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CHANGES IN PERMEABILITY OF *STAPHYLOCOCCUS AUREUS* AND DERIVED LIPOSOMES WITH VARYING LIPID COMPOSITION

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SUMMARY

The physical and barrier properties of the phospholipids phosphatidylglycerol and lysylphosphatidylglycerol were studied in membrane model systems. Packing in monolayers at the air–water interface showed a larger area per molecule for lysylphosphatidylglycerol than for phosphatidylglycerol. The non-electrolyte permeability of liposomes prepared with lysylphosphatidylglycerol was higher than of those prepared with phosphatidylglycerol. On the other hand, the permeability of $^{86}Rb^+$ was higher for liposomes of phosphatidylglycerol than for those of lysylphosphatidylglycerol. Valinomycin was able to increase the permeability of this cation in the phosphatidylglycerol liposome only.

Studies on the effect of the environmental pH on the lysylphosphatidylglycerol to phosphatidylglycerol ratio in intact cells of *Staphylococcus aureus* showed that the total amount of lysylphosphatidylglycerol, cardiolipin and neutral lipids in the membrane did not change when the pH of the medium was varied between pH 6.5 and 5.0; but the total amount of phosphatidylglycerol decreased when the pH of the medium was lowered. The permeability of the intact cells for erythritol appeared to increase with increasing lysylphosphatidylglycerol to phosphatidylglycerol ratio; whereas the valinomycin mediated exchange of $^{86}Rb^+$ over the cell membrane appeared to decrease when this ratio was increased.

From the correlation between the permeability properties of cells and liposomes the conclusion is drawn that in *S. aureus* the chemical nature of the phospholipids determines to a great extent the properties of the permeability barrier.

INTRODUCTION

Aminoacyl derivatives of phosphatidylglycerol have been found in many Gram-positive bacteria. Lysine, ornithine, glycine and alanine are the amino acids identified as esters of phosphatidylglycerol[1–4]. Lysylphosphatidylglycerol has been found in *Staphylococcus aureus*[3], *Bacillus megaterium*[4], *Bacillus subtilis*[5], *Clostridium welchii*[1] and *Streptococcus faecalis*[6]. The structure of lysylphosphatidylglycerol of *S. aureus* has been established by HOUTSMULLER AND VAN DEENEN[6]. As regards to the physiological role of the amino acid esters of phosphatidylglycerol, it has been speculated that they may play a role in: cell wall or protein synthesis[8]; amino acid

transport[7]; or that they may have a function in regulation of the ion permeability of the cells[3,6]. The latter possibility was suggested by HOUTSMULLER AND VAN DEENEN, who studied the effect of acidity of the medium on the phospholipid composition of *S. aureus* and found that at pH 7.0 phosphatidylglycerol was the main phospholipid, whereas lysylphosphatidylglycerol prevailed at pH values below 5.0. The positively charged phospholipid lysylphosphatidylglycerol could play a role in inhibiting protons to enter the cells from acidic media[18].

The aim of this study was to evaluate this possibility. Therefore, we studied permeability properties of *S. aureus* cells with varying phosphatidylglycerol to lysylphosphatidylglycerol ratios and of liposomes prepared from these phospholipids.

MATERIALS AND METHODS

Organism and growth conditions

S. aureus was grown in a medium containing 10 g pepton, 10 g yeast extract, 5 g NaCl and 0.8 g Na_2HPO_4 per l of water. The pH of the medium was 6.8. A preculture of *S. aureus* cells was inoculated 1:100 into fresh medium and aerobically grown by shaking at 37° untill semilogarithmic phase (pH 6.5). For some experiments the medium was supplemented with 10 g of glucose per l of growth medium. These cells were grown for 16 h.

Phospholipid analysis

Cells of *S. aureus* were grown until semi-logarithmic phase ($A_{550\ nm}$ approx. 1.0) in 100 ml of the growth medium, supplemented with 100 μC $^{32}P_i$. The cells which were grown without glucose at pH 6.5 were 10 times concentrated suspended in a solution of 0.3 M sucrose, containing 0.01 M citrate phosphate buffer (pH 5.0). The cells grown with glucose until pH 4.7 were 10 times concentrated suspended in a solution of 0.3 M sucrose, containing 0.01 M citrate phosphate buffer (pH 7.0).

After incubation for varying times at 37° in these media, the cells were acidified with HCl to pH 2 and immediately extracted as described by BLIGH AND DYER[9]. The total lipid extracts were separated on silica gel plates in chloroform–methanol–water–acetic acid (65:25:5:1, by vol.) and the plates were scanned for radioacitvity. Percentages of the different phospholipids were determined by counting the radioactivity of the different spots.

Characterization of the fatty acid constituents

About 5 mg of lipid was converted to methyl esters by transesterification with 20 ml of a solution of 5 % H_2SO_4 in methanol at 70° for 2 h under N_2. After adding an equal volume of water, fatty acid methylesters were extracted with two portions of 20 ml of pentane. The combined pentane layers were washed with 20 ml of water and dried over Na_2SO_4. The Na_2SO_4 was removed by filtration and the pentane was evaporated. The fatty acid methylesters were solved in 2 or 3 drops of trimethylpentane and determined by gas–liquid chromatography in a Carlo Erba gaschromatographic apparatus with a 2-m column of 8 % PEGA on gas-chrom Q (100–120 mesh) using N_2 as carrier gas. The temperature of the column was 180°. The fatty acid methylesters were identified by their retention times, as compared with standard solutions and literature[14] and by plotting the logarithm of the retensions *versus* the number of carbon atoms in the fatty acid methylesters.

Purification of phosphatidylglycerol and lysylphosphatidylglycerol

Cells of *S. aureus* from a 6-l culture were centrifuged, suspended in 50 ml of water acidified with HCl to pH 2, and immediately extracted by the method of BLIGH AND DYER[9]. The lipids were purified by chromatography on a column of 5 g of silica (140–200 mesh) using 500-ml volumes of the following mixtures as eluent: 0, 2, 4, 8, 15 and 25 % methanol in chloroform (v/v). The fractions were tested for purity by thin-layer chromatography using chlorform–methanol–water–acetic acid (65:25:5:1, by vol.) as solvent system. Lysylphosphatidylglycerol was identified with the ninhydrine reagent and phospatidylglycerol with the periodate Schiff reagent. The 4 and 8 % methanol fractions contained mainly phosphatidylglycerol. If necessary a further purification was obtained by preparative thin-layer chromatography. The 25 % methanol fraction contained pure lysylphosphatidylglycerol. The fractions were evaporated *in vacuo* and taken up in dry diethyl ether. Traces of silica gel were removed by centrifugation. The diethyl ether was evaporated *in vacuo*. The lipids were stored at −20° in chloroform–methanol (90:10, v/v) at a concentration of 10 mg lipid per ml.

Monolayer and liposome studies

Monolayers of the isolated lipids at the air–water interface were studied according to the methods described by DEMEL *et al.*[10]. Multilayered liposomes were prepared by gentle dispersion of the pure lipids into 50 mM KCl (ref. 11). The ζ potential of the obtained liposomes was determined by electrophoresis[25]. The permeability of the bilayers to glycol, glycerol and erythritol was measured by optical registration of the swelling rate of the liposomes in isotonic solutions of these non-electrolytes. The leak of $^{86}Rb^+$ out of the liposomes was measured according to BANGHAM *et al.*[13]. For the detection of the leak of glucose from the vesicles the procedure of KINSKY *et al.*[12] was used. In this case the liposomes were prepared in a solution containing 150 mM glucose and 75 mM KCl.

Measurement of [^{14}C]erythritol leakage from S. aureus cells

A 2-l culture of *S. aureus* cells was harvested in the semi-logarithmic phase at pH 6.5 by centrifugation. One part of the cell pellet was suspended in 0.3 M erythritol buffered with 0.01 M Tris buffer (pH 7.0). After stirring of the suspension for 20 min at 37°, the cells were centrifuged and the washing procedure was repeated 2 times. After centrifugation 0.5 ml of the thick cell pellet was added to 5 μC of [^{14}C]erythritol and incubated at room temperature for 1 h. The other part of the cell pellet was treated in the same way with the exception that 0.01 M Tris buffer was replaced by 0.01 M dimethylglutaric acid adjusting the pH at 5.0. From the thick cell suspensions loaded with [^{14}C]erythritol 50-μl samples were brought in 25 ml of 0.15 M NaCl of 3°. These diluted suspensions were stirred vigorously and at various times, 1-ml samples were filtrated on membrane filters (Sartorius, effective diameter 0.45 μm) and washed with 5 ml of cold 0.15 M NaCl. Filters were dried at room temperature and brought in a scintillation vial containing 15 ml of a solution containing 5 g PPO, 0.7 g POPOP and 100 g naphthalene in 1 l of dioxane. The radioactivity of the filter was then determined using a Packard-Tricarb liquid-scintillation counter.

Measurement of $^{86}Rb^+$ uptake by S. aureus cells

S. aureus cells were harvested by centrifugation and washed with a solution

of 0.3 M sucrose buffered with 0.01 M Tris buffer (pH 7.0). In one type of experiments semi-logarithmic cells, grown at pH 6.5 ($A_{550\ nm}$ approx. 1.0), were 10 times concentrated suspended in a solution of 0.3 M sucrose and 0.01 M dimethylglutaric acid adjusted at pH 5.0. In the other type of experiments cells, grown untill pH 4.7 were 10 times concentrated suspended in a solution of 0.3 M sucrose buffered with 0.01 M Tris buffer (pH 7.0).

At different times after suspending the cells in 0.3 M sucrose aliquots of 2.5 ml of these suspensions were added to 25 ml of a stirred solution, which was buffered at pH 7.0 with 0.05 M Tris, and contained 0.3 M sucrose and in addition 0.01 ml ^{86}RbCl (1 ml = 1 mC = 12.66 mg). The temperature of the incubation mixture was kept at 37°. The cells in 1 ml of the suspensions were collected by filtration through membrane filters and washed with 5 ml of cold 0.3 M sucrose solution. The first three samples were collected within 2 min. After 2 min 1 μg of valinomycin per ml of incubation solution was added. The radioactivity was measured in the liquid-scintillation counter.

Measurement of $^{86}Rb^+$ leakage from S. aureus cells

Cells of *S. aureus* were grown in 200 ml medium supplemented with 1 ml of ^{86}RbCl (1 ml = 1 mC = 12.66 mg). The cells were centrifuged and washed with cold solution of 0.3 M sucrose, buffered with 0.01 M Tris buffer (pH 7.0). The cells were 10 times concentrated suspended in a solution of 0.3 M sucrose, buffered with 0.01 M Tris buffer (pH 7.0). Aliquots of 2.5 ml of this suspension were diluted 10-fold with the same buffer and incubated at 37°. Residual radioactivity in the cells was measured as described above.

RESULTS

Lipid analyses

Cells of *S. aureus* harvested in the semi-logarithmic phase (pH 6.5) were analysed for their phospholipid composition and were found to contain mainly phosphatidylglycerol, lysylphosphatidylglycerol and cardiolipin. When the cells were incubated for increasing times at pH 5.0 decreasing percentages of phosphatidylglycerol in the membrane could be observed (*cf.* Table I). Fig. 1 shows that the absolute amounts of lysylphosphatidylglycerol and cardiolipin did not change by incubation at pH 5.0. The decrease in the amount of phosphatidylglycerol in the membrane was rapid until 30–45 min and then only slowly. Thin-layer-chromatography demonstrated that other major lipids as diglycerides and glucosyldiglycerides did not change by incubation at pH 5.0.

When the cells of *S. aureus* were grown in a medium with 1% glucose for 16 h a decrease in the pH of the growth medium to pH 4.7 could be noticed. These cells containing a relative high concentration of lysylphosphatidylglycerol were incubated for increasing times in a buffer of pH 7 and as a consequence a decrease in the percentage of lysylphosphatidylglycerol and an increase in phosphatidylglycerol could be observed. This is also illustrated in Table I.

The fatty acid compositions of the total lipids from the cells harvested at pH 6.5 and these cells incubated for 1 h at pH 5.0 are shown in Table II. From the results it can be concluded that the fatty acid pattern of the cell lipids did not

TABLE I

CHANGES IN PHOSPHOLIPID COMPOSITION OF *S. aureus* DUE TO CHANGES IN THE pH OF THE INCUBATION MEDIUM

Data are expressed as percentages of the total phospholipids.

Cells harvested at pH 6.5				*Cells harvested at pH 4.7*			
Time of incubation at pH 5.0 (min)	*Lysyl-phospha-tidyl glycerol*	*Phospha-tidyl glycerol*	*Cardiolipin*	*Time of incubation at pH 7.0 (min)*	*Lysyl-phospha-tidyl glycerol*	*Phospha-tidyl-glycerol*	*Cardiolipin*
0	38	57	5	0	79	14	7
15	57	36	7	15	60	33	7
30	67	26	7	30	56	37	7
60	75	19	6	60	54	39	7
120	83	12	5	120	50	42	8

TABLE II

FATTY ACID COMPOSITIONS OF THE LIPIDS FROM *S. aureus*

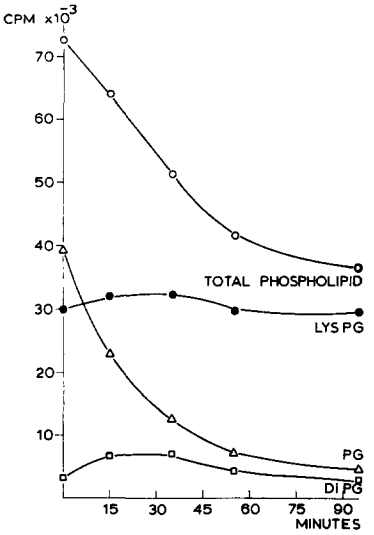
Data are expressed as percentages of the total fatty acids.

Fatty acid	*Total lipids of cells harvested at pH 6.5*	*Total lipids of cells harvested at pH 6.5 and incubated for 1 h at pH 5.0*	*Lysylphosphatidyl-glycerol fraction of cells harvested at pH 6.5*	*Phosphatidyl-glycerol fraction of cells harvested at pH 6.5*
15:0	—	—	+	+
iso 13:0	—	—	+	+
13:0	—	—	+	+
iso 14:0	2.8	2.0	1.4	2.0
14:0	2.9	1.4	1.4	1.8
iso 15:0	55.9	57.3	8.3	7.5
anteiso 15:0			39.3	36.0
15:0			1.2	1.7
iso 16:0	0.7	1.2	1.0	1.0
16:0	6.9	7.3	6.1	7.2
16:1	0.3	0.5	+	+
iso 17:0	2.2	2.8	2.8	2.7
anteiso 17:0	1.9	2.4	5.8	6.0
17:0	—	—	2.1	2.1
iso 18:0	—	—	+	+
18:0	17.9	17.0	15.4	15.3
18:1	0.3	0.7	1.5	3.2
iso 19:0	—	—	0.5	0.7
anteiso 19:0	2.3	2.6	0.9	1.1
19:0	—	—	3.5	3.7
20:0	6.1	5.4	8.4	7.6

change significantly during the incubation at pH 5.0. Table II shows that the fatty acid compositions of the phosphatidylglycerol and lysylphosphatidylglycerol fractions obtained by chromatographic purification are almost identical.

Monolayer and liposome studies

Phosphatidylglycerol and lysylphosphatidyl glycerol fractions isolated by column chromatography from the total lipids of cells harvested at pH 6.5 have been used for the monolayer and liposome experiments. In Fig. 2 the force–area



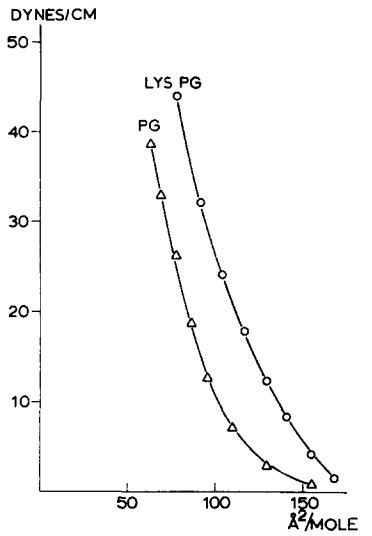


Fig. 1. Alterations in the amount of phospholipids of *S. aureus*. Cells were harvested at pH 6.5 from normal growth medium containing $^{32}P_i$ and were incubated for increasing periods in 0.3 M sucrose, buffered at pH 5.0 with dimethylglutaric acid. Abreviations used are:LYS PG = lysylphosphatidylglycerol; PG = phosphatidylglycerol; DiPG = cardiolipin. CPM = counts/min.

Fig. 2. Force–area curves of phospholipids isolated from *S. aureus*.

curves of both lipids are given, showing a significant smaller surface area per molecule for phosphatidylglycerol than for lysylphosphatidylglycerol at all pressures. From both phospholipid fractions multilayered liposomes could be prepared by suspending 15 μmoles of dry phospholipid into 1 ml of 50 mM KCl of pH 5.5. Examination of the dispersions with the polarization microscope showed in both preparations particles with the characteristic birefringence and no apparent difference in mean particle size could be observed. The ζ potential as measured from the electrophoretic mobility of the particles in 50 mM KCl (pH 5.5) was found for phosphatidylglycerol particles about –60 mV and for lysylphosphatidylglycerol liposomes +60 mV.

Furthermore, the amount of glucose trapped inside the vesicles per μmole of lipid appeared to be practically identical. Therefore it is justified to consider the two liposome preparations identical in architecture and size. Fig. 3 shows the optically measured volume changes of the liposomes when they are equilibrated in NaCl solutions of various concentrations. In earlier investigations $1/A$ was found for liposomes, to be proportional with the volume[11]. The linear relationship between this parameter and the reciprocal of the osmolarity indicate that the osmotic swelling of these liposomes obeys Boyle–van 't Hoff's law. Furthermore, the identical slope of the two lines suggest that at pH 5.5 the osmotic swelling of the phosphatidylglycerol and lysylphosphatidylglycerol liposomes is comparable. In Fig. 4 the swelling rates of the two liposome preparations are given in isotonic glycol, glycerol and ery-

thritol solutions. At any temperature the swelling rate of lysylphosphatidylglycerol liposomes is faster than of those prepared of phosphatidylglycerol. The conclusion that can be made from these results is that the positively charged lysylphosphatidylglycerol liposomes are better permeable to non-electrolytes than the phosphatidylglycerol liposomes. Confirmation of these findings has been obtained from experiments concerning the leakage of glucose from the two types of vesicles. After incubation at 40° during 90 min 43 ± 4 % of the trapped glucose appeared to leak from the lysylphosphatidylglycerol liposomes and only 29 ± 3 % from the phosphatidylglycerol liposomes.

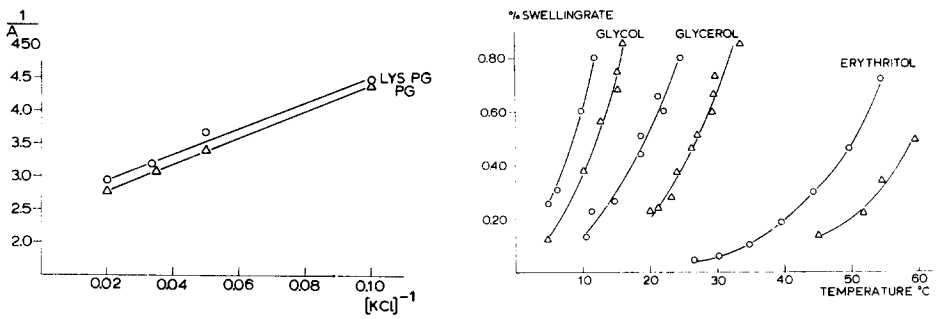


Fig. 3. Osmotic behaviour of liposomes prepared of phosphatidylglycerol and lysylphosphatidylglycerol. Liposomes were prepared in 50 mM KCl. 50-μl samples of the concentrated dispersion were added to 5 ml of KCl solutions of various concentrations. After 1 h equilibration the absorbance (A) of the diluted dispersion was measured.

Fig. 4. Initial swelling rates of liposomes prepared of phosphatidylglycerol and lysylphosphatidylglycerol in isotonic non-electrolyte solutions. Aliquots of 50 μl of a concentrated dispersion of liposomes prepared in 50 mM KCl were added to 5 ml of stirred and thermostated solution of 100 mM glycol, glycerol and erythritol, respectively. The swelling rate was measured optically as the percental change in d $1/A/dt$.

To study the cation permeability, liposomes of phosphatidylglycerol and lysylphosphatidylglycerol were prepared with $^{86}Rb^+$ trapped inside the bilayer structures. Fig. 5 shows the results of the spontaneous and valinomycin-induced leak of $^{86}Rb^+$ from the liposomes. It can be concluded that the permeability with respect to the Rb^+ is the reverse of non-electrolyt permeability. The positively charged liposomes of lysylphosphatidylglycerol being less permeable for the cation $^{86}Rb^+$ than the liposomes of the negatively charged phosphatidylglycerol. In the valinomycin-induced permeability experiments 0.01 μg valinomycin per 1 mg of phospholipid was present. The results indicate that valinomycin has a very limited effect on the Rb^+ penetration through the positive lysylphosphatidylglycerol bilayers, but has a strong promoting effect on the permeability through the negatively charged phosphatidylglycerol bilayers.

Permeability studies on intact S. aureus cells

To elucidate as to how far the results obtained on the liposome model system can be extrapolated to the natural membrane, we attempted to collect permeability data on intact *S. aureus* cells with low and high phosphatidylglycerol to lysylphosphatidylglycerol ratios. Fig. 6 concerns the non-electrolyte permeability. As described in MATERIALS AND METHODS cells harvested at pH 6.5 were incubated at, respectively,

pH 5.0 and 7.0 and subsequently loaded with [^{14}C]erythritol whereafter the leak into an inactive medium was measured at 3°. The result shows that cells with high phosphatidylglycerol to lysylphosphatidylglycerol ratio retained better the trapped [-^{4}C]erythritol and consequently are less permeable to this non-electrolyte than cells with a low phosphatidylglycerol to lysylphosphatidylglycerol ratio.

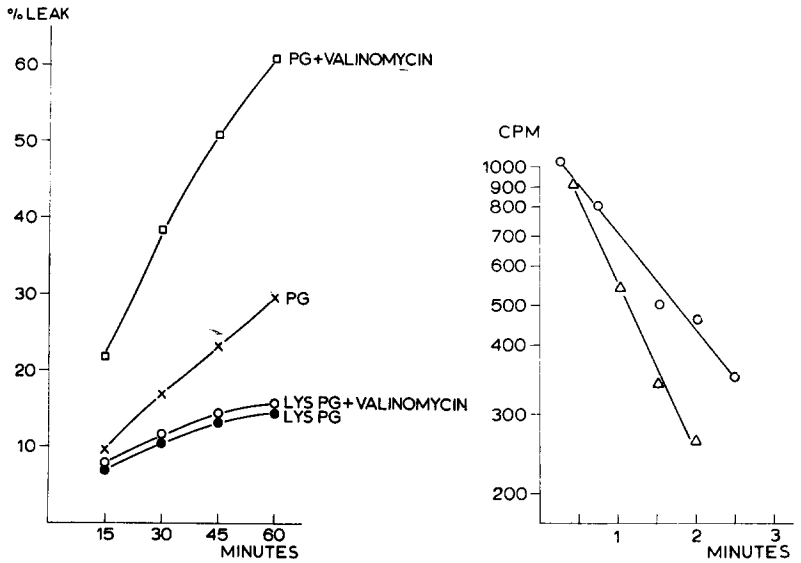


Fig. 5. Spontaneous and valinomycin-induced leakage of $^{86}Rb^+$ from liposomes prepared of phosphatidylglycerol and lysylphosphatidylglycerol. Liposomes were prepared in the presence of $^{86}Rb^+$. Activity in the outside medium was removed by rapid dialyses. Small aliquots of the liposomes were incubated in small dialyses bags in 10 ml of 50 mM KCl which was refreshed every 15 min. The leak at 25° was measured as radioactive counts in the 10-ml incubation samples and calculated as percentage of the total amount of $^{86}Rb^+$ trapped inside the liposomes.

Fig. 6. [^{14}C]Erythritol leakage from *S. aureus* cells with different phosphatidylglycerol to lysylphosphatidylglycerol ratios. *S. aureus* cells were harvested at pH 6.5 and incubated at respectively pH 5.0 and 7.0 as described in MATERIALS AND METHODS. Table I shows that this incubation results in cells with respectively higher and lower lysylphosphatidylglycerol to phosphatidylglycerol ratios. The cells were loaded with [^{14}C]erythritol and subsequently the leak from the cells into an inactive medium at 3° was measured. Δ-Δ, radioactivity in cells preincubated at pH 5.0; ○—○, radioactivity in cells preincubated at pH 7.0.

The influence of the differences in the polar head groups of the phospholipids on charged permeants was studied in measurements concerning the uptake of $^{86}Rb^+$ in the presence of valinomycin. The importance of charged groups for such an ion transport is obvious already from the effect of the pH on the ion uptake. Fig. 7 shows the $^{86}Rb^+$ uptake by cells with the same phosphatidylglycerol to lysylphosphatidylglycerol ratio at various pH's. With decreasing pH, that means with increasing protonation of the amino groups in the membrane proteins and lipids, there is a strong reduction in $^{86}Rb^+$ uptake. In the experiments illustrated in Fig. 8 such an uptake was measured at the same pH but with cells in which by preincubations variations in the phosphatidylglycerol to lysylphosphatidylglycerol ratios were obtained. Cells harvested at pH 6.5 were preincubated for various times in buffer of pH 5.0 to decrease

the ratio phosphatidylglycerol to lysylphosphatidylglycerol (*cf.* Table I) and cells harvested at pH 4.7, were incubated at pH 7.0 to increase this ratio. The results show that with decreasing phosphatidylglycerol content the uptake of the positive

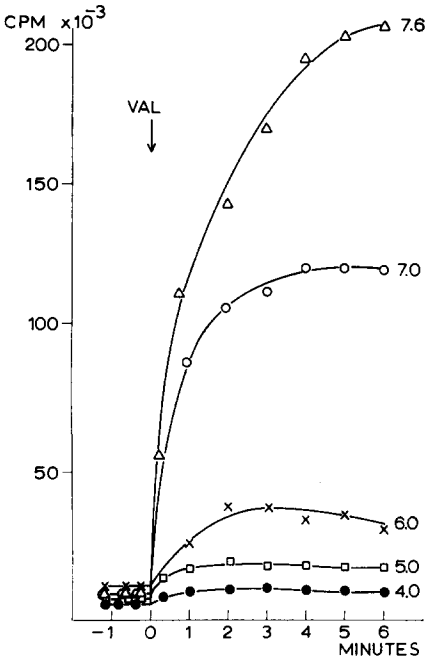


Fig. 7. Valinomycin-induced $^{86}Rb^+$ uptake of *S. aureus* cells in media of various pH's. The cells were harvested at pH 6.5 and 10 times concentrated suspended in 0.3 M sucrose. Samples of this suspension were added to 0.3 M sucrose containing 0.04 mM $^{86}RbCl$ and buffered at various pH's as indicated in the figure. Three samples of cells were separated from the medium by filtration before and seven after the addition of valinomycin (VAL). The radioactivity on the filter was counted.

ion is reduced (Fig. 8a), whereas with increasing phosphatidylglycerol to lysylphosphatidylglycerol ratio the uptake increases (Fig. 8b). The results show a satisfactory correlation between the permeability properties of model systems and natural membranes. Both liposomes and intact cells with high concentrations of lysylphosphatidylglycerol are better permeable to nonelectrolytes and less permeable to positively charged electrolytes, when compared with phosphatidylglycerol liposomes and cells with relatively low concentrations of lysylphosphatidylglycerol. The measured permeations through the bilayer of the liposomes can be considered as simple diffusion driven by defined concentration gradients, but in the experiments with the intact cells complicating factors may play a role. In the first place, the preincubations of cells at various pH values might induce notable changes in the cation gradients (H^+, K^+) over the cell membrane. To find out the importance of this factor, the following experiments were performed. Fig. 9 shows that when cells, harvested at pH 6.5, are incubated at pH 5.0, the intracellular pH decreased rapidly during the first 15 min and then only slowly. The valinomycin induced uptake of Rb^+ in these cells, however, changes progressively also after the first 15 min of preincubation

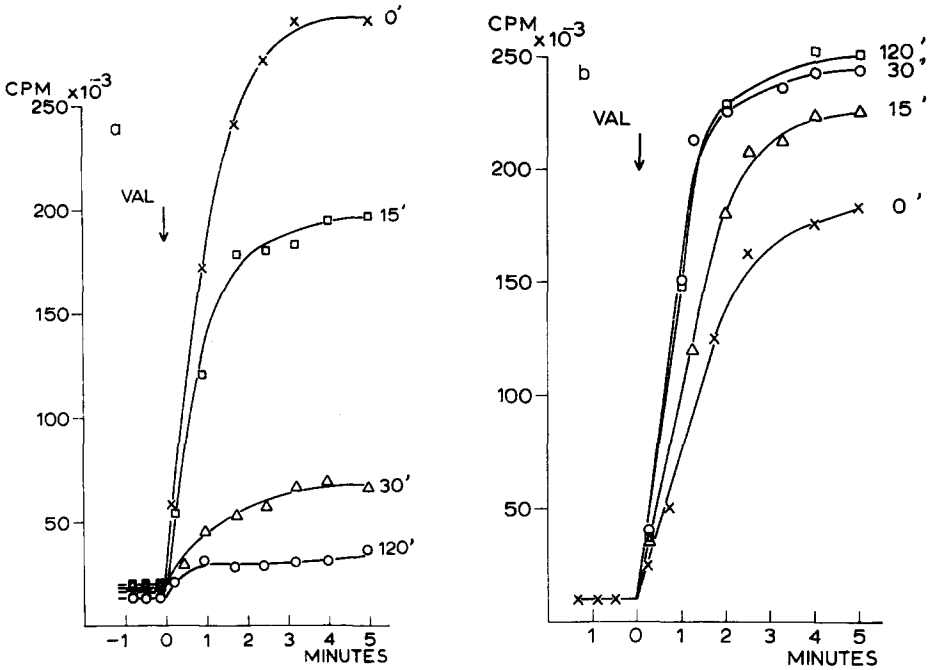


Fig. 8. Valinomycin-induced $^{86}Rb^+$ uptake of *S. aureus* cells with different phosphatidylglycerol to lysylphosphatidylglycerol ratios.
(a) Cells were harvested at pH 6.5 and preincubated for various times at pH 5.0. (b) Cells were harvested at pH 4.7 and preincubated for various times at pH 7.0. For the consequences of these incubations for the phospholipid composition see Table I. At different times after dispersion in sucrose pH 7.0 containing $^{86}RbCl$, samples were taken for filtration as described in the legend of Fig. 7.

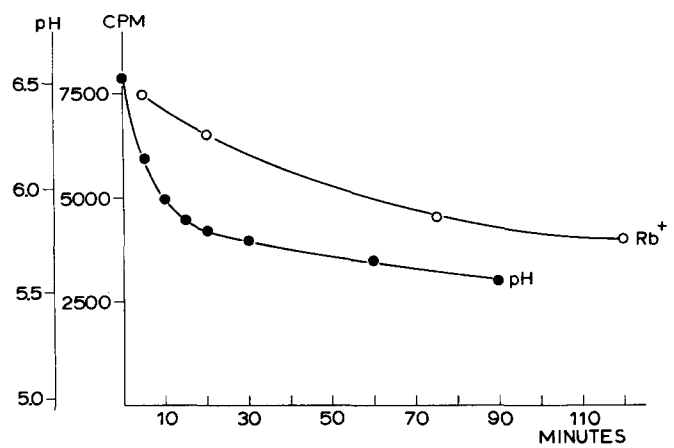


Fig. 9. Change in the intracellular pH of *S. aureus* and $^{86}Rb^+$ release from these cells during incubation. Cells were grown in the presence of $^{86}RbCl$ and harvested at pH 6.5 and suspended in 0.3 M sucrose pH 5.0. After various times of incubation at 37° samples were taken for filtration and determination of $^{86}Rb^+$ activity. For the measurement of the change in intracellular pH cells were harvested at pH 6.5 by centrifugation, washed with unbuffered water of pH 6.5 and centrifuged at 16000 × *g* for 15 min. 8 g of cells (wet wt.) were suspended in 40 ml unbuffered water of pH 5 at 37°. The pH of the suspension was kept at pH 5 with 0.02 MHCl at a pH stat. At different times 5-ml samples were pipetted in a tube and 0.5 ml of lysostaphin (1 mg/ml) was added. To lyse the cells the suspension was shaken for 1 h at 37° and the pH was measured.

as shown in Fig. 9. Therefore, these data indicate that the initial decrease in internal pH of the cell is not correlated with the observed changes in Rb^+ uptake. Also a change in the K^+ gradient during preincubation seems not to be very important. When we assume that the leak of K^+ from the cells during the preincubation is comparable to the leak of Rb^+, then Fig. 9 shows that this leak is limited and about linear with the gradient even after 45 min.

A second complicating factor, which has to be considered when a comparison is made between the Rb^+ uptake of the model systems and the bacterial cells is the possible contribution of an active transport mechanism in the biological cell membrane. Fig. 11 shows the influence of dicyclohexylcarbodiimide, an inhibitor of bacterial transport ATPase[22,23]. The total uptake of Rb^+ by the cells after addition of valinomycin is reduced in the presence of dicyclohexylcarbodiimide, but there is still a marked difference in valinomycin induced $^{86}Rb^+$ uptake between the two types of cells. Remarkable was the effect of 2,4-dinitrophenol on the valinomycin induced $^{86}Rb^+$ uptake. After an initial $^{86}Rb^+$ uptake, showing the characteristic difference between the two types of cells, there is a leak of activity from the cells.

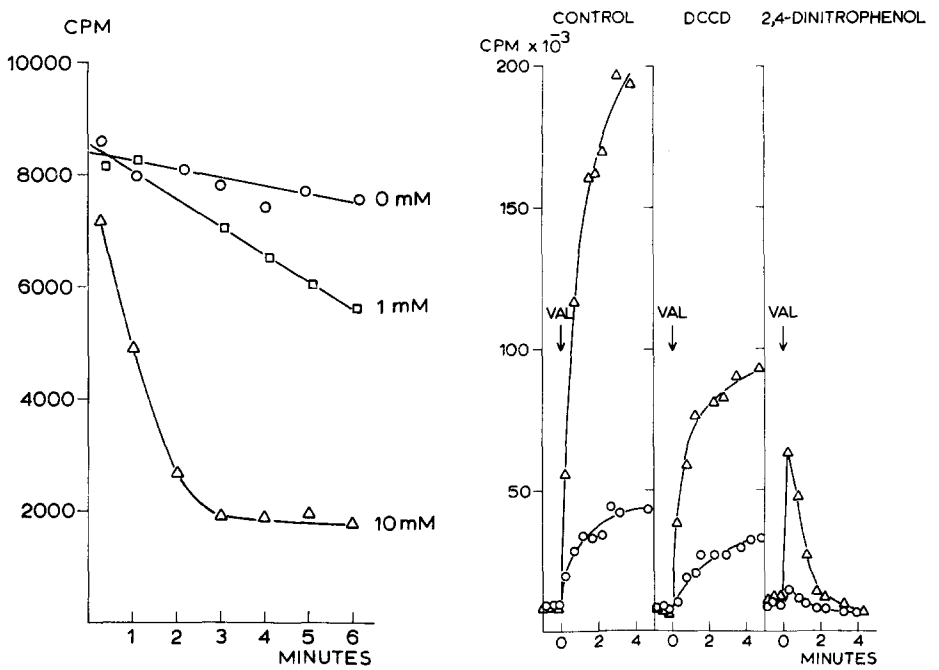


Fig. 10. Valinomycin-induced $^{86}Rb^+$ leakage from *S. aureus* cells in media with different KCl concentrations. Cells were grown in a medium, supplemented with $^{86}RbCl$ and harvested at pH 6.5. The cells were washed with 0.3 M sucrose buffered with 0.01 M Tris buffer (pH 7.0). The $^{86}Rb^+$ leakage was measured in the same sucrose medium, containing different KCl concentrations, as indicated in the figure.

Fig. 11. Effect of 2,4-dinitrophenol and dicyclohexylcarbodiimide (DCCD) on the valinomycin induced $^{86}Rb^+$ uptake of *S. aureus*. Cells harvested at pH 6.5 were suspended in 0.3 M sucrose buffered at pH 7.0 (△) or at pH 5.0 (○) and preincubated for 2 h at 37° with and without 2,4-dinitrophenol (1 mM) or dicyclohexylcarbodiimide (10 μM). The valinomycin-induced $^{86}Rb^+$ uptake was measured as described in the legends of Fig. 7.

Fig. 10 supports the view that valinomycin facilitates predominantly the passive exchange over the biological cell membrane, showing that the $^{86}Rb^+$ leak from cells grown in the presence of this radioactive ion, into a K^+-free medium is only limited even in the presence of valinomycin, whereas a rapid release takes place when K^+ is present in the outside medium.

DISCUSSION

HOUTSMULLER AND VAN DEENEN[6] found that the content and composition of phospholipids in *S. aureus* were greatly dependent on the pH of the medium. They suggested that the changes in the polar head groups of the phospholipids might help the cell to control its permeability at variations in the environmental pH. In order to study transport processes in relation to the change in phosphatidylglycerol to lysylphosphatidylglycerol ratios, we looked for optimal conditions to obtain cells with varying percentages of phosphatidylglycerol and lysylphosphatidylglycerol in the membrane.

The cells of *S. aureus* harvested at pH 4.7, contained high percentages of positively charged lysylphosphatidylglycerol (about 80 %). The percentage of lysylphosphatidylglycerol in these cells declined rapidly during the first 30 min of incubation at pH 7.0 and thereafter only slowly. The cells of *S. aureus* harvested at pH 6.5, during semilogarithmic growth, contained high percentages of negatively charged phosphatidylglycerol (about 55 %). When these cells were incubated at pH 5.0 the amount of phosphatidylglycerol decreased rapidly during the first 30 min of incubation and then only slowly. The decrease in the total amount of phospholipid in the membrane when cells harvested at pH 6.5, were incubated at pH 5.0 was due to a loss of phosphatidylglycerol. This is in agreement with the observations of GOULD AND LENNARZ[15], who showed that the decrease in phosphatidylglycerol was due to a decrease in synthesis and an increase in catabolism of phosphatidylglycerol at pH around 5.

To obtain a clear picture of the difference in the barrier properties of phosphatidylglycerol and lysylphosphatidylglycerol, we studied the isolated and purified phospholipids in membrane model systems. Monolayer experiments showed that, although the fatty acid constituents were nearly identical, a significant bigger surface area per molecule was occupied by lysylphosphatidylglycerol than by phosphatidylglycerol. This indicates that the more bulky polar group of the lysylphosphatidylglycerol makes the packing less close than of phosphatidylglycerol molecules. In agreement with these findings the bilayers of the liposomes appeared to be better permeable for the non-electrolytes glycol, glycerol, erythritol and glucose when prepared with lysylphosphatidylglycerol than with phosphatidylglycerol. In this respect it is relevant to mention experiments[16] on monolayers and liposomes prepared of various lecithins. The more unsaturated lecithins demonstrated more loosely packed films in monolayers than saturated ones and the permeability of the liposomes of unsaturated phhospholipids was increased when compared with saturated ones.

However, with respect to the ion permeability the conformity between increased unsaturation and more bulky head group is lost. In earlier experiments we studied[19] valinomycin-induced $^{86}Rb^+$ leak out of liposomes of various lecithins and found that apart from an increased non-electrolyte permeability also the ion leak was increased in the more unsaturated liposomes. The present results show that despite

the looser packing, the lysylphosphatidylglycerol liposomes exhibit a considerably reduced permeability for Rb^+, when compared with the phosphatidylglycerol liposomes. This has to be explained by the positive charge on the polar headgroup of the lysylphosphatidylglycerol. These results are in agreement with findings of PAPAHADJOPOULOS *et al.*[17] who studied the cation permeability of liposomes containing various polar headgroups. These authors found that negatively charged bilayers were more permeable for cations than positively charged bilayers. Using the black film system, HOPFER *et al.*[18] showed that the negatively charged bilayers of phosphatidylglycerol were cation selective whereas the positively charged bilayers of lysylphosphatidylglycerol were anion selective.

Valinomycin showed a marked increase in the $^{86}Rb^+$ leak from the liposomes of phosphatidylglycerol but was unable to affect significantly the release of these ions from the liposomes of lysylphosphatidylglycerol. The results are in agreement with experiments and considerations of MCLAUGHLIN *et al.*[21] and of CIANI *et al.*[20]. These authors found that the membrane conductance in the presence of nonactin and valinomycin in KCl solutions for bilayers was in the order: negative > amphoteric > no charge > positive charge. They concluded that molecular carriers of cations can be used as "probes" to distinguish between effects of charge and of other variables, such as the fluidity of the bilayer interior and the lipid solubility of the complex.

Discussing the valinomycin-induced uptake of $^{86}Rb^+$ by *S. aureus* we can consider this uptake primarily as a passive exchange of Rb^+ and K^+ over the membrane, facilitated by the valinomycin which can act as an efficient and selective carrier for these two ions. Such an exchange is supported by the finding that valinomycin is capable of promoting $^{86}Rb^+$ leak out of the cells only when in the outside medium K^+ is present (Fig. 10). A small contribution from an active transport mechanism cannot be excluded. Such a contribution could be concluded from the effect of the transport ATPase inhibitor dicylohexylcarbodiimide on the uptake. On the other hand, we have to realize that the prolonged preincubation with dicyclohexylcarbodiimide might have resulted in loss of K^+ from the cells, diluting that radioactive exchangeable ion concentration in the outside medium. Furthermore, it has been demonstrated that in Gram-positive[27] and Gram-negative[26] bacteria valinomycin did not stimulate the K^+ pump. The remarkable release of Rb^+ from the cells in the presence of 2,4-dinitrophenol after an uptake showing the characteristic difference between the two types of cells (see Fig. 11) can be explained by a net leak of K^+ and Rb^+ from the cells in the presence of this uncoupler. An enormous leak of K^+ from cells in the presence of both valinomycin and an uncoupler is quite common for biological cells and also liposomes containing potassium acetate demonstrate a rapid release of their ion content in the presence of the two agents[24].

Summarizing, the present results show a good correlation between the permeability properties of cells containing a high and low lysylphosphatidylglycerol to phosphatidylglycerol ratio and liposomes prepared of the two purified phospholipids. Both in the model system and in the intact cells the non-electrolyte permeability appears to be dependent on the packing of lipid molecules, whereas the cation permeability is mainly ruled by the charges on the polar headgroups. This enables us to conclude that also in *S. aureus* the chemical nature of the phospholipids determines to a great extent the properties of the permeability barrier.

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