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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-tetrahydronaphthalene, 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 (proteo)liposomes. 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* K-12 (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 CHCl$_3$-methanol (9:1, vol/vol) were mixed together in appropriate quantities and dried under a stream of N$_2$ 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 (Sonicor; Sonicor 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 N$_2$ 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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* Corresponding author.

mM potassium phosphate (pH 7.0) were cosonicated till clarity under a constant stream of N_2 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 tetralin. The partitioning of tetralin over membrane and buffer phases was determined in a liposome-buffer system (11). Increasing amounts of tetralin 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-H_2-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 tetralin was monitored in liposomes labeled with the fluorescent fatty acid octadecyl rhodamine β-chloride (R_{18}; 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 R_{18} 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 tetralin 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 tetralin.

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.

Cytoplasmic 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, Oreg.). 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 ($\Delta\psi$) 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 $\Delta\psi$ 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. $\Delta\psi$-induced proton flux measurements in cytochrome c oxidase-containing proteoliposomes were performed in the presence of increasing concentrations of tetralin 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 $A_{560} - A_{610}$ (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)-tetralin 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 tetralin in water and in water-DMF mixtures was determined either spectrophotometrically at
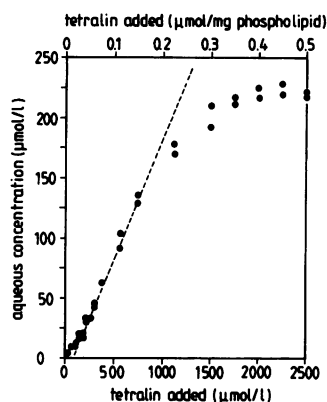
FIG. 1. Concentration of tetralin in the water phase as determined in the presence of liposomes (5.0 mg of phospholipid per ml). The partition coefficient of 1,100 was determined in the linear part of the curve.

FIG. 2. Effect of tetralin on the relief of fluorescence self-quenching of R_{18}-labeled liposomes. The reaction mixture contained liposomes (0.33 mg of E. coli phospholipid per ml) labeled with fluorescent probe (4 mol%) in potassium phosphate buffer (50 mM, pH 7.0). The temperature of the solution was kept at 30°C.

270 nm using $\varepsilon_{270} = 740$ M^{-1} 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 $\varepsilon_{550-540} = 19.5$ mM^{-1} 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 μmol/liter added, the concentration of tetralin in the aqueous phase increased linearly, but then slowly curved to a maximum of approximately 200 μmol/liter. Above 200 μmol 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
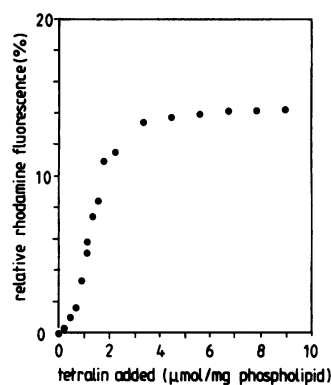
the amount of tetralin added exceeded the solubility of tetralin in buffer, the excess of tetralin was contained by the membrane at least up to 2,500 μmol of tetralin added per liter (Fig. 1).

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 μmol 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 μmol 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 μmol/mg of phospholipid) did not reveal substantial rhodamine fluorescence in the supernatant. At 0.1, 0.3, and 0.6 μmol/mg of phospholipid, extraction of fluorescent probe could maximally account for 1% of the observed increase in R_{18} fluorescence. At 3.0 μmol/mg, 5.6%, and at 7.5 μmol/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
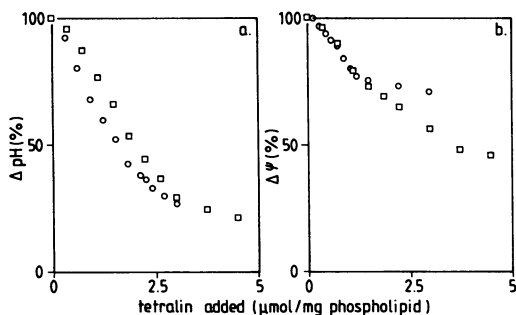
FIG. 3. Effect of tetralin on ΔpH (a) and Δψ (b) generated by cytochrome c oxidase-containing proteoliposomes. The effect of tetralin on the ΔpH was measured by monitoring the change in intensity of pyranine fluorescence in the presence of the potassium ionophore valinomycin (1 μM, final concentration). The effect of tetralin on the Δψ was measured by observing the distribution of TPP⁺ across the membrane by using a TPP⁺-sensitive electrode in the presence of the ionophore nigericin (20 nM, final concentration). The electron donor system to energize the proteoliposomes 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; ○, 0.25 mg of phospholipid per ml.

force-generating mechanism. At pH 7.0, cytochrome c oxidase-containing proteoliposomes generated a ZΔpH and Δψ of −54 and −60 mV, respectively, in the presence of the electron donor system ascorbate-TMPD-cytochrome c. Incubation of the proteoliposomes with increasing concentrations of tetralin resulted in a decrease of both ΔpH and Δψ, the effect being more pronounced on the ΔpH than on the Δψ (Fig. 3). ΔpH and Δψ were measured in the presence of valinomycin and nigericin, respectively, to estimate 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, when the amount of tetralin added is expressed as micromoles per milligram of phospholipid, the effects are (almost) independent of the phospholipid concentration (Fig. 3).

Site(s) of action of tetralin. The decrease in ΔpH and Δψ could be due to inhibition of cytochrome c oxidase activity or to increased ion permeability of the membrane in the presence of tetralin or both. Addition of tetralin to cytochrome c oxidase-containing proteoliposomes led to a partial inhibition of enzyme activity, i.e., a maximum of 50% inhibition at 15 μmol/mg of phospholipid. The effect of a lowered cytochrome c oxidase activity on the generation of the pH gradient and electrical potential in proteoliposomes was assessed by incubating cytochrome c oxidase-containing liposomes with different concentrations of sodium azide. At 0.12 mM NaN₃, cytochrome c oxidase was 50% inhibited; the enzyme, however, was able to generate a ΔpH and Δψ that were reduced by 17 and 12%, respectively, relative to incubations in the absence of NaN₃.

To test whether the decrease in the ΔpH and Δψ by tetralin was due to an increased proton permeability of the membranes, potassium-loaded liposomes were diluted into potassium-free medium in the presence of valinomycin, and the initial rates of H⁺ influx in the absence and presence of different amounts of tetralin were determined. As shown in
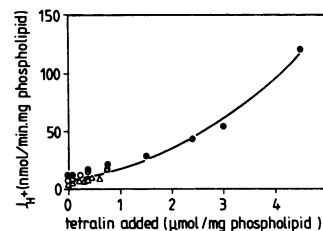

FIG. 4. Influence of tetralin on the proton permeability of liposomal membranes. Liposomes (0.25 mg of phospholipid per ml) were washed and resuspended in a medium in which sodium ions were substituted for potassium ions and additional phenol red (20 μg/ml) was added. To initiate the potassium diffusion potential, we added valinomycin (2 μM, final concentration). Subsequently, absorbance changes were measured at A_{560} minus A_{610} 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 the 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-
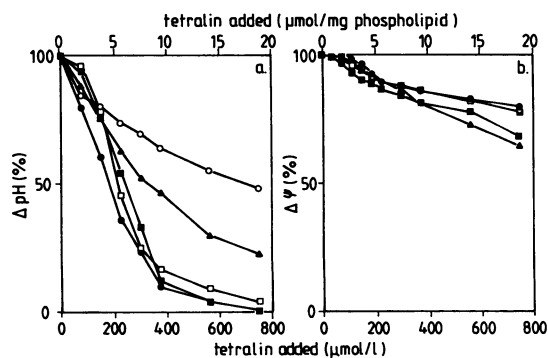

FIG. 5. Effect of tetralin on ΔpH (a) and on Δψ (b) as generated by intact cells. The incubation mixture contained 5 g of sodium succinate per liter in 50 mM potassium phosphate buffer (pH 7.0) and 0.25 mg of cell protein per ml. ●, *E. coli*; ○, *B. subtilis*; ▲, *Arthrobacter* strain T2; ■, *Acinetobacter* strain T5; and □, *Corynebacterium* strain C125.
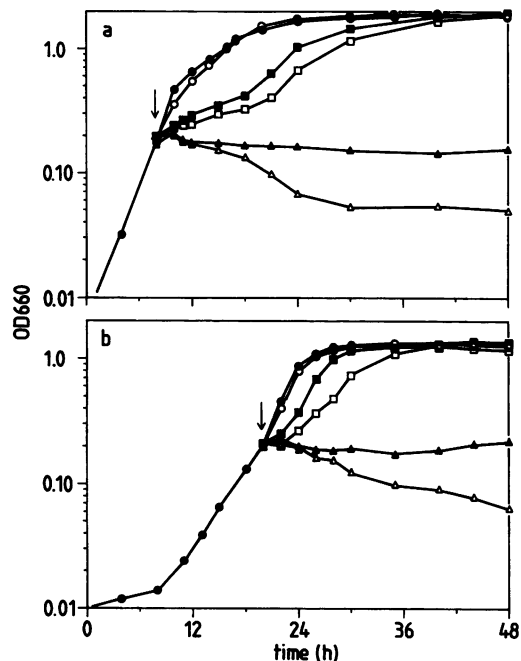
FIG. 6. Effect of tetralin on the growth of *E. coli* (a) and *Arthrobacter* strain T2 (b). Cells were grown in mineral medium (pH 7.0) supplemented with sodium succinate (5 g/liter) and yeast extract (0.5 g/liter) to a cell density of 0.2 at 660 nm. Subsequently, different concentrations of tetralin were added (indicated by arrows), and growth was observed for additional generation times. ●, control, without tetralin added; tetralin added (micromoles per liter): ○, 75; ■, 375; □, 750; ▲, 1,875; △, 3,750. OD, optical density.
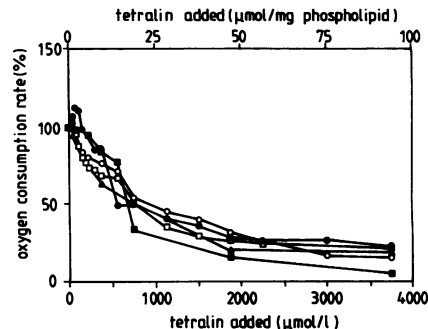

FIG. 7. Effect of tetralin on the rate of oxygen consumption. Cells were grown on succinate-yeast extract medium, and oxygen consumption rates were determined in the presence of succinate (20 mM). The incubation mixture contained 0.25 mg of cell protein per ml. Rates are expressed relative to the rate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

lipids 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-

tem, it will preferentially partition to the nonaqueous phase. From the experimentally obtained partition coefficient of 1,100 for tetralin in a liposome-buffer system ($P_{membrane}$), at a maximum aqueous solubility of 124 μmol/liter (26), a tetralin concentration in the membrane of 0.136 μmol/mg of membrane lipid can be calculated using the following equation: $[solvent_{membrane}] = P_{membrane} \times [solvent_{aqueous}]$. This means that approximately 18 nl of tetralin is dissolved in 1 mg of membrane lipid, corresponding to 1 molecule of tetralin per 10 molecules of phospholipid. The equation, however, is only valid at low concentrations, i.e., when the behavior of the solute can be considered ideal, as described by Raoult's law. At higher concentrations, the nonideal behavior of the solute may lead to a deflection in the partition coefficient (Fig. 1).

One of the principal functions of the cytoplasmic membrane is energy transduction, which involves the generation and maintenance of a Δp. The Δp is composed of a proton gradient (ΔpH) and an electrical gradient ($\Delta \psi$). Dissipation of these gradients leads to a situation in which the cell is unable to screen its cytoplasm from the surrounding medium. The dissipation of the Δp by tetralin can be caused either by leakage of protons and/or other ions or by inhibition of cytochrome *c* oxidase. Although inhibition of cytochrome *c* oxidase occurred, the decrease in cytochrome *c* oxidase activity could not fully account for the decrease in the pH gradient and electrical potential. Since tetralin also affected the proton (ion) permeability of the membrane (Fig. 4), the decrease may in fact be due to an increased proton (ion) permeability and the partial inhibition of cytochrome *c* oxidase. Since more protons have to be translocated across a membrane to generate a pH gradient than to generate an electrical potential of similar magnitude (16), the observation that tetralin affects the pH gradient more severely than the $\Delta \psi$ suggests that the proton influx must partly be balanced by cation efflux or anion influx or both. The effect of tetralin in intact cells also appeared to be more pronounced on ΔpH than on $\Delta \psi$. Analogous to the situation with the cytochrome *c* oxidase-containing proteoliposomes, the effect of tetralin on ΔpH and $\Delta \psi$ in intact cells is likely due to an increase in permeability for protons (ions) and the impairment of the ion-translocating (and/or electron carrier) enzymes. Apart from the effect on the Δp, dissipation of the ΔpH can severely impair normal functioning of the cell because of the

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 Δp (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 Δp 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.

ACKNOWLEDGMENTS

REFERENCES

1. **Bar, R.** 1987. Phase toxicity in a water-solvent two-liquid phase microbial system, p. 147–153. *In* C. Laane, J. Tramper, and M. D. Lilly (ed.), Biocatalysis in organic media. Elsevier Biomedical Press, Amsterdam.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Cartwright, C. P., J.-R. Juroszek, M. J. Beavan, F. M. S. Ruby, S. M. F. De Morais, and A. H. Rose.** 1986. Ethanol dissipates the proton-motive force across the plasma membrane of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **132:**369–377.
4. **Clement, N. R., and J. M. Gould.** 1981. Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) as a probe of internal aqueous hydrogen ion concentration in phospholipid vesicles. Biochemistry **20:**1534–1538.
5. **de Vrij, W., R. A. Bulthuis, and W. N. Konings.** 1988. Comparative study of energy-transducing properties of cytoplasmic membranes from mesophilic and thermophilic *Bacillus* species. J. Bacteriol. **170:**2359–2366.
6. **Enomoto, S., M. Kashiwayanagi, and K. Kurihara.** 1991. Liposomes having high sensitivity to odorants. Biochim. Biophys. Acta **1062:**7–12.
7. **Gaydos, R. M.** 1981. Naphthalene, p. 698–719. *In* M. Grayson and D. Eckroth (ed.), Kirk-Othmer encyclopedia of chemical technology, 3rd ed. John Wiley & Sons, Inc., New York.
8. **Hinkle, P. C., J. J. Kim, and E. Racker.** 1972. Ion transport and respiratory control in vesicles formed from cytochrome oxidase and phospholipids. J. Biol. Chem. **247:**1338–1339.
9. **Hoekstra, D., T. de Boer, K. Klappe, and J. Wilschut.** 1984. Fluorescence method for measuring the kinetics of fusion between biological membranes. Biochemistry **23:**5675–5681.
10. **Kagawa, Y., and E. Racker.** 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. J. Biol. Chem. **235:**769–775.
11. **Katz, Y., and J. M. Diamond.** 1974. A method for measuring nonelectrolyte partition coefficients between liposomes and water. J. Membr. Biol. **17:**69–86.
12. **Leao, C., and N. van Uden.** 1984. Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **774:**43–48.
13. **Leo, A., C. Hansch, and D. Elkins.** 1971. Partition coefficients and their uses. Chem. Rev. **71:**525–616.
14. **Lolkema, J. S., K. J. Hellingwerf, and W. N. Konings.** 1982. The effect of 'probe binding' on the quantitative determination of the proton-motive force in bacteria. Biochim. Biophys. Acta **681:** 85–94.
15. **Mishra, P., and S. Kaur.** 1991. Lipids as modulators of ethanol tolerance in yeast. Appl. Microbiol. Biotechnol. **34:**697–702.
16. **Mitchell, P.** 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. **41:**445–502.
17. **Molenaar, D., T. Abee, and W. N. Konings.** 1991. Continuous measurement of the cytoplasmic pH in *Lactococcus lactis* with a fluorescent pH indicator. Biochim. Biophys. Acta **1115:**75–84.
18. **Neidhardt, F. C.** 1987. Chemical composition of *Escherichia coli*, p. 3–6. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
19. **Osborne, S. J., J. Leaver, M. K. Turner, and P. Dunnill.** 1990. Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. Enzyme Microb. Technol. **12:**281–291.
20. **Poolman, B., A. J. M. Driessen, and W. N. Konings.** 1987. Regulation of solute transport in streptococci by external and internal pH values. Microbiol. Rev. **51:**498–508.
21. **Prausnitz, C.** 1922. Uber die Natur des d'Hérelle'schen Phänomens. Klin. Wochenschr. **1:**1639–1642.
22. **Roth, S., and P. Seeman.** 1972. Anesthetics expand erythrocyte membranes without causing loss of K^+. Biochim. Biophys. Acta **255:**190–198.
23. **Rottenberg, H.** 1990. Decoupling of oxidative phosphorylation and photophosphorylation. Biochim. Biophys. Acta **1018:**1–17.
24. **Sarkar, H. K., B. Thorens, H. F. Lodish, and H. R. Kaback.** 1988. Expression of the human erythrocyte glucose transporter

in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:**5463–5467.

25. **Schraa, G., B. M. Bethe, A. R. W. van Neerven, W. J. J. van den Tweel, E. van der Wende, and A. J. B. Zehnder.** 1987. Degradation of 1,2-dimethylbenzene by *Corynebacterium* strain C125. Antonie Leeuwenhoek **53:**150–170.

26. **Schreiber, A. F.** 1983. Zur bakteriellen Verwertbarkeit von Tetralin. Ph.D. thesis. Ruhruniversität, Bochum, Germany.

27. **Seeman, P.** 1972. The membrane actions of anesthetics and tranquilizers. Pharmacol. Rev. **24:**583–655.

28. **Seeman, P., W. O. Kwant, T. Sauks, and W. Argent.** 1969. Membrane expansion of intact erythrocytes by anesthetics. Biochim. Biophys. Acta **183:**490–498.

29. **Seeman, P., S. Roth, and H. Schneider.** 1971. The membrane concentrations of alcohol anesthetics. Biochim. Biophys. Acta **225:**171–184.

30. **Sikkema, J., and J. A. M. de Bont.** 1991. Isolation and initial characterization of bacteria growing on tetralin. Biodegradation **2:**15–23.

31. **Stoye, W.** 1924. Uber Einwirkung von Fettsäureestern auf Bakterien. Z. Hyg. Infektionskr. **103:**97–105.

32. **Uribe, S., J. Ramirez, and A. Pena.** 1985. Effects of β-pinene on yeast membrane functions. J. Bacteriol. **161:**1195–1200.

33. **Uribe, S., P. Rangel, G. Espínola, and G. Aguirre.** 1990. Effects of cyclohexane, an industrial solvent, on the yeast *Saccharomyces cerevisiae* and on isolated yeast mitochondria. Appl. Environ. Microbiol. **56:**2114–2119.