Effects of the Membrane Action of Tetralin on the Functional and Structural Properties of Artificial and Bacterial Membranes

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Tetralin is toxic to bacterial cells at concentrations below 100 μmol/liter. To assess the inhibitory action of tetralin on bacterial membranes, a membrane model system, consisting of proteoliposomes in which beef heart cytochrome c oxidase was reconstituted as the proton motive force-generating mechanism, and several gram-positive and gram-negative bacteria were studied. Because of its hydrophobicity, tetralin partitioned into lipid membranes preferentially (lipid/buffer partition coefficient of tetralin is approximately 1,100). The excessive accumulation of tetralin caused expansion of the membrane and impairment of different membrane functions. Studies with proteoliposomes and intact cells indicated that tetralin makes the membrane permeable for ions (protons) and inhibits the respiratory enzymes, which leads to a partial dissipation of the pH gradient and electrical potential. The effect of tetralin on the components of the proton motive force as well as disruption of protein-lipid interaction(s) could lead to impairment of various metabolic functions and to low growth rates. The data offer an explanation for the difficulty in isolating and cultivating microorganisms in media containing tetralin or other lipophilic compounds.

Interest in the application of water-immiscible organic compounds in fermentations has increased in the last decade. Many lipophilic compounds are harmful to microorganisms, impair growth, and even inhibit other biological reactions. Knowledge of the toxic action of lipophilic compounds on bacterial cells is mainly restricted to the relation between the hydrophobicity (13) of a compound and its effect on a specific enzyme (19). In most studies, the cytoplasmic membrane is mentioned as a possible target, but information about the nature of the toxic action is not presented (1, 19).

A correlation between the hydrophobicity of a compound and its effects on cells was first observed for anesthetics (27–29), which provided a basis for calculating a dose-effect relationship. In addition, the uncoupling effects of lipophilic compounds on energy transduction have been studied in animal cells (23). For microorganisms, only a few studies on the toxic effects of lipophilic compounds on various membrane functions have been performed (3, 32, 33). These studies showed that hydrocarbons, e.g., β-pinene (32) and cyclohexane (33), impaired energy transduction in both mitochondrial and plasma membranes of yeast cells. Studies on the toxic effects of ethanol on yeast cells indicated that the Δp across the plasma membrane was dissipated in the presence of ethanol (3), probably due to an increased influx of protons (3, 12).

In this investigation, the toxic action of the lipophilic compound tetralin on bacteria was studied. Tetralin was found to be toxic to bacterial cells at concentrations below 100 μmol/liter (30). Tetralin, 1,2,3,4-tetrahydroxynaphthalene, is a bicyclic molecule that consists of an aromatic and an alicyclic moiety. The compound is widely applied as an industrial solvent and as a substitute for turpentine (7). In the early 1920s, it was already known that tetralin was toxic to bacteria (21, 22) and bacteriophages (21), and it was therefore applied as a biocide. Since detailed information about the mode of interaction of lipophilic compounds with membranes is difficult to obtain with intact cells, the effects of tetralin were first studied in (proteoliposomes). The inhibitory action of tetralin in intact cells was addressed in both tetralin-utilizing and nonutilizing bacterial strains.

MATERIALS AND METHODS

Organisms. Acinetobacter strain T5 and Arthrobacter strain T2 were isolated from the environment by selective enrichment on tetralin as described previously (30). Corynebacterium strain C125 was kindly provided by G. Schraa, Department of Microbiology, Agricultural University Wageningen, Wageningen, The Netherlands (25). Escherichia coli K12 (ATCC 25404) and Bacillus subtilis ATCC 6633 were obtained from the American Type Culture Collection (Rockville, Md.).

Cultivation conditions. Cells were grown in 1-liter Erlenmeyer flasks with 200 ml of mineral medium containing 0.5% sodium succinate and 0.05% yeast extract (Difco Laboratories, Detroit, Mich.). The flasks were incubated in a shaker incubator (200 rpm) at 30°C, except for E. coli and B. subtilis, which were grown at 37°C.

Preparation of liposomes. Lipids dissolved in CHCl3-methanol (9:1, vol/vol) were mixed together in appropriate quantities and dried under a stream of N2 gas. Traces of solvent were then removed under vacuum for 1 h. Dried lipid was suspended in 50 mM potassium phosphate (pH 7.0) at a concentration of 20 mg of lipid per ml and dispersed by ultrasonic irradiation, using a bath sonicator (Sonicator; Sonicator Instruments, New York, N.Y.). Liposomes were obtained by sonication (probe-type sonicator; MSE, West Sussex, United Kingdom) for 300 s at maximal amplitude, using intervals of 15 s of sonication and 45 s of rest, at 4°C under a constant stream of N2 gas.

Reconstitution of cytochrome c oxidase into proteoliposomes. Forty milligrams of acetone-ether-washed E. coli lipid and 18 mg of n-octyl-β-D-glucopyranoside in 2 ml of 50

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mM potassium phosphate (pH 7.0) were cosonicated till clarity under a constant stream of N2 gas at 4°C with a probe sonicator. Cytochrome c oxidase (9 nmol of heme a) was added, and the suspension was dialyzed at 4°C for 4 h against a 500-fold volume of 50 mM potassium phosphate (pH 7.0). Dialysis was repeated for another 4 h and continued overnight at 4°C (8).

Partitioning of tetratin. The partitioning of tetratin over membrane and buffer phases was determined in a liposome-buffer system (11). Increasing amounts of tetratin were added to 50 mM potassium phosphate (pH 7.0) containing liposomes (5.0 mg of phospholipid per ml) (final volume, 0.5 ml). After equilibration (30 min), the liposomes were spun down in an Airfuge (Beckman Instruments, Inc., San Ramon, Calif.) for 30 min at 135,000 × g. The supernatant was removed with a Pasteur pipette and subsequently extracted twice with an equal volume of diethyl ether (containing 0.1% n-decane as an internal standard). The pellet was dried and resuspended in 100 μl of diethyl ether (containing 0.1% n-decane as an internal standard). Both the pellet and the supernatant were analyzed quantitatively by gas chromatography.

Gas chromatography. Gas-liquid chromatographic analysis was performed on a CP-9000 gas chromatograph with an on-column injector (Chrompack BV, Middelburg, The Netherlands) fitted with a fused silica WCOT CP-Sil 8 CB column (25 m by 0.32 mm) (Chrompack). Gas flow rates were: He-H2-air, 30:20:300 ml/min, each; temperature of the flame ionization detector was 300°C. The column oven was programmed from 80°C initial temperature to 200°C at a rate of 10°C/min.

Membrane expansion and extraction of phospholipids. The expansion of liposomal membranes and extraction of phospholipids from the liposomes resulting from the addition of tetratin was monitored in liposomes labeled with the fluorescent fatty acid octadecyl rhodamine β-chloride (R18, Molecular Probes Inc.) or the fluorescent phospholipid analog N-(lissamine rhodamine β-sulfonyl)phosphatidylethanolamine (N-Rh-PE; Avanti Polar Lipids Inc., Alabaster, Ala.). The method is based on the relief of fluorescence self-quenching (9) of rhodamine β-chloride as a result of expansion of the membrane and/or extraction of the probe from the membrane. The fatty acid probe was incorporated into liposomal membranes at a concentration of 4 mol% phospholipid phosphorus. Maximum R18 fluorescence was determined upon the addition of 1% (vol/vol) Triton X-100. Fluorescent changes were measured in a spectrofluorometer by using the excitation-emission pair 560 and 590 nm. To discriminate between fluorescence increase due to expansion of the membrane or due to extraction of membrane constituents, we centrifuged incubation mixtures with different concentrations of tetratin at 135,000 × g (Beckman Airfuge, 30 min). Subsequently, the fluorescence of the supernatant was determined relative to that of the supernatant of an incubation mixture without added tetratin.

Internal pH of cytochrome c oxidase-containing proteoliposomes. Internal pH changes were measured by monitoring the fluorescence of entrapped pyranine (Eastman Kodak Co., Rochester, N.Y.) (4). To incorporate pyranine into proteoliposomes (20 mg of phospholipid per ml), 100 nmol of pyranine was added to 0.5 ml of proteoliposomes and rapidly mixed. The suspensions were rapidly frozen in liquid nitrogen and subsequently thawed slowly (approximately 30 min) at room temperature. The suspension was sonicated for 8 s with a probe-type sonicator at an amplitude of 4. To remove external pyranine, the proteoliposomes were washed in 10 ml of 50 mM potassium phosphate (pH 7.0) and centrifuged for 45 min at 55,000 rpm (maximally 280,000 × g) in a Beckman type Ti 75 rotor at 4°C. Fluorescent changes were measured at excitation and emission wavelengths of 460 and 508 nm, respectively, using a spectrofluorometer (The Perkin-Elmer Corp., Norwalk, Conn.). Calibration was performed by titration with acid or base upon the addition of nigericin to a final concentration of 20 nM.

Cytoplastic pH of intact cells. The internal pH of cells was measured by monitoring the pH-dependent fluorescence of BCECF (2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein) (Molecular Probes Inc., Junction City, Ore.). Cells were loaded with BCECF as described by Molenaar et al. (17) and stored on ice. Fluorescent changes were measured by using the excitation-emission pair 502 and 525 nm with a Perkin-Elmer spectrofluorometer. The values obtained were corrected for efflux of BCECF as described previously (17).

Electrical potential across membranes of proteoliposomes and intact cells. The transmembrane electrical potential (Δψ) generated in cytochrome c oxidase-containing proteoliposomes in the presence of the electron donor system ascorbate-TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine)-cytochrome c was determined by monitoring the distribution of tetraphenylphosphonium ion (TPP+) across the membrane with a TPP+-sensitive electrode as previously described (14). To estimate Δψ in E. coli and Acinetobacter sp., cells were treated with EDTA before TPP+ distribution was monitored as described by Sarkar et al. (24). A correction for concentration-dependent probe binding was applied according to the model of Lolkema et al. (14). Specific reaction conditions are specified in the figure legends.

Proton fluxes through liposomal membranes. Δψ-induced proton flux measurements in cytochrome c oxidase-containing proteoliposomes were performed in the presence of increasing concentrations of tetratin in a well-stirred thermostat-equipped 2-ml cuvette, using phenol red (20 μg/ml, final concentration) as the indicator of the external pH. The rate of change of external pH, monitored by A560 − A510 (5), was converted into H+ flux by using pulses of calibrated amounts of oxalic acid or KOH.

Valinomycin-induced potassium diffusion potentials were imposed across the liposomal membrane by 100-fold dilution of the liposomes (20 mg of phospholipid per ml) in the same medium, in which sodium ions were substituted for potassium ions and phenol red (20 μg/ml) was added. Generation of the electrical potential was initiated by adding valinomycin (2 μM, final concentration).

Oxygen consumption measurements. Cells grown on succinate medium were harvested in the exponential growth phase, washed twice with potassium phosphate buffer (50 mM, pH 7.0), and resuspended in this buffer to a density of 7.5 mg of cell protein per ml. Succinate-induced oxygen consumption was subsequently measured in a 3-ml incubation vessel fitted with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio). The reaction was started by injecting 100 μl of the cell suspension into the vessel containing 60 μl of a dimethylformamide (DMF)-tetratin mixture in 2.84 ml of potassium phosphate buffer (50 mM, pH 7.0). The measurements were performed at 30°C. The oxygen concentration of air-saturated buffer was 0.25 mM.

Miscellaneous. E. coli phospholipids, obtained from Sigma Chemical Co. (St. Louis, Mo.), were washed with acetone-ether as described by Kagawa and Racker (10).

The concentration of tetratin in water and in water-DMF mixtures was determined either spectrophotometrically at
The partition coefficient of tetralin was determined in the presence of liposomes (5.0 mg of phospholipid per ml). The partition coefficient of 1,100 was determined in the linear part of the curve.

270 nm using $e_{270} = 740 \text{ M}^{-1} \text{ cm}^{-1}$ (26) or by gas chromatography of ether extracts. Tetralin was prepared as a solution in DMF. In all cases, the amount of DMF added was 2% (vol/vol) of the total volume. DMF had no effect on any parameter studied except for the binding of TPP$^+$ to membranes (binding of TPP$^+$ was less in the presence of DMF); $\Delta \Psi$ values were corrected accordingly (14).

Cytochrome $c$ oxidase activity was measured spectrophotometrically by monitoring the decrease in the absorbance of the alpha peak of cytochrome $c$, using an extinction coefficient (reduced minus oxidized) of $e_{550-540} = 19.5 \text{ mM}^{-1} \text{ cm}^{-1}$ (5).

Protein was determined by the method of Bradford (2), using bovine serum albumin as a standard.

Growth was assessed by observing the increase in $A_{660}$ of the cell suspension.

**Chemicals.** 1,2,3,4-Tetrahydronaphthalene was purchased from Janssen Chimica (Beerse, Belgium). DMF was obtained from Merck GmbH (Darmstadt, Germany). All other chemicals were analytical grade.

**RESULTS**

**Partitioning of tetralin between membrane and aqueous phases.** The solubility of tetralin in the buffer used was estimated spectrophotometrically by the method of Schreiber (26). Up to 130 $\mu$mol/liter added, the concentration of tetralin in the aqueous phase increased linearly, but then slowly curved to a maximum of approximately 200 $\mu$mol/liter. Above 200 $\mu$mol of tetralin per liter, a distinct second phase was formed.

In studying the effect of tetralin on the functional and structural properties of membranes, the partitioning of the compound between a membrane and the aqueous phase needs to be established. Liposomes prepared from *E. coli* phospholipids were used as a model system to determine the partitioning of tetralin. At subsaturating concentrations, the tetralin added distributed over the lipid and buffer phases, from which the partition coefficient could be estimated. The partition coefficient was calculated on the basis of the respective weights of the membrane and buffer fractions present. For the liposomes, a partitioning coefficient of 1,100, on a weight basis, was obtained (Fig. 1). The preferential partitioning of tetralin to the membrane phase significantly lowered its concentration in the water phase. When the amount of tetralin added exceeded the solubility of tetralin in buffer, the excess of tetralin was contained by the membrane at least up to 2,500 $\mu$mol of tetralin added per liter (Fig. 1).

**Expansion of liposomal membrane in the presence of tetralin.** Accumulation of tetralin in the phospholipid bilayer of liposomes could cause an expansion of the membrane. To analyze the effect of tetralin on the swelling of the membrane, we labeled liposomes prepared from *E. coli* phospholipid with R$_{18}$ or N-Rh-PE. The rationale is that expansion of the membrane should lead to a dilution of the probes in the membrane which can be observed as a relief in fluorescence self-quenching. Since the fluorescence signal is related to the lipid concentration (9), a change in fluorescence will be proportional to a change in surface area. Alternatively, relief of self-quenching might be observed as a consequence of extraction of the fluorescent probe from the membrane by tetralin. Addition of tetralin to a liposome suspension resulted in a partial relief of self-quenching and reached a maximum when 2 to 3 $\mu$mol of tetralin per mg of phospholipid was added (Fig. 2). Further addition of tetralin did not result in an extra increase of fluorescence. However, a discrete tetralin phase was not observed at concentrations up to 10 $\mu$mol of tetralin per mg of phospholipid.

Ultracentrifugation of liposomes equilibrated with various amounts of tetralin (0.1, 0.3, 0.6, 3.0, and 7.5 $\mu$mol/mg of phospholipid) did not reveal substantial rhodamine fluorescence in the supernatant. At 0.1, 0.3, and 0.6 $\mu$mol/mg of phospholipid, extraction of fluorescent probe could maximally account for 1% of the observed increase in R$_{18}$ fluorescence. At 3.0 $\mu$mol/mg, 5.6%, and at 7.5 $\mu$mol/mg, 9.7% of the increase in fluorescence could be attributed to extraction of the fluorescent compounds. Experiments performed with liposomes labeled with the fluorescent phospholipid N-Rh-PE yielded results similar to those with R$_{18}$ (fatty acid)-labeled liposomes. The results indicate that the observed increase in fluorescence was primarily due to swelling of the membrane and not to extraction of probe from the membrane.

**Effect of tetralin on pH gradient and electrical potential in proteoliposomes.** To analyze the effect of tetralin on the generation of the pH gradient and the electrical potential in artificial membranes, we reconstituted beef heart cytochrome $c$ oxidase into liposomes as a proton motive
Fig. 3 shows the effect of tetralin on ΔpH (a) and Δψ (b) generated by cytochrome c oxidase-containing proteoliposomes. The ΔpH effect on tetralin was measured by monitoring the change in intensity of pyranine fluorescence in the presence of the potassium ionophore valinomycin (1 μM, final concentration). The Δψ effect on tetralin was determined by observing the distribution of TPP⁺ across the membrane using a TPP⁺-sensitive electrode in the presence of the ionophore nigericin (20 nM, final concentration). The sucrose donor system energized the proteoliposomes and was composed of cytochrome c (20 μM), potassium ascorbate (10 mM, pH 7.0), and TMPD (400 μM). 0.1 mg of phospholipid per ml; 0, 0.25 mg of phospholipid per ml.

The electron donor was due to the presence of cytochrome c. ΔpH and Δψ were measured in the presence of valinomycin and nigericin, respectively, to determine the maximal gradients; the effects of tetralin were similar in the absence of the ionophores (data not shown). Because of the high partition coefficient, the extent of the inhibition depended on the liposome (phospholipid) concentration in the incubation mixture, i.e., when the tetralin concentration was expressed as micromoles added per liter (data not shown). However, the amount of tetralin added is expressed as micromoles per milligram of phospholipid, the effects are (almost) independent of the phospholipid concentration (Fig. 3).

Fig. 4 shows the influence of tetralin on the proton permeability of liposomal membranes. Liposomes (0.25 mg of phospholipid per ml) were washed and resuspended in a medium in which sodium ions were substituted for potassium ions and additional phenol red (20 μg/ml) was added. To initiate the potassium diffusion potential, we added valinomycin (2 μM, final concentration). Subsequently, absorbance changes were measured at A660 minus A410 to determine the external pH changes caused by proton influx as a compensatory effect on the imposed diffusion potential.

Fig. 4, the proton permeability increased with increasing tetralin concentrations. The proton permeability was highest at concentrations at which the ΔpH and Δψ were maximally affected. These results suggest that the lowering of ΔpH and Δψ by tetralin is primarily caused by an increased H⁺ permeability of the membrane and to a lesser extent by the inhibition of cytochrome c oxidase.

Effect of tetralin on the ΔpH and Δψ of intact cells. The results obtained with (proteo)liposomes indicate that membranes are an important target of the toxic action of tetralin. Therefore, the effect of tetralin on the pH gradient and electrical potential was also studied in bacterial cells. Five strains were chosen for these experiments: three strains that are able to grow on tetralin (Acinetobacter strain T5, Arthrobacter strain T2, and Corynebacterium strain C125) and two strains that cannot utilize tetralin (E. coli K-12 and B. subtilis ATCC 6633).

The ΔpH generated by B. subtilis and Arthrobacter strain T2 was significantly less affected by tetralin than the pH gradient generated by the other organisms (Fig. 5a). The inhibitory effect of tetralin on Δψ of intact cells was less pronounced (Fig. 5b). As for the (proteo)liposomes, the effect on the ΔpH and Δψ was dependent on the amount of membrane lipid (biomass) present (data not shown). Assuming that a bacterial cell is composed of protein and phospho-
Arthrobacter strain without tetralin added; tetralin well over growth was concentrations of tetralin 7.0)
was observed for additional generation times. control, without tetralin added; tetralin added (micromoles liter): ○, 75; ■, 375; □, 750; △, 1,875; ▽, 3,750. OD, optical density.

**FIG. 6.** Effect of tetralin on the growth of *E. coli* (a) and *Arthrobacter* strain T2 (b). Cells were grown in mineral medium (pH 7.0) supplemented with sodium succinate (5 g/liter) and yeast extract (0.5 g/liter) to a cell density of 0.2 at 660 nm. Subsequently, different concentrations of tetralin were added (indicated by arrows), and growth was observed for additional generation times. ○, control, without tetralin added; tetralin added (micromoles liter): ○, 75; ■, 375; □, 750; △, 1,875; ▽, 3,750. OD, optical density.

**FIG. 7.** Effect of tetralin on the rate of oxygen consumption. Cells were grown on succinate-extracted medium, and oxygen consumption rates were determined in the presence of succinate (20 mM). The incubation mixture contained 0.25 mg of cell protein per ml. Rates are expressed relative to the rate in the absence of tetralin. ○, *E. coli*; ○, *B. subtilis*; △, *Arthrobacter* strain T2; ■, *Acinetobacter* strain T5; and □, *Corynebacterium* strain C125.

lipsids constituting 55 and 9.1%, respectively, of the total cellular mass (data for *E. coli* [18]), a tetralin concentration of 500 μmol/liter corresponds to approximately 10 μmol/mg of phospholipid for the experiment presented in Fig. 5.

**Effects of tetralin on the growth rate and metabolic activities of bacteria.** (i) **Growth rate.** Inhibitory effects of tetralin on the growth rates of bacteria growing on succinate were observed upon the addition of tetralin at concentrations of well over 100 μmol/liter, approximately 20 μmol/mg of phospholipid at an optical density at 660 nm of 0.2 (Fig. 6). As can be seen for *E. coli* (Fig. 6a) and *Arthrobacter* strain T2 (Fig. 6b), growth was also impaired at tetralin concentrations lower than 100 μmol/liter; however, despite the reduced growth rate, cells could easily overcome the initial effect. Eventually, the cells could grow at the same rate as the uninhibited cells. Similar results were obtained with the other three organisms. Emulsification of the growth medium was observed during growth of *Arthrobacter* strain T2 and *Corynebacterium* strain C125 in the presence of tetralin.

(ii) **Respiration.** Succinate-dependent oxygen consumption by intact cells was monitored in the presence of increasing amounts of tetralin. The different bacteria differed only slightly in their sensitivity of respiration toward tetralin (Fig. 7). Interestingly, the gram-negative *E. coli* and *Acinetobacter* strain T5 showed elevated oxygen uptake rates at low tetralin concentrations.

**DISCUSSION**

Tetralin is a lipophilic compound with a low solubility in water. Therefore, in an aqueous-nonaqueous two-phase sys-

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![Graph](image-url)
lowered internal pH (20). The observation that tetralin reached its maximum effect at concentrations which just saturated the membrane (2.5 μmol/mg of phospholipid) indicates that tetralin is toxic at the molecular level (1). The interaction of tetralin with the membrane (Fig. 2) did not result in full dissipation of the Δψ (Fig. 3), not even when the amount of tetralin present exceeded the concentration that saturated the membrane, indicating that tetralin does not fully disrupt the membrane. Therefore, the effects of tetralin must be caused by toxicity of dissolved molecules and not by the presence of a phase transition due to the water-immiscible second phase (1).

The effect of tetralin on membrane functioning has consequences for the overall metabolism and growth characteristics of the cell. Both the lag phase and growth rate of the strains examined were affected by tetralin. Interestingly, cells did eventually overcome the inhibition by low concentrations of tetralin (concentrations of 750 μmol/liter and lower), which most likely is due to an initial slow increase in biomass which subsequently lowers the medium concentration of tetralin as a consequence of the high membrane/buffer partition coefficient. Furthermore, the presence of an additional substrate (succinate) provides the cell with an energy source that may fuel the protection and adaptation of the organism. In the absence of succinate, growth was already inhibited at a tetralin concentration of 100 μmol/liter (30). The inhibition of growth by tetralin could be due to the lowered Δψ and the more acidic internal pH. On top of this, tetralin could affect various membrane enzymes directly by disrupting protein-lipid interactions (at maximal inhibitory concentrations, approximately 2 molecules of tetralin are present per molecule of phospholipid). The inhibition of cytochrome c oxidase in the proteoliposomes and of respiration in intact cells are indicative of this.

Arthrobacter strain T2 was less susceptible to tetralin, most likely because of the observed production of emulsifying compounds. Another opportunity for a cell to overcome toxic effects of lipophilic compounds is altering the fatty acid composition of the membrane (6, 15). This could lead to different sensitivities, probably as a result of altered partition characteristics of the lipophilic compounds (6). Future studies will be directed toward the effect of lipid composition on the partitioning of lipophilic compounds in biological membranes.

Our observation of the high membrane/buffer partition coefficient for tetralin has important implications for the isolation and cultivation of microorganisms on lipophilic compounds. The results suggest that the partitioning of lipophilic compounds in biological membranes could be an important parameter for the choice of the concentration range at which organisms can be isolated.

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