature dependence. According to the results described in a preceeding section the transfer of large anions (lactate, phosphate) becomes only enhanced by amphotericin B at high concentrations of the antibiotic. Even this effect is only obtained at temperatures between 0 and 10 °C. At higher temperatures almost no alteration of lactate permeability can be demonstrated (Fig. 9a). Obviously, the effectivity of amphotericin diminishes with increasing temperature. A similar phenomenon was also observed with erythritol, giving rise to an apparent inverse temperature dependence of erythritol transfer in the presence of amphotericin B (Fig. 9b, broken lines).

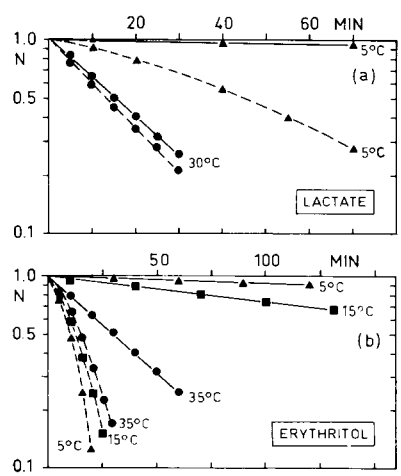

Fig. 9. Effect of amphotericin B on the lactate and erythritol efflux from pig erythrocytes at different temperatures. ——, controls; – – –, amphotericin B, 10 μM.

In order to evaluate more precisely the temperature dependence of the amphotericin-induced permeability of the erythrocyte membrane, Arrhenius diagrams were drawn for the rate constants of erythritol and lactate fluxes in normal and amphotericin-treated erythrocytes (Fig. 10). In case of the normal cells linear regression lines are obtained when log k is plotted versus $1/T$. The negative slopes of these lines correspond to μ/R, where μ is the Arrhenius activation energy and R the gas constant. μ values amount to 20 kcal/mole for erythritol and 22 kcal/mole for lactate in the pig erythrocytes. It should be noted that the activation energy for erythritol is constant between 5 and 50 °C.

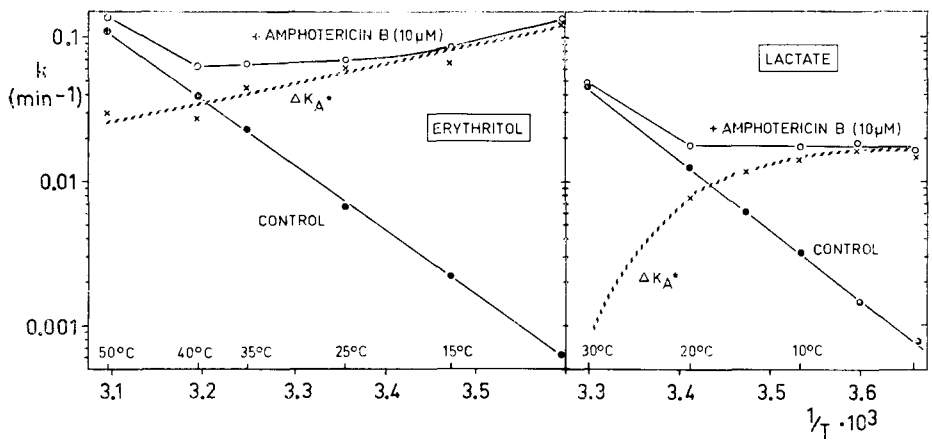

Fig. 10. Arrhenius diagrams for the temperature dependence of erythritol and lactate transfer in normal and amphotericin B-treated pig erythrocytes. From the slope of the curves activation energies can be calculated. Δk_A^* values determined as in Fig. 8.

In the presence of amphotericin B, however, non-linear relationships between log k and $1/T$ are obtained, which below a critical temperature (approx. 40 °C for erythritol, approx. 20 °C for lactate) do not have a "normal" negative slope but run parallel to the abscissa or even change to a positive slope. A positive slope also characterizes the relationship between $1/T$ and log Δk_A^*, the k value for the transfer proceeding *via* the amphotericin-induced pathway. In the range of temperatures accessible in our experiments, log Δk_A^* increases linearly with $1/T$ for erythritol, whereas in case of lactate a non-linear increase is observed.

The anion and nonelectrolyte permeabilities induced by amphotericin B in porcine erythrocyte membranes thus have apparent negative activation energies, equivalent to negative temperature coefficients. A similar phenomenon was reported for the nystatin-induced conductance of artificial lipid membranes[21], and has also been demonstrated for the leakage of potassium from amphotericin B-treated human erythrocytes[11]. Such negative temperature coefficients cannot be ascribed to the transmembrane diffusion of the permeant *per se*. It seems more plausible to assume that the number of transfer sites created by a given concentration of amphotericin B decreases with rising temperature, probably due to thermal forces inhibiting the formation and favouring the disintegration of polyene–cholesterol complexes[20].

Amphotericin-induced selectivity versus normal selectivity of the erythrocyte membrane

From studies of the nonelectrolyte and water permeabilities it has been concluded that the transfer sites induced by polyene antibiotics in artificial membranes have the properties of aqueous pores with a radius of about 4–4.5 Å[22]. On the basis of measurements of water permeability and nonelectrolyte permselectivity, similar pores have previously been claimed[25] in the normal erythrocyte membrane as pathways for the noncatalyzed transmembrane diffusion of water and hydrophilic nonelectrolytes. Recently, a virtual identity of the properties of the two aqueous pathways has been proposed[24].

The present investigations provided the possibility to compare the selectivities of the normal and the amphotericin-treated erythrocyte membrane. If channels produced in the erythrocyte membrane by amphotericin also have the same properties as the normal aqueous pathways of this membrane, amphotericin should increase the permeability for different permeants using this pathway, but should leave the ratio of permeabilities unaltered. As is evident from Table III, the latter is not true at least for the two pairs of permeants tested as yet: In both cases the unaltered membrane discriminates more sharply between two homologous molecules of different molecular size than does the amphotericin-induced pathway. Thus, the "pathways" induced in the erythrocyte membrane by polyene antibiotics—in contrast to the channels induced in lipid membranes—seem to differ from the normal pathway of the erythrocyte membrane.

TABLE III

RATE CONSTANTS ($k \cdot 10^3(\text{min}^{-1})$) FOR THE TRANSFER OF ANIONS AND NONELECTROLYTES IN NORMAL AND AMPHOTERICIN B-TREATED PIG ERYTHROCYTES

	Normal pathway ($k_{control}$)	*Amphotericin-induced pathway* ($k_{amphotericin} - k_{control}$)	*Experimental conditions*
Glycollate (C_2)	10.5	38.0	5 °C
Lactate (C_3)	1.5	18.0	amphotericin 10 μM
Ratio	7.0	2.1	
Glycerol (C_3)	13.0	150.0	5 °C
Erythritol (C_4)	0.7	44.6	amphotericin 5 μM
Ratio	18.6	3.4	

CONCLUSIONS

On the basis of the data presented in the foregoing, the questions posed in the Introduction may now be answered as follows:

(1) Amphotericin B enhances solute transfer across the erythrocyte membrane by a process which has a number of peculiarities (cholesterol requirement, time dependence, exponential concentration dependence, negative temperature dependence) in common with the polyene-induced formation of transfer sites in artificial lipid membranes. These similarities suggest that basically the same events occur in presence

of amphotericin B in natural and artificial membranes. Therefore, the transfer pathway induced by amphotericin B in the erythrocyte membrane seems not to differ in its principal molecular organisation from the "channels" in the artificial lipid membranes, in spite of the presence in the biomembrane of a considerable amount of proteins. These proteins could be responsible for some of the quantitative differences between the effects of amphotericin B on artificial and on biological membranes and on different biological membranes.

The polyene-induced pathway in the erythrocyte membrane is presumably arranged in parallel to the normal pathways of passive solute transfer. Whether it really consists of a pore in the sense of a water-filled continuum remains an open question[50], especially in view of the finding[51], that amphotericin B seems not to enhance the hydraulic conductivity of the erythrocyte membrane.

(2) Our data do not yet allow a detailed functional characterisation of the amphotericin-induced pathways in the erythrocyte membrane in terms of equivalent pore radii, reflection coefficients or parameters of anion-cation discrimination. It seems at present justified to state that the amphotericin-induced pathways in the erythrocyte membrane are less selective than the normal ones. Although both pathways discriminate hydrophilic nonelectrolytes and organic anions according to size, only a partial similarity but not a functional identity of the amphotericin-induced transfer pathway in the erythrocyte membrane with the normal pathways may thus be postulated.

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REFERENCES

1 Mechlinski, W., Schaffner, C. P. , Ganis, P. and Avitabile, G. (1970) *Tetrahedron Lett.* 44, 3873–3876
2 Kinsky, S. C., Luse, S. A. and van Deenen, L. L. M. (1966) *Fed. Proc.* 25, 1503–1510
3 Lampen, J. O. (1966) *Symp. Soc. Gen. Microbiol.* 16, 111–130
4 Kinsky, S. C. (1967) in *Antibiotics I: Mechanism of Action* (Gottlieb, D. and Shaw, P. D., eds), p.p. 122–141, Springer, Heidelberg–New York
5 Steinmetz, Ph. R. and Lawson, L. R. (1970) *J. Clin. Invest.* 49, 596–601
6 Wizemann, V. and Schulz, I. (1972) *Pflügers Arch.* 332, R 23
7 Lippe, C., Giordana, B. and Carpraro, V. (1968) in *Membrane Models and the Formation of Biological Membranes* (Bolis, L and Pethica, B. A., eds), p.p 160–165, North Holland, Amsterdam
8 Kinsky, S. C., Avruch, J., Permutt, M., Rogers, H. B. and Schonder, A. A. (1962) *Biochem. Biophys. Res. Commun.* 9, 503–507
9 Butler, W. T., Alling, D. W. and Cotlove, E. (1965) *Proc. Soc. Exp. Biol. Med.* 118, 297–300
10 Blum, St. F., Shohet, St. B., Nathan, D. G. and Gardner, F. H. (1969) *J. Lab. Clin. Med.* 73, 980–987
11 Butler, W. T. and Cotlove, E. (1971) *J. Infect. Dis.* 123, 341–350
12 Kim, H. D. and McManus, T. J. (1971) *Biochim. Biophys. Acta* 230, 1–11

13 Weber, M. M. and Kinsky, S. C. (1965) *J. Bacteriol.* 89, 306–316
14 Feingold, D. S. (1965) *Biochem. Biophys. Res. Commun.* 19, 261–267
15 Kinsky, S. C. (1962) *Proc. Natl. Acad. Sci. U.S.* 48, 1049–1052
16 Weissmann, G. and Sessa, G. (1967) *J. Biol. Chem.* 242, 616–625
17 Lippe, C. (1968) *J. Mol. Biol.* 35, 635–637
18 Andreoli, Th. E. and Monahan, M. (1968) *J. Gen. Physiol.* 52, 300–325
19 Andreoli, Th. E., Dennis, V. W. and Weigl, A. M. (1969) *J. Gen Physiol.* 53, 133–156
20 Dennis, V. W., Stead, N. W. and Andreoli, Th. E. (1970) *J. Gen. Physiol.* 55, 375–400
21 Cass, A., Finkelstein, A. and Krespi, V. (1970) *J. Gen. Physiol.* 56, 100–124
22 Holz, R. and Finkelstein, A. (1970) *J. Gen. Physiol.* 56, 125–145
23 Van Zutphen, H., Demel, R. A., Norman, A. W. and Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* 24, 310–330
24 Solomon, A. K. and Gary-Bobo, C. M. (1972) *Biochim. Biophys. Acta* 255, 1019–1021
25 Solomon, A. K. (1968) *J. Gen. Physiol.* 51, 335_s–364_s
26 Kinsky, S. C. (1963) *Arch. Biochem. Biophys.* 102, 180–188
27 Funder, J. and Wieth, J. O. (1966) *Scand. J. Clin. Lab. Invest.* 18, 151–166
28 Dalmark, M. and Wieth, J. O. (1972) *J. Physiol.* 224, 583–610
29 Lippe, C. and Giordana, B. (1967) *Biochim. Biophys. Acta* 135, 966–972
30 Wieth, J. O., Dalmark, M., Gunn, R. B. and Tosteson, D. C. (1973) in *Erythrocytes, Thrombocytes, Leukocytes: Recent Advances in Membrane and Metabolic Research* (Gerlach, E., Moser, K., Deutsch, E. and Wilmanns, W., eds), p.p 71–76, Thieme, Stuttgart
31 Tosteson, D. D., Gunn, R. B. and Wieth, J. O. (1973) in *Erythrocytes, Thrombocytes, Leukocytes: Recent Advances in Membrane and Metabolic Research* (Gerlach, E., Moser, K., Deutsch, E. and Wilmanns, W., eds), p.p. 71–76, Thieme, Stuttgart
32 Lassen, U. V. (1972) in *Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status* (Rørth, M. and Astrup, P., eds), p.p. 291–303, Munksgaard, Copenhagen
33 Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 672–676
34 Henderson, P. J. F., McGivan, J. D. and Chappell, J. B. (1966) *Biochem. J.* 111, 521–535
35 Harris, E. J. and Pressman, B. C. (1967) *Nature* 216, 918–920
36 Myers, V. B. and Haydon, D. A. (1972) *Biochim. Biophys. Acta* 274, 313–322
37 Hladky, S. B. and Haydon, D. A. (1970) *Nature* 225, 451–453
38 Bonner, D. P., Mechlinski, W. and Schaffner, C. P. (1972) *J. Antibiot.* 25, 261–262
39 Norman, A. W., Demel, R. A., De Kruyff, B., Geurts van Kessel, W. S. M. and van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* 290, 1–14
40 Kinsky, S. C., Luse, S. A., Zopf, D., van Deenen, L. L. M. and Haxby, J. (1967) *Biochim. Biophys. Acta* 135, 844–861
41 Nelson, G. J. (1972) in *Blood Lipids and Lipoproteins. Quantitation, Composition and Metabolism* (Nelson, G. J., ed.), p.p. 318–386, Wiley Interscience, New York–London–Sydney–Toronto
42 Gruber, W. and Deuticke, B. (1973) *J. Membrane Biol.*, in the press
43 Uhlenbruck, G. (1969) *Naturwissenschaften* 56, 422
44 Triplett, R. B. and Carraway, K. L. (1972) *Biochemistry* 11, 2897–2903
45 Hamaguchi, H. and Cleve, H. (1972) *Biochem. Biophys. Res. Commun.* 47, 459–463
46 Deuticke, B. and Zöllner, Chr. (1972) *Biochim. Biophys. Acta* 266, 726–731
47 Murphy, J. R. (1962) *J. Lab. Clin. Med.* 60, 86–109
48 Schroeder, F., Holland, J. F. and Bieber, L. L. (1972) *Biochemistry* 11, 3105–3111
49 Bittman, R. and Fischkoff, S. A. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 3795–3799
50 Verkleij, A. J., De Kruijff, B., Gerritsen, W. F., Demel, R. A., van Deenen, L. L. M. and Ververgaert, P. H. J. (1973) *Biochim. Biophys. Acta* 291, 577–581
51 Rich, G. T., Sha'afi, R. I., Romualdez, A. and Solomon, A. K. (1968) *J. Gen. Physiol.* 52, 941–954