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Mechanisms of Membrane Toxicity of Hydrocarbons

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INTRODUCTION

Microbial transformation of cyclic hydrocarbons is important in nature as well as in technological applications of microorganisms, such as wastewater and waste gas treatment, bioremediation, and biocatalysis. In these processes, cyclic hydrocarbons such as terpenes, aromatics, and cycloalkanes are present either as pollutant or as starting material for biological transformation reactions.

Terpenes are plant-based organic compounds that consist of isoprene units. In plants, they are present mainly as anti-feedants. Industrial applications of terpenes include fragrances, solvents (turpentine), and food preservatives. As a result of biological synthesis, terpenes have been present in the environment for a very long time. Today, other cyclic hydrocarbons enter the environment as a result of human activities, e.g., transport and application of mineral oils and products derived thereof. Aromatics are especially abundant because of applications such as fuels, industrial solvents (benzene, toluene), polymer synthesis (styrene), and starting materials for chemical syntheses. The cycloalkanes are less abundant although biologically more-persistent compounds. In particular, cyclohexane is becoming increasingly important as an industrial solvent replacing benzene, which is known to be carcinogenic.

Many cyclic hydrocarbons are known to be transformed by microorganisms, which may lead to complete mineralization of these compounds. The metabolic pathways by which these compounds are degraded have been elucidated for a great number of compounds in various microorganisms (72, 104, 291, 292). More recently, genetic aspects of the metabolic routes have received considerable attention (10, 117, 360). During studies on the transformation of cyclic hydrocarbons by microorganisms, many researchers observed that these compounds were toxic to the cells (102, 151, 271, 274). In fact, one of the major problems encountered in the application of microorganisms in the transformation of cyclic hydrocarbons is the low stability of the desired activity, which is mainly due to inactivation of cells. The impact of cyclic hydrocarbons on microorganisms and the environmental and economical consequences are clear. However, the mechanism of the toxicity of these compounds has been documented relatively poorly. The available data show that as a result of the lipophilic character of these compounds, interactions with hydrophobic parts of the cell play an important role in the mechanism of the toxic action. The literature on the toxic action of cyclic hydrocarbons on microorganisms contains an enormous number of reports

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on similar effects caused by other groups of organic compounds. The most important characteristic that these compounds have in common is that they are more or less hydrophobic. Hydrophobicity or, perhaps better in the context of this review, lipophilicity is a physicochemical feature of a compound. The lipophilicity of a compound depends on various physical and chemical characteristics, e.g., molecular surface area, molecular volume, and polarity (177). Biological data on the toxic effects of lipophilic compounds on microorganisms in general may be helpful in postulating a mechanism for the toxic action of cyclic hydrocarbons. An excellent review by Harold (118) compiled a wealth of information on various antimicrobial agents that affect membrane functions. A large variety of antibacterial agents were listed by Hugo, but in his review their primary site of action was not analyzed (133).

In this review, we present an overview of data on the toxicity of cyclic hydrocarbons to microorganisms. Microorganisms that are able to transform such compounds and microorganisms that do not have the transforming capability are both discussed. Furthermore, the aspect of bioavailability of the usually poorly soluble cyclic hydrocarbons will be treated with respect to the balance between availability for metabolic processes on the one hand and toxicity to the cell on the other. A few years ago, microorganisms that withstand extremely high concentrations of cyclic hydrocarbons were discovered. These strains are discussed in connection with potential adaptation mechanisms. The examples are compiled from studies in the fields of applied microbiology, bioprocess technology, ecotoxicology, environmental technology, food microbiology, and medical microbiology. Furthermore, examples from anesthesiology, pharmacology, biochemistry, and physical chemistry, pertaining to mechanisms of interaction of lipophilic compounds with cells and cell constituents, are discussed.

TABLE 1. Physical data for some cyclic hydrocarbons discussed in this review[a]

Compound	Formula	M_w	Solubility (mmol/liter at 25°C)	Log *P*
Terpenes				
α-Pinene	$C_{10}H_{16}$	136.24		4.46
Limonene	$C_{10}H_{16}$	136.24	0.101	4.46
Aromatics				
Benzene	C_6H_6	78.11	22.9	2.13
Toluene	C_7H_8	92.14	6.28	2.69
Ethylbenzene	C_8H_{10}	106.17	1.27	3.15
o-Xylene	C_8H_{10}	106.17	2.02	3.12
Naphthalene	$C_{10}H_8$	128.17	0.797	3.37
Tetralin	$C_{10}H_{12}$	132.21	0.125	3.86
Biphenyl	$C_{12}H_{10}$	154.21	0.126	4.04
Anthracene	$C_{14}H_{10}$	178.23	0.040	4.45
Phenanthrene	$C_{14}H_{10}$	178.23	0.025	4.46
Cycloalkanes				
Cyclohexane	C_6H_{12}	84.16	0.683	3.44
Decalin	$C_{10}H_{18}$	138.25		4.83

[a] Adapted from reference 275.

BIOAVAILABILITY OF CYCLIC HYDROCARBONS

At present, it is believed that only molecules of cyclic hydrocarbons that are dissolved in the aqueous phase are available for intracellular metabolism (351, 352). Transfer of lipophilic and also hydrophilic substrates proceeds via dissolution in the aqueous phase and subsequent uptake (actively or passively) by the cell. Direct contact between lipophilic compounds and the hydrophobic part of the cell membrane is prevented by the presence of the cell wall and/or the hydrophilic parts of the outer membrane. Moreover, as a result of the hydrophilic head groups of the cell membrane phospholipids, direct interaction seems unfavorable. The involvement of intracellular cyclic hydrocarbon-metabolizing enzymes implies that the substrates must enter the cell prior to their metabolism. Although this subject has not been studied in detail, the general opinion is that the uptake of cyclic hydrocarbons is a passive process. An important mechanism in the uptake of lipophilic compounds is the partitioning of these molecules into the lipid bilayer of the cytoplasmic membrane. Therefore, it is valuable to consider the different steps that are involved in the uptake of the cyclic hydrocarbons.

Dissolution of Cyclic Hydrocarbons

Cyclic hydrocarbons are poorly soluble in water (85, 180, 193) (Table 1). Benzene, the most polar cyclic hydrocarbon, is soluble up to 22 mM at 25°C (85). Compounds with a higher molecular weight and/or a higher degree of saturation have a lower solubility. Many of these compounds are often reported to be insoluble in water (180), although traces of the hydrocarbon will always dissolve. Nevertheless, for biotransformation reactions, the availability of such a compound can become limiting (200). Work by Wodzinski and coworkers in the early 1970s showed that microorganisms utilize only hydrocarbon molecules that are dissolved in the aqueous phase (351, 352). The growth kinetics of some bacteria growing on polycyclic aromatic hydrocarbons, e.g., naphthalene, phenanthrene, and anthracene, were studied. These hydrocarbons dissolve only slowly in the aqueous phase (295). Recently, Volkering et al. showed that the growth rate of a *Pseudomonas* strain growing on naphthalene depended on the particle size of the solid naphthalene in the incubation mixture (333). Thomas et al. (311) showed that a close correlation exists between the dissolution rate, which is a function of the solute surface area, and the degradation rate of insoluble compounds such as naphthalene. For biphenyl, anthracene, and phenanthrene, a dependence of the growth rate of bacteria on the solubilization rate has been reported (302). Because of their high molecular weight, hydrophobicity, and solid state, polycyclic aromatic hydrocarbons are particularly known to adsorb to surfaces (194, 196, 209). Both microorganisms that live in the suspended state and those that are attached to surfaces utilize molecules that are dissolved (224, 327). Therefore, the rate of dissolution of a compound is a critical measure of the bioavailability of such a compound in all environments (199). The dissolution rate is very critical for both growth and toxicity, since it governs the transfer of a compound to the microorganism. The rate of transfer of a compound is dependent on the difference between the equilibrium concentration and the actual concentration and on the surface area between the bulk phase and the aqueous phase (Fig. 1). For a compound such as naphthalene, which is solid under normal cultivation conditions, the limiting step in the transfer is related to the small surface area of the naphthalene particles. Therefore, Volkering et al. (333) could decrease mass transfer limitation by selecting smaller naphthalene particles. An alternative method to decrease or even prevent mass transfer limitation is the use of a cosolvent (Fig. 1) or a surfactant. A cosolvent enables the substrate surface area to increase by dispersion of the particles. Ideally, the cosolvent is miscible with both the aqueous phase and the substrate. Cosolvents that are often used in biochemical studies include dimethyl formamide, dimethyl sulfoxide, acetone, and ethanol. The application of surfactants to increase the aqueous concen-

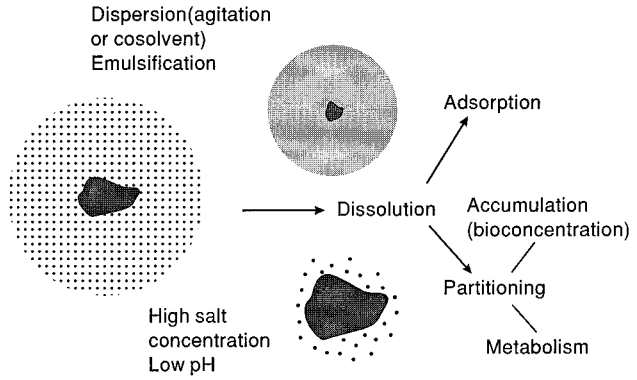

FIG. 1. Dissolution and fate of solid hydrocarbons as affected by some environmental parameters. An increase in the dissolution rate can result from an increase in the surface area of the hydrocarbon bulk, which may occur by mechanical dispersion (e.g., stirring, agitation, wave movement), chemical dispersion (cosolvents), or emulsification (surface-active compounds). In environments with high salt concentrations or extreme pH values, the solubility may be significantly lowered (332). Dissolved molecules may (i) partition to biological systems, resulting in either transformation or accumulation (bioconcentration), (ii) adsorb to surfaces, or (iii) remain in the solid-dissolved equilibrium.

tration of poorly water-soluble compounds such as polycyclic aromatic hydrocarbons has been investigated in relation to environmental remediation (9, 89, 123, 312). Although some synthetic surfactants have been shown to be effective, many of these compounds are not applicable either, since they are degraded by the microorganisms or are toxic to the cells (312). The mechanism of this toxicity is discussed below. Also, the application of strains that produce surfactants of their own has been investigated (89, 92). Such biosurfactants not only emulsify the lipophilic compounds but may also allow adhesion of the cells to the hydrophobic substrate (222, 364, 365). Despite promising results obtained with biosurfactants in laboratory-scale experiments (222), the application of biosurfactants and surfactant-producing strains appears to be less satisfactory (92, 96).

Data obtained by Thomas et al. (311) indicated that dissolution rates of solid hydrocarbons were higher in the presence of bacteria than in sterile controls. Also, for microorganisms growing on long-chain alkanes, the dissolution rates have been shown to be increased by the presence of bacteria. In most instances, the effects were a result of surface-active compounds produced by the hydrocarbon-degrading microorganisms (96, 364). These compounds, usually large amphiphilic molecules, e.g., lipopolysaccharides (LPS) and rhamnolipids, are able to form and/or stabilize hydrocarbon emulsions in aqueous systems (132, 363). As a result of emulsification, the exchange surface is increased, which facilitates higher dissolution rates and prevents mass transfer limitation. Furthermore, the involvement of outer membrane LPS in the dissolution of hydrocarbons by gram-negative bacteria may facilitate attachment of the cell to hydrocarbon droplets (349).

As a consequence of mass transfer limitation, the amount of hydrocarbon available may be limiting growth, but, on the other hand, toxic effects may be minimal. When microorganisms that can metabolize these compounds are studied, the rate of metabolism sometimes exceeds the rate of mass transfer from the environment to the cell. This leads not only to limitation of growth rate (333) but also to the absence of toxic effects. When compounds with a low dissolution rate, e.g., naphthalene, biphenyl, and phenanthrene, are allowed to equilibrate (47) or are added with a cosolvent (275, 276, 293, 320, 321), toxic effects are observed. Also, other physical parameters such as temperature, pH, and salt concentration have a significant effect on dissolution and solubility (332). However, interpretation of the corresponding data is often difficult, since these parameters also affect physiological processes directly.

Cell Envelope

The cell envelope of microorganisms basically consists of a cell wall and one or two lipid membranes (26). In addition, many eubacteria and archaea are surrounded by a crystalline surface layer (S-layer), which forms the outermost component of the cell envelope (290). The cell envelope can differ significantly from one organism to another, and even within a strain it can change as a result of physiological adaptation to the environment (245, 252).

For the eubacteria, two major groups can be discerned on the basis of their cell envelope composition: the gram-positive and gram-negative bacteria. Gram-positive bacteria have only one membrane, the cytoplasmic membrane, which is surrounded by a thick, rigid cell wall (Fig. 2). Gram-negative bacteria have, in addition to the cytoplasmic membrane, an outer membrane that consists of phospholipids and LPS (221) (Fig. 2). Between the outer membrane and the cytoplasmic membrane is a thin peptidoglycan layer. The outer membrane functions as a molecular sieve through which molecules with a molecular mass of greater than 600 to 1,000 Da cannot penetrate (221). Despite the presence of porins with low specificity, the outer membrane shows a very low permeability toward hydrophobic compounds, which has been ascribed to the presence of the lipophilic LPS (219–221). However, it has been demonstrated that highly lipophilic compounds such as steroids penetrate relatively easily through the outer membrane of several bacteria (235). Recently, it has been reported that the outer membrane of several *Brucella* strains does not function as an effective barrier to hydrophobic drugs (191).

The cell wall consists of a variety of sugar polymers, the most common group of which are the peptidoglycans, and forms the support layer of the cell envelope. As a result of external stimuli, cells may develop an outer cell wall core that is more adapted to the specific circumstances, e.g., changes in hydrophobicity of the cell surface to attach to surfaces (327). Crystalline S-layers, consisting of protein and glycoprotein subunits, have been found in different groups of bacteria (26). S-layers have been shown to determine and maintain cell shape and to promote adhesion (289). Furthermore, a role in the protection of cells against antimicrobial agents can be envisaged.

The cytoplasmic membrane of cells consists of a phospholipid bilayer (108) and forms a matrix in which enzymes and transport proteins are embedded (68, 284). The carrier (transport protein) molecules allow the selective uptake and excretion of solutes (239). In addition to its role in solute transport, the cytoplasmic membrane plays a crucial role in maintenance of the energy status of the cell (127), regulation of the intracellular environment (33), turgor pressure (131), signal transduction (297), and other energy-transducing processes. Under physiological conditions, the enzyme-containing bilayer can be best described as a liquid crystal (283, 284). Although the lipid molecules constitute only a part of the total membrane mass, they do form the matrix in which the other components are embedded (142, 253, 358). The physical properties of the cytoplasmic membrane thus not only reflect the lipid bilayer but

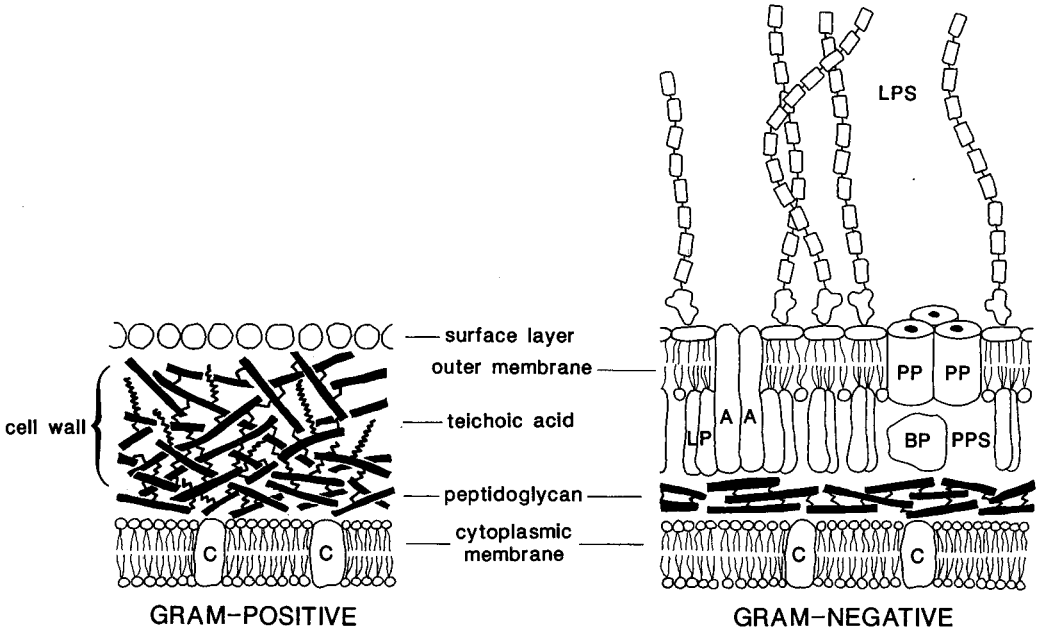

FIG. 2. Schematic presentation of the cell envelope of gram-positive and gram-negative bacteria. PP, porin; C, cytoplasmic membrane-embedded protein (e.g., carrier); BP, binding protein; PPS, periplasmic space; A, outer membrane protein; LP, lipoprotein.

also influence the structure and functioning of the other components (30). The fluidity of the cytoplasmic membrane is one important parameter in the "overall" homeostasis of the cell. Consequently, cells adapt to external stimuli by altering the lipid composition such that the bilayer fluidity remains relatively constant (30, 245, 252, 281, 282). This aspect will be discussed below in more detail when the effects of lipophilic compounds on biological membranes are addressed.

The cytoplasmic membrane has a low permeability for polar and charged molecules. Apolar compounds, such as cyclic hydrocarbons, can easily penetrate the lipid bilayer. The transfer of such molecules across the membrane, therefore, is most probably a diffusion process.

It should be noted that specific transport processes could play a role in the mineralization of low concentrations of cyclic hydrocarbons but that the transport activities may not easily be revealed because of passive diffusion of the compounds. On the other hand, if energy-requiring transport processes take place in addition to passive diffusion, the cells may be confronted with an energy-dissipating futile cycle; i.e., when carrier-mediated uptake occurs, metabolism of the hydrophobic compound does not match the rate of transport. Under those conditions, part of the compound may leak out of the cell by passive diffusion. The permeability of the membrane is dependent on the hydrophobicity of the solutes that have to cross the membrane (60, 84, 181). In addition, Lieb and Stein (181) demonstrated that the size of solute molecules plays a role in the permeability. Bateman et al. (18), who studied the uptake of naphthalene by a *Pseudomonas* species, showed that neither ATP nor an electrical potential was required for the uptake of this apolar compound. Other reports on the physiology of (cyclic) hydrocarbon metabolism only briefly discussed the nature of the uptake process that precedes the metabolism of these compounds. Although the uptake of hydrocarbons could essentially be a passive transport process, different adaptations have evolved to increase the uptake rates. For the uptake of alkanes, Witholt et al. (349) postulated that LPS related to outer membrane LPS are released and encapsulate hydrocarbon droplets, thus increasing the efficiency of mass transfer (349). Studies of bacteria and yeasts, growing mainly on aliphatic hydrocarbons, show that these cells contain inclusions of unmodified hydrocarbon substrate (262). This has also been observed for a *Pseudomonas* strain growing on naphthalene (262). Further studies on the hydrocarbon inclusions in an *Acinetobacter* strain, cultivated on hexadecane, showed that the inclusion was surrounded by a lipid-rich monolayer membrane with a phospholipid composition that is qualitatively similar to the composition of the cytoplasmic membrane (94, 263, 264). These observations indicate that the hydrophobic core of lipid bilayers or micelles is a perfect matrix for lipophilic molecules such as cyclic hydrocarbons.

Partitioning of Solutes into the Membrane

The accumulation of lipophilic compounds into lipid bilayers may enhance their availability to the cell but may also cause toxicity problems (275, 276). The partitioning of hydrocarbons in membrane buffer systems has been studied by some groups (4–7, 78–80, 186, 242, 275, 276). However, most researchers have determined partition coefficients of hydrocarbons and other lipophilic compounds in octanol/water, hexadecane/water, diethyl ether/water, olive oil/water, etc. The partition coefficients thus obtained were used to predict effects of the compounds on intact cells, e.g., bioconcentration (56), biodegradation, toxicity (228), and anesthetic effects (265). Although these methods, especially the octanol/water partition coefficient (176), showed good correlations with biological effects (172, 228, 265, 275), quantitative estimations were impossible as a result of differences in membrane composition. As shown by the results of Antunes-Madeira and Madeira (4–7), the specific lipid composition of a lipid bilayer can strongly influence the partitioning behavior of a compound (Table 2) and, consequently, its biological effect.

De Young and Dill studied the partitioning of benzene and

TABLE 2. Partition coefficients of lipophilic compounds between membrane and aqueous phases, depending on the composition of the membrane[a]

Lipophilic compound	Partition coefficient in membrane[b]:			Reference
	DMPC	DPPC	DSPC	
Malathion	225	135	48	7
Parathion	1,950	650	270	4
Lindane	2,450	600	50	5
DDT	336,000	180,000	88,000	6

[a] Data from references 4–7.
[b] DMPC, dimyristoylphosphatidylcholine (14:0); DPPC, dipalmitoylphosphatidylcholine (16:0); DSPC, distearoylphosphatidylcholine (18:0).

hexane in dilauroylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), and dipalmitoylphosphatidylcholine (DPPC) membranes as a function of the membrane surface density (78, 79). Their results showed that partition coefficients of these compounds between the membrane and the aqueous phases depended not only on simple partitioning behavior as observed for oil-water systems but also on ordering constraints of the lipid bilayer. Different methods to alter the surface density were applied, with no significant differences in the results. The major reason for the observed incompatibility of organic-solvent/bulk phase partitioning and membrane partitioning is that in a membrane a solute will not be distributed homogeneously but, rather, a gradient is formed that varies with the composition and geometry of the membrane (189). The high degree of ordering of solutes in a lipid bilayer compared with a bulk liquid phase also significantly changes the thermodynamics of the partitioning (280, 348). Nevertheless, a good correlation between the partition coefficient of various lipophilic compounds in membrane/buffer and octanol/water two-phase systems has been observed (186, 275) (Fig. 3). These partition coefficients can be applied to estimate admissible aqueous concentrations of lipophilic compounds, which can be useful for setting up enrichment cultures used for isolating novel microorganisms. Since the octanol/water partition coefficients (log P) are known for a large number of chemicals and can even be estimated from group contributions, they can be very useful for assessing toxicity hazards of chemicals by applying empirical relations determined in model systems (275). Despite the good correlation between toxicity and the log P observed in many instances (172, 173, 228, 331), application of the log P toxicity relationship to assess upper limits for concentrations of chemicals in biological systems should be done carefully. Because of differences in membrane composition, environmental conditions (e.g., pH, temperature, ion concentration), metabolization rate, and/or characteristics of the chemical, large variations in the distribution of the lipophilic molecules can occur. Therefore, determination of the partition coefficients between the membrane of the microorganism(s) of interest and the relevant aqueous environment will provide more reliable information (4–7, 275, 276).

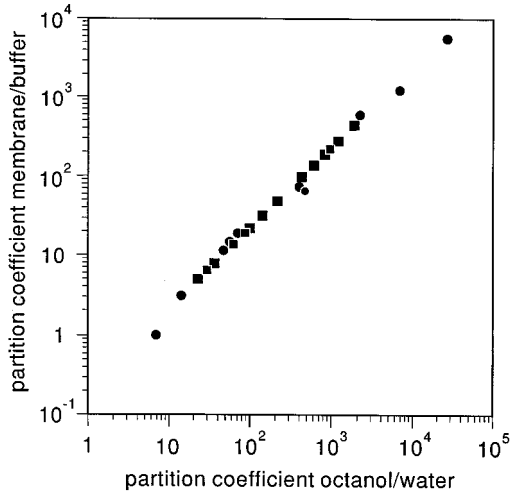

FIG. 3. Correlation between partition coefficients of lipophilic compounds in membrane/buffer systems and partition coefficients in a standard octanol/water system. Symbols: ●, cyclic hydrocarbons in *E. coli* phospholipid liposomes (276); ◆, substituted phenols in erythrocyte membranes (186).

MEMBRANE TOXICITY OF LIPOPHILIC COMPOUNDS

Cyclic Hydrocarbons

As long ago as 1921, Jentsch reported that cyclic hydrocarbons impaired the growth of bacteria and fungi (152). Cyclic hydrocarbons, e.g., turpentine constituents and tetralin, were consequently added to cleansers as antibacterial agents (243). Baier (12) concluded, on the basis of elegant experiments with suspended and filter-dried bacteria, that the antibacterial action of petroleum compounds was a function of their solubility in water. Furthermore, he observed that *Escherichia coli* was more susceptible to low- than to high-boiling-point fractions (e.g., paraffin oil). This is in agreement with other observations that refined oil is more toxic to microorganisms than crude oil (336), which indicates that volatile hydrocarbons (e.g., benzene and xylenes) are more toxic than the high-molecular-weight alkanes. Walbum, who studied the toxic effect of a number of hydrocarbons including benzene, toluene, ethylbenzene, and xylene on microorganisms, observed that the increase in toxicity paralleled the decrease in viscosity of the hydrocarbon added (334). Observations on the toxicity of cyclic hydrocarbons were also reported by microbiologists who attempted to isolate new strains of microorganisms that were able to grow at the expense of these compounds. Examples of toxic effects of cyclic hydrocarbons, as observed during isolation and cultivation of microorganisms growing on these compounds, are listed below. In addition, studies on the mechanism of the toxicity of these compounds are included. Despite the toxicity of cyclic hydrocarbons, many researchers have been able to isolate new strains of hydrocarbon-metabolizing microorganisms. They minimized toxicity problems by supplying the hydrocarbon substrate via the vapor phase (65, 103). This method results in a controlled aqueous concentration of the hydrocarbon.

Terpenes. Terpenes are hydrocarbon compounds consisting of multiple isoprene units and may or may not be cyclic. In contrast to the numerous reports that have been published on the toxicity of essential oils, knowledge of the inhibition action of purified terpene hydrocarbons is limited. The most common representative of cyclic terpenes, α-pinene, together with β-pinene, limonene, and terpinolene, was shown to inhibit bacterial growth in agar plate diffusion tests (149). Andrews et al. (3) studied the toxic effects of α-pinene and some other terpenes produced by the Douglas fir on some *Bacillus* strains and on *Saccharomyces cerevisiae* (3). It was shown that α-pinene, limonene, camphene, and isobornyl acetate were inhibitory to the microorganisms at concentrations normally present in the fir needle diet of Douglas fir tussock moth larvae. The presence of such terpenes in the diet of these

insects was found to strongly influence the infectivity of *Bacillus thuringiensis* spores for the Douglas fir tussock moth larvae. α-Pinene destroyed the cellular integrity and inhibited respiratory activity in yeast mitochondria.

Similar effects were observed for the structural isomer, β-pinene, on yeast cells (320). β-Pinene inhibited the respiration of both intact cells of *S. cerevisiae* and mitochondria isolated from this yeast. The extent of the inhibitory effect depended strongly on the ratio between the β-pinene concentration and the biomass. Addition of β-pinene resulted in an inhibition of proton and potassium ion translocation, whereas no effect on ATPase activity was observed. The inhibitory effects of β-pinene were stronger with ethanol than with glucose as the substrate, suggesting that the effects are exerted at the level of metabolic energy conservation, i.e., the mitochondrial membrane. The studies on isolated mitochondria showed a series of effects, starting with the disappearance of the respiratory control and deenergization of the organelle, followed by an inhibition of respiration at higher concentrations of the terpene. The effect on respiration could be attributed to the cytochrome *b* region of the electron transport chain. No effect on the activity of the mitochondrial ATPase could be detected. Other observations in rat liver mitochondria by Uribe et al. showed that β-pinene stimulated the passive efflux of potassium ions and decreased the transmembrane electrical potential (318). Furthermore, a strong increase in ATPase activity was observed, which may be indicative of an elevated proton leakage through the membrane (260). Increasing the extracellular β-pinene concentration to above 600 μM resulted in an apparent resealing of the rat liver mitochondrial membrane, as was concluded from observation of a decrease in the potassium permeability (318). This resealing effect was also observed for yeast mitochondria at β-pinene concentrations of 600 μM, whereas uncoupling effects were observed at 100 to 200 μM and respiration was inhibited at 400 μM (320). Similar effects on energy transduction in mitochondria were observed with limonene but not with other hydrophobic molecules (320). The toxic effects of β-pinene and limonene on *S. cerevisiae* were proportional to the size of the monoterpene droplets in the suspension (319). Experiments with β-pinene and limonene dissolved in different cosolvents and added to yeast cell cultures were performed to determine the effects of a decrease in the droplet size and enhancement of the toxicity of either monoterpene. Studies with liposome model systems confirmed that cyclic terpene hydrocarbons accumulate in the membrane, which causes a loss of membrane integrity and dissipation of the proton motive force (275).

Aromatics. The toxicity of benzene to a strain of *Pseudomonas putida* was observed by Gibson et al. (102). These authors reported that addition of benzene to the culture medium prevented growth of this bacterium whereas addition of the substrate in the vapor phase sustained normal growth. In later work on the biochemistry of aromatic hydrocarbon metabolism, the volatile aromatic substrates were always added indirectly, thus preventing substrate inhibition (103). In bioconversion experiments with cyclic hydrocarbons as substrates for the formation of interesting fine chemicals, toxic effects of the hydrocarbon substrates were observed. Van den Tweel et al. (324) observed a significant decrease in the rate of conversion of benzene into *cis*-3,5-cyclohexadiene-1,2-diol when the amount of benzene added to the incubation was increased. These observations are in agreement with work published by Yarmoff et al. (357) that indicated that benzene impaired growth and hampered the production of *cis*-3,5-cyclohexadiene-1,2-diol. Benzene, in concentrations higher than 0.15% (wt/vol), also impaired succinate-supported growth and catechol formation of a mutant *Pseudomonas* strain (271). An inhibitory effect of toluene under comparative conditions was observed for a strain of *P. putida* (151). Parallel to an inhibitory effect on cellular growth, a decrease of the adenylate energy charge by over 50% was observed. Since the decrease of the energy charge was matched by a significant increase in AMP levels and not by an increase of extracellular levels of adenine nucleotides, the lower energy status of the cell was most probably a result of metabolic energy losses. Since toluene has often been applied by microbiologists and cell biologists for permeabilization of cells, a number of studies have been performed on the mechanism of the permeabilizing action (44), and effects of toluene on the ultrastructure and physiology of cells have been observed. The galactose permease system became totally inactive in the presence of low concentrations of toluene (147), possibly as a result of impaired energy transduction (275, 279). It was shown by Jackson and DeMoss (147) that toluene impaired growth of *E. coli*, caused leakage of macromolecules (e.g., RNA and proteins), and altered the ultrastructure of the cells (353). Electron micrographs showed that the cells are not completely lysed. de Smet et al. (76) showed that treatment of *E. coli* cells with toluene resulted in an increased permeability of the cytoplasmic membrane. Electron-microscopic studies confirmed that the cytoplasmic membrane is considerably damaged, whereas the outer membrane is still intact. These studies also indicate that magnesium ions protect the membrane against deleterious effects of toluene.

Hartmans et al. (121) were able to isolate 16 different strains of styrene-utilizing microorganisms from 12 soil samples by supplying styrene to the vapor phase (concentration in the aqueous phase, approximately 1.5 mM). Omori et al., who added styrene directly to the aqueous medium in a concentration of approximately 175 mM, obtained no styrene utilizers from 101 soil samples (226). These results suggest that the amount of styrene added was critical for isolating new strains of styrene utilizers.

Attempts made by us (273, 274) to obtain microorganisms capable of growing on tetralin were successful only when tetralin was applied at low (subsaturating) concentrations. It appeared that tetralin was toxic to microorganisms when it was present at concentrations of ≥125 μM. Further studies with both intact bacterial cells and liposomes revealed that tetralin strongly interacted with the membrane. As a result of this interaction, the membrane surface area was increased and the passive flux of protons across the membranes was increased, thereby causing dissipation of the components of the proton motive force (276). It was also observed that tetralin accumulated in the membrane ($P_{\text{membrane/buffer}}$ = 1,100), which lowered the actual extracellular concentration of tetralin. This latter aspect provided a rationale for difficulties met by us and others in isolating pure cultures of microorganisms growing on tetralin; i.e., at the onset of the experiments, the biomass concentration is low and the cell experiences the actual tetralin concentration; once sufficient biomass has been obtained, the external tetralin concentration is lowered and the inhibition may be less pronounced. The high $P_{\text{m/b}}$ value may explain the effectiveness of tetralin as a biocide against moths (62) and bacteria (241, 298). In addition, tetralin was shown to be toxic to *Salmonella typhimurium* in an Ames test that was performed to screen mutagenic and toxic effects of tobacco smoke constituents (95).

Cerniglia et al. (52) studied the toxicity of naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene and their oxygenated derivatives to cells of the cyanobacterium *Agmenellum quadruplicatum*. No significant inhibition of the growth of the cyanobacterium by the hydrocarbons was observed. In con-

trast, the phenolic and quinonic naphthalene derivatives inhibited the growth of the cells (52). A possible explanation for the absence of a toxic effect by these hydrocarbons is that the mass transfer of the molecules to the cells is limited by the small surface area of the solid particles (333, 351, 352), whereas the phenols and quinones have a higher solubility. Data obtained on toxic effects of naphthalene, biphenyl, anthracene, and phenanthrene on energy transduction in liposomes indicated that the solid hydrocarbon became toxic only after long periods of incubation, whereas addition of the hydrocarbon dissolved in a cosolvent (dimethyl formamide) was instantaneously toxic (275). This could also offer an alternative explanation for the observed synergistic toxicity of ethylbenzene and biphenyl to a *Pseudomonas* strain (293).

Toxic effects of the highly lipid soluble polycyclic aromatic hydrocarbons (15, 325) on microorganisms are not well documented. In contrast, a wealth of information is available on the mutagenicity and carcinogenicity in mammals of the polycyclic aromatic hydrocarbons and derivatives thereof (230, 345). A careful set of experiments on the toxicity of polycyclic aromatic hydrocarbons on microorganisms was reported by Calder and Lader (47), who demonstrated that increasing amounts of naphthalene, 2-methylnaphthalene, pyrene, and others resulted in an increased lag phase and lowered growth rate of two bacteria growing on these compounds. Unlike in other studies, the compounds were allowed to equilibrate prior to inoculation. It has also been demonstrated that an increase in cultivation temperature results in an increased inhibition of growth of the pyrene-utilizing *Rhodococcus* strain (337). This suggests a toxic effect of pyrene as a result of an increase of the mass transfer rate at elevated temperatures. Unfortunately, the authors did not include data on the temperature sensitivity of this strain in the absence of pyrene. Biotoxic effects of eluates of polycyclic aromatic hydrocarbon-contaminated soil were observed in bioluminescence assays (342). The toxicity greatly depended on the adsorption characteristics of the eluted soil and was prevented by the addition of activated carbon. Mahaffey et al. (187) reported that benz[*a*]anthracene added to an incubation formed a fine particulate film at the air-water interface when no cells were present. When benz[*a*]anthracene was added to a cell suspension, the initially formed film disappeared within 1 h of incubation. The authors explained this observation by possible partitioning of the hydrophobic compound into the cytoplasmic membrane and other parts of the cell envelope. Recent studies by Brodkorb and Legge on the mineralization of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium* in oil tar-contaminated soils indicated that growth of this organism was significantly impaired by the presence of the pollutants (41). However, in addition to some polycyclic aromatic hydrocarbons, significant quantities of other oil components were present. The observed toxicity might therefore have been the result of the presence of either the unidentified oil components, the polycyclic aromatic hydrocarbons, or the simultaneous presence of both (41).

In general, polycyclic aromatic hydrocarbons are expected to have aspecific toxic effects on cells, although this effect may be negligible in comparison with the enormous toxic potential of oxidized derivatives of these compounds (51, 53, 54, 304). Recent studies on the aspecific toxicity of naphthalene, biphenyl, anthracene, phenanthrene, and others, performed with liposomes as a model system, showed that in the absence of mass transfer limitation, these compounds do affect the energy transduction across the biological membranes (275).

Cycloalkanes. The degradation of cycloalkanes by pure cultures of microorganisms has been attempted for a long time. In 1919, Tausz and Peter reported the isolation of a bacterium capable of utilizing cyclohexane as the sole source of carbon and energy (307). However, this claim and other claims of pure cultures growing on alicyclic compounds have been disputed (314). Meanwhile, transformation of cycloalkanes by mixed cultures (19, 75) or by pure cultures in the presence of cosubstrates (227, 238) was observed. The first undisputed isolation of a pure culture growing on cyclohexane was reported in 1977, when Stirling et al. obtained a *Nocardia* strain, which could grow on cyclohexane as the sole source of carbon and energy, from mud flats near Sittingbourne, England, by selective enrichment on methylcyclohexane that was added to the vapor phase (296). Ooyama and Foster (227), on the other hand, were not able to isolate or cultivate microorganisms in the presence of alicyclic compounds added to the aqueous medium (cyclohexane concentrations of 31 and 140 mM for isolation and cultivation, respectively). Recently, Uribe et al. reported the toxic effects of cyclohexane on the energy transduction in *S. cerevisiae* (321). Cyclohexane inhibited oxygen uptake in intact cells and isolated mitochondria. Studies on isolated mitochondria showed that ATP synthesis was impaired whereas ATP hydrolysis was slightly increased. Uptake of potassium ions was impaired, and dissipation of the mitochondrial membrane potential was observed (321). These studies indicate that the permeability barrier of the inner mitochondrial membrane was disrupted by cyclohexane.

Pelz and Rehm tried to isolate microorganisms capable of utilizing decalin (decahydronaphthalene) as the sole source of carbon and energy, but none of their 250 enrichment cultures, containing decalin at a concentration of 100 mM, led to the isolation of a decalin-utilizing strain (232). The concentration of decalin applied was probably enough to kill any biological activity present in their enrichments. In contrast to the statement made by Pelz and Rehm that decalin is resistant to microbial attack, the removal of this compound from mixtures of hydrocarbons by different microorganisms has been reported (294, 299). In 1960, Colla and Treccani reported the isolation of a *Flavobacterium* species capable of growing on decalin as the sole source of carbon and energy (61).

Other Lipophilic Compounds

Toxic effects of various lipophilic compounds have been observed. Compounds that are used as food preservatives and disinfectants, as well as a wide variety of drugs, have been shown to affect membrane functions (55, 251, 258). Food preservatives such as lactic acid, benzoic acids (338, 339), and other lipophilic acids (101) act predominantly by dissipating the pH gradient (ΔpH) across the cytoplasmic membrane (254); however, enzymes and DNA have also been mentioned as possible targets (55). Since the undissociated acids act as uncouplers, the inhibitory effect of the weak acids strongly depends on pH (182). The essential oils that are used as flavor compounds in food have also been studied for their antimicrobial activities. This group comprises a wide range of mainly terpenoid compounds, e.g., limonene, carvone, pinene, ocimene, and verbenol. Present studies are directed mainly at obtaining qualitative data on the toxicity of such compounds isolated from various plants (74, 82, 130, 163–165, 212, 213).

Applications of lipophilic compounds as disinfectants range from soaps and detergents to antiseptics used in facilities for potable water (347) and agents used to prevent biodeterioration. Since the beginning of this century, compounds with a wide antimicrobial spectrum have been used as disinfectants (20, 306); these include coal tar liquids (such as tetralin and decalin), lysol-type disinfectants (cresols), chlorinated compounds, and quaternary ammonium compounds (315). As a

result of their lipophilic character, these compounds most probably act on the cytoplasmic membrane (97, 156). Detergents, a heterogeneous class of surface-active compounds, are able to disrupt phospholipid membranes by disturbing the hydrophobic interactions between the lipids and proteins (126). In addition to their application as disinfectants, detergents are used in biochemical studies for the permeabilization of cells (157, 201) and the release of membrane-embedded proteins (126, 355). As a result of the diversity of surface-active compounds, their effects on biological membranes vary significantly depending on the characteristics of the surfactants and the incubation conditions used (126, 156, 161). In model studies with liposomes in seawater to assess the effectiveness of detergents as shark repellents, the anionic detergent sodium dodecyl sulfate (SDS) and the nonionic detergent Triton X-100 were compared (156). SDS formed pores in the membranes at concentrations below the critical micelle concentration (70 μM in seawater), whereas Triton X-100 was minimally effective below its critical micelle concentration (60 μM in seawater). Above its critical micelle concentration, Triton X-100 caused immediate solubilization of the liposomes, as was demonstrated by the fast release of carboxyfluorescein (156). With SDS, no direct lysis of the liposomes was observed, although the release of carboxyfluorescein increased significantly after longer incubation times, indicating solubilization of the membranes. Also, electron-microscopic studies of *E. coli* treated with SDS revealed that after prolonged incubation with SDS, solubilization of the cytoplasmic membrane and release of cell constituents (DNA, RNA, and cellular protein) occurred (354). From the observations on toxic effects of detergents, it can be concluded that the effectiveness of detergents is determined largely by the critical micelle concentration and the partition coefficient of the compound. These parameters are strongly dependent on the medium and temperature for all detergents and also on pH, ionic strength, and ionic composition for ionic detergents (156).

Observations that various nonantibiotic drugs exhibit antimicrobial activity have been reported for over 60 years (170). At the conference *Antimicrobial Activity of Non-Antibiotics*, held in Copenhagen in May 1990, contributions from bacteriologists and physicians revealed that a wide variety of drugs show antimicrobial activities (170). Local and general anesthetics, antimalarial agents, probenecid, antihistamine drugs, barbiturates, acetylsalicylic acids, procaine, diuretics, steroids, etc., were found to be active against microorganisms. The microorganisms studied were mainly pathogenic strains, e.g., *Listeria monocytogenes*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Vibrio cholerae*, and *Mycobacterium tuberculosis* (170). Effects on microorganisms include changes in morphology, impaired growth, reduced metabolic activities, and inhibition of DNA synthesis. Silva et al., who studied the effect of local anesthetics on bacterial cells (278), demonstrated that growth was impaired and that respiration was inhibited at the level of the membrane-embedded electron transfer enzymes. Leakage of potassium ions and changes in the appearance of the cytoplasmic membrane (electron-microscopic studies) were also observed. The extent of the effects depended on the amount of anesthetic added and on their hydrophobicity.

In the last decade, efforts have been made by bioprocess technologists to find solvents that are suitable for continuous extraction during fermentation processes (40, 43, 173, 356). These studies contain information on the biodegradability, biocompatibility, and applicability of the solvents as extraction media for a wide variety of organic (lipophilic) compounds. For this review, the biocompatibility and/or toxicity of these compounds is of importance. Since bioprocess technologists are interested in extraction processes, the data pertain to situations in which the amount of solvent added by far exceeds the aqueous solubility. Usually a specific solvent is added at 10% (vol/vol), resulting in the formation of a distinct second phase. As a result of the presence of a distinct second phase, effects of the phase transition, leading to mechanical stress on the cell envelope, may also occur. To enable classification of solvents for extractive uses in bioconversion reactions, Bar discriminated between molecular toxicity and phase toxicity (14). To be able to predict the biocompatibility of a solvent compound, correlations between physical characteristics of the solvents and toxicity have been made. The most appropriate parameter for this purpose appeared to be the partition coefficient of a solvent in a standard octanol/water system (log P or log K_{OW}) as a measure of the hydrophobicity of the compound (172, 173). Recently, Osborne et al. studied the effects of a great number of organic solvents, applied in subsaturating and saturating amounts, on the activity of a membrane-bound dehydrogenase (228). From the data obtained on the dependence of the enzyme activity on the amount of solvent applied, they were able to derive a critical solvent concentration in the aqueous phase. The critical concentration in the aqueous phase is related to a critical membrane concentration by an estimated partition coefficient (265). This membrane/buffer partition coefficient was estimated from the above-mentioned octanol/water partition coefficient and an empirically derived conversion factor. The approach followed by Osborne et al. (228) enables the prediction of the biocompatibility of an organic solvent with the specific enzyme used in their investigations. However, extrapolation to solvents with "out-of-range" log P values, as well as extension to other organisms and even other cellular functions in the same organism, will be inaccurate as a result of membrane characteristics (4–7, 348).

Also, in the field of ecotoxicology, the effects on microorganisms of lipophilic compounds that are present in the environment as pollutants have gained considerable attention. This attention was stimulated by the growing awareness that microorganisms play an essential role in the removal of pollutants from the environment (322, 323). Inhibition of the biodegrading capacity will result in a prolonged waste removal period. Examples of pollutants that have been shown to impair microbial activity in the environment are mineral oils (285, 286, 336), polycyclic aromatic hydrocarbons, industrial solvents (160, 234), agrochemicals (27, 28, 71, 233), and surface-active compounds that are applied in soaps and detergents (156, 315). Parameters that were affected included growth, transport systems, CO_2 fixation in cyanobacteria, and gas production by anaerobic bacteria (234, 272). Inhibition of growth and effects on bioluminescence, respiration, and methanogenesis have all been used to assess the toxicity of various environmental pollutants on microorganisms (16, 39, 113, 158, 301, 335). Data on the toxic effects of xenobiotic compounds have been gathered and, together with knowledge of physicochemical characteristics of such compounds, can be used to predict the fate of pollutants in the environment. These so-called quantitative structure-activity relationships (QSARs) were originally developed for pharmaceutical applications (115) but have been successfully used in environmental research to predict bioconcentration (63) and environmental toxicity in general (116, 129, 166). Although quantitative structure-activity relationships can be helpful in assessing general features of the toxicity of specific groups of chemicals (e.g., phenols, chlorinated aromatics), they do not provide further information on the mechanisms of the toxic effects.

A few groups of noncyclic hydrocarbons are treated in more detail below. The examples provide additional information on the membrane toxicity exerted by lipophilic compounds.

Alkanes. Similarly to cyclic hydrocarbons, aliphatic hydrocarbons may be toxic to microorganisms (11, 350). As demonstrated by Gill and Ratledge (105), the toxicity of these compounds is related to their chain length, which correlates perfectly with their solubility in water and their hydrophobicity. Experiments have been performed with two *Candida* strains which are able to utilize *n*-alkanes above C_8 and *Saccharomyces carlsbergensis*, which cannot grow on any alkane. The growth and respiration rates of the three yeasts were monitored in the presence of an alkane and in the presence and absence of an additional carbon and energy source. The results showed that the *Saccharomyces* strain was sensitive to a larger range of *n*-alkanes than were the *Candida* strains. The authors suggested that this might be a result of the ability of the *Candida* strains to metabolize the hydrocarbons. Alternatively, it can be postulated that the *Candida* strains are better adapted to survive in an environment containing lipophilic compounds. Further studies by these authors revealed that addition of *n*-decane to cells of one of the two *Candida* strains resulted in inhibition of the glucose transport system of the cell (106). Studies of the application of the alkane-hydroxylating system of *Pseudomonas oleovorans* showed that aliphatic substrates, e.g., *n*-octane, caused loss of biocatalyst stability but did not fully inactivate the cells (349, 350). Also, recent data on the effect of various organic solvents on the viability of cells of *Flavobacterium dehydrogenans* show that alkanes only partially inhibit cellular activity (29).

Alcohols. Ethanol is probably the most commonly applied antimicrobial compound. It has been known for a long time that ethanol prevents the deterioration of food and is extremely effective as an antiseptic (155). In line with the expectations, production of ethanol in fermentation processes is impaired by product inhibition. As a result of the commercial importance of ethanol, the mechanism of the toxicity of ethanol has been studied rather extensively. A great deal of knowledge gathered on the toxicity of ethanol, as well as on the mechanisms of adaptation of microorganisms to this compound, has been treated in an excellent review by Ingram and Buttke (138). As to the mechanism of ethanol toxicity, it is known that ethanol interacts with biological membranes, resulting in a decreased membrane integrity. This causes an increased passive flux of protons across the membrane, leading to the dissipation of the proton motive force (50, 174). This effect was also observed with other alkanols, and it was shown that the effective concentrations decreased with increasing lipid solubility of the alkanols. This indicates that the toxic action of alkanols is exerted at a hydrophobic site, most probably the cytoplasmic membrane (49). Studies on the inhibitory action of butanol on cells of the butanol-producing bacterium *Clostridium acetobutylicum* confirmed the close relationship between effective concentration and lipid solubility (37). For this reason, solvent production in an acetone-butanol-ethanol fermentation depends on the concentration of butanol and not on the amounts of ethanol or acetone present (154). In addition to the increase in proton flux, a partial inhibition of AT Pase activity was observed (37). As a result of both effects, the ΔpH is diminished in these fermentative cells, which may lead to a reduction of transport and various other activities (237).

In general, the observations made for cyclic hydrocarbons mimic those made for alcohols and other lipophilic compounds. Phenethyl alcohol, which is a bacteriostatic agent, impairs growth but also inhibits DNA synthesis in *E. coli* (23, 246, 313). Further studies on the mechanism of these effects showed that phenethyl alcohol affected the cytoplasmic membrane, resulting in the loss of intracellular potassium (279). Parallel experiments with toluene, conducted by these researchers, showed that the two compounds act via a similar mechanism (147, 279). In conclusion, the degree of toxicity of the alcohols is directly proportional to the molecular chain length (135, 138) and parallels the partitioning behavior of the alcohols in *n*-octanol/water (181).

Phenols. Since the 19th century, phenol has been used as a biocide (114). Recently, the effects of phenol and its substituted derivatives have been the subject of renewed attention because of the widespread presence of these compounds in the environment. Phenolic compounds are commonly found in wastewaters of industrial origin (35). Because of their relatively high aqueous solubility, these compounds are also readily available. As a consequence of the bacteriostatic action of these compounds, the microbial degradation of phenolic compounds is often minor (24, 93, 343). Also, phenolic food additives such as butylated hydroxytoluene and butylated hydroxyanisole, used as antioxidants, are known to have antimicrobial effects (73). Phenolic compounds probably exert their toxic effects at the level of the membrane, as can be deduced from the high correlation between the toxicity and hydrophobicity of different phenolic compounds (22, 272). This conclusion is supported by observations that phenol changes membrane functioning and influences protein-to-lipid ratios in the membrane (159). In addition, Heipieper et al. demonstrated that addition of phenol and 4-chlorophenol to suspensions of *E. coli* induced efflux of potassium ions (124).

In conclusion, in the studies in which the toxic effects of lipophilic compounds have been analyzed in some detail, effects on the cytoplasmic membrane and/or membrane-embedded enzymes have been demonstrated (3, 124, 128, 228, 275, 276, 320, 321, 347). Furthermore, the inhibitory action is in most cases directly related to the partitioning behavior of the lipophilic compounds in *n*-octanol/water, again suggesting that the (cytoplasmic) membrane is the primary site of toxic action. This of course does not rule out additional sites of toxic action. For instance, oxidizing agents and weak acids can be more toxic to a cell than expected on the basis of their lipophilic nature as a result of the specific properties of the molecules. In the next section, an outline of studies on the mechanism of the toxic action of lipophilic compounds is presented.

SITE(S) OF TOXIC ACTION OF CYCLIC HYDROCARBONS

The mechanism of the inhibitory action of lipophilic compounds has been studied for many years, beginning with studies on the action of anesthetics by Meyer (197) and Overton (229) almost a century ago. Their hypothesis was that anesthetics accumulated in lipoid parts of the human body and that the action of the anesthetics was a result of this accumulation. The experimental proof for this was that the anesthetic efficacy of a compound showed an excellent correlation with the partitioning of that compound in olive oil. The theory that developed from this hypothesis is the so-called lipid theory of anesthesia. As a result of accumulation of lipophilic compounds in lipid membranes, loss of membrane integrity has been observed in several instances (134, 265, 276, 320). In addition to observed changes in membrane structure and function, alterations in enzyme activities have been observed (98, 153, 228). Since the late 1970s, Franks and Lieb have advocated proteins as the primary site of the action of lipophilic compounds (98–100, 192). Effects of lipophilic compounds on the "model protein" firefly luciferase perfectly correlated with anesthetic ef-

fects in animals (98–100). However, the important phenomenon of pressure reversal, which is generally observed in anesthesia, cannot be observed for firefly luciferase (211). Since the protein targets of lipophilic compounds are often membrane-embedded enzymes, it has recently been postulated that the toxic effects could be at the level of lipid-protein interactions as well as lipid-lipid interactions, which are likely to be disturbed when a molecule accumulates in the lipid bilayer (148, 256, 275).

In this section the state of knowledge about the action of lipophilic compounds on lipid bilayers and/or membrane-embedded proteins is evaluated and effects are related to observations made for microorganisms.

Changes in Membrane Structure

As a result of partitioning of lipophilic compounds into lipid bilayer membranes, one could expect significant changes in the structure and integrity of the membranes. The changes that are induced differ greatly for the various solutes interacting with membranes. This is, to a large extent, the result of differences in the polarity of the compounds and consequently of differences in the location in the bilayer. As mentioned above, membranes do not behave as bulk liquid phases with respect to partitioning. A concentration gradient of lipophilic compound exists (189), as a result of variations in polarity of the lipid bilayer, when looking at cross-sections of the membranes (257). Also, lateral heterogeneity and cluster formation of membrane lipids can be affected by accumulation of lipophilic compounds in the lipid bilayer (70). Therefore, solutes that interact with the membrane will cause different perturbations of the bilayer depending on their preferential site of accumulation (146). This effect may be accentuated by an asymmetric distribution of the phospholipids. Sheetz and Singer suggested that different amphipathic drugs accumulate in either the outer or the inner layer of the membrane depending on their charge, which affects the interaction with phospholipids that are specifically abundant in either layer (268). However, intrinsic elasticity of the bilayer may largely compensate for the changes induced by the amphipaths (90). By using fluorescent amphiphilic molecules such as TMA-DPH {1-[4-(trimethylamino)-phenyl]-6-phenylhexa-1,3,5-triene}, it has been shown that the cationic molecules accumulate rapidly into the outer leaflet of the membrane but that the flip-flop to the inner leaflet is a relatively slow process (66). This may also lead to an asymmetric distribution of the compounds in the membrane, especially when the rate of metabolism of the compounds in the cytoplasm exceeds the flip-flop of the molecules. On the basis of changes in ultrastructure induced by the amphipathic drugs, Sheetz and Singer (268) discerned crenating (anionic) and cup-forming (cationic) drugs. Further work by Singer's group showed that these amphipathic drugs caused a marked redistribution of protein and lipid components in the plane of the membrane (188). These effects may include restructuring of domains of specific constituents, such as supramolecular protein complexes and lipid patches that exist in biological membranes (30). Also, vesiculation of the membrane may occur as a result of the intercalation of lipophilic molecules with the bilayer (112).

Studies by Seeman on the interaction of various drugs and anesthetics with erythrocyte membranes revealed that these compounds have significant impact on the volume of the membrane bilayer (265). The expansion of the membrane is the result of the accumulation of the lipophilic compounds in the lipid bilayer, which appears to correlate with the hydrophobicity of the molecules. This is in agreement with observations made by Skou (287) on lipid monolayers which indicate that the surface pressure of the monolayer increases linearly with the amount of solute that penetrated into the monolayer.

The effects of solutes on membranes, as mentioned above, pertain to compounds that possess a polar function (e.g., a hydroxyl group) or have a charged group (e.g., anionic, cationic, or zwitterionic drugs). For hydrocarbons, the principal site of accumulation in the membrane is probably the central, aliphatic part of the bilayer. Therefore, hydrocarbons will probably not induce crenating or cup-forming effects as observed by Sheetz and Singer for amphipathic drugs (268). Hydrocarbons have been found to reside either in the area of the acyl chains of the phospholipids or in the area between the opposing monolayers (195, 346). The major change in membrane dimension as a result of interaction with small lipid-soluble compounds, e.g., hexane, is an increase in the area occupied per phospholipid molecular rather than the bilayer thickness (64). This result can be explained by interaction of these solutes with the acyl chains of the phospholipids, since the area occupied by a phospholipid molecule is determined largely by the volume occupied by the acyl chains (38, 326). Studies on the interaction of *n*-alkanes with lipid bilayers (195) show that *n*-alkanes, from hexane to hexadecane, enter the hydrocarbon region of the lipid bilayer. It was demonstrated by X-ray diffraction for the longer alkanes (dodecane to hexadecane) that accumulation causes an increase in bilayer width. Also, an increase in the transition temperature of DPPC membranes was observed, probably as a result of an increase in hydrocarbon chain interactions. The increase in hydrocarbon chain interactions is probably due to a parallel alignment of the longer alkanes ($>C_{12}$) and the lipid acyl chains, which also reduces the area occupied per phospholipid molecule (38, 326). Shorter alkanes ($<C_{10}$), which also partition into the hydrophobic part of the lipid bilayer (346), lower and broaden the transition temperature. The observation that the presence, in the bilayer, of approximately one hexane molecule per phospholipid does not cause any increase in membrane volume (162, 346) suggests that the hexane molecules align perfectly with the fatty acid acyl chains. The interaction between the hexane molecules and the acyl chains disturbs the interactions between the acyl chains and, consequently, reduces the ordering of the lipid bilayer. Long-chain alkanes probably also disturb the interactions between the acyl chains within one monolayer but compensate for this loss by interacting with acyl chains of both leaflets of the bilayer, thus increasing the overall degree of ordering of the membrane.

Studies on the interaction of δ-hexachlorocyclohexane with human erythrocyte membranes showed that the basic structure of the lipid bilayer is altered by subsaturating concentrations of this compound (329, 330). Cell lysis is observed, and changes in lipid-protein interactions in the annulus or boundary lipids surrounding membrane-embedded proteins have been postulated. It is suggested that the observed cell lysis is caused by the combined effect of the δ-hexachlorocyclohexane on both the lipid-lipid interactions and the interaction of boundary lipids with the transmembrane components of the protein cytoskeleton. Also, interaction of γ-hexachlorocyclohexane (lindane) with liposomal membranes resulted in disordering of the membrane structure (8).

The interaction of cyclic hydrocarbons with *E. coli* phospholipid liposomes, as assessed by studies of liposomes labeled with fluorescent probes, showed that these hydrocarbons interacted primarily with the bilayer interior (275). Addition of the lipophilic cyclic hydrocarbons caused a decrease in fluorescence polarization of DPH, which is located in the lipophilic interior of the bilayer (66, 175, 270). Fluorescence polarization of TMA-DPH, which is located in the head group region of the

membrane (66), was not observed (275). The decrease in DPH polarization paralleled an increase in fluorescence of rhodamine-labeled fatty acids and phospholipids, which reflects an increase in bilayer surface area caused by the interaction of the cyclic hydrocarbons with the acyl chains (64, 275).

The interaction of lipophilic compounds with the phospholipid bilayer causes dramatic changes in the structure of the membrane. Accumulation of such compounds in the hydrophobic part of the membrane will disturb the interactions between the acyl chains of the phospholipids, leading to modification of membrane fluidity, and eventually may result in swelling of the bilayer. Furthermore, the lipid annulus which surrounds membrane-embedded proteins will also change, which may cause altered protein conformations. The presence of alcohols and amphipathic molecules will affect primarily the outer part of the bilayer, leading to invagination of the membrane and eventually to vesicle formation. Additionally, binding of alcohols and amphipaths will affect the hydration characteristics of the membrane surface.

Changes in Membrane Function

The changes in the integrity of the membrane, as a result of the interactions of lipophilic solutes with different components of the membrane, also affect the functioning of the membrane. The principal functions of the cytoplasmic membrane involve (i) barrier function and energy transduction, which allows the membrane to form ion gradients that can be used to drive various endergonic processes, and (ii) formation of a matrix for proteins (enzymes).

Energy transduction. The functioning of the membrane as a selective barrier is of special importance for protons and some other ions (e.g., sodium), since the gradients of these ions can be used in secondary transport processes to drive the selective uptake of solutes (substrates) and excretion of (metabolic end) products (167, 236, 238). The chemical proton potential (ΔpH) and the electrical potential ($\Delta\psi$) together form the proton motive force (Δp). The chemiosmotic hypothesis, which was established by Mitchell (205, 206), proposes that the proton motive force forms an energy intermediate for the cell that can be used to drive processes such as solute transport, ATP synthesis, and others (127). More recently, it has been shown that not only a proton motive force but also a sodium motive force plays a role in the energy transduction across the cytoplasmic membrane (81). The proton motive force and sodium motive force are interlinked with each other via special transport proteins but are also interlinked with the phosphate potential (ΔG_P) via H^+,Na^+-ATPases (239).

An increase in the passive flux of protons or ions across the membrane may lead to lowering of Δp (or sodium motive force), which will impair proper functioning of the membrane in energy transduction and screening the cytoplasm from the environment. Proton (ion) leakage occurs in every biological membrane, but the endogenous proton (ion) flux is relatively low. The passive proton (ion) flux can increase as a result of alterations in membrane structure caused by an increase in temperature, mechanical stress, interaction with lipophilic molecules, and other factors. Studies on the proton leakage in membrane vesicles from mesophilic and thermophilic bacilli have shown that the passive flux increases with increasing temperature but that leakage in membranes from the thermophilic *Bacillus* strain was affected at higher temperatures than that of the mesophilic organisms (77). Apparently, the phospholipid composition of the membrane of the thermophilic *Bacillus* strain has been adapted to life at higher temperatures. Bangham et al. showed that various anesthetics stimulated the permeability of a phospholipid bilayer to potassium ions (13). The concentrations at which increased permeability was observed correlated perfectly with chemical activities inducing narcosis in biological systems. Stimulation of the leakage of protons and potassium ions has been observed in various membranes upon addition of lipophilic compounds such as ethanol (50, 174), butanol (37), fatty acids (36, 91, 249), β-pinene (320), and dolichol (208), as well as cyclohexane (321), tetralin (276), and other cyclic hydrocarbons (275). For some fragrance compounds, decreases of the transmembrane electrical potential have been shown (87). The changes in membrane potential paralleled the fluidity changes of the membrane, as was also observed for the effects of cyclic hydrocarbons in proteoliposomes (275).

Dissipation of the proton motive force indirectly affects other gradients across the cytoplasmic membrane (134); these gradients can be formed by specific antiport systems that use H^+ as the coupling ion (238). As a result of linkage of the Δp to the ΔG_P (via the F_0F_1-ATPases), lipophilic compounds may indirectly influence the pool of adenine nucleotides. Depletion of ATP upon addition of lipophilic compounds has been observed in several instances (151, 158). The observation that lipophilic compounds stimulate the activity of proton- and/or ion-translocating ATPases (107) may originate from the removal of the ion motive force that limits ATP hydrolysis by the corresponding ATPase in intact cells. In addition to dissipation of the proton motive force, loss of the barrier function of the membrane to protons impairs pH homeostasis (33). This results in altered (transport) enzyme activities (237), which may also lead to impairment of cellular viability.

In addition to the effect of the energy status of the cell, the maintenance of cellular homeostasis is affected by the increased membrane leakage (33, 34). The inability to control the intracellular pH may severely impair proper functioning of the cell (237) and may be even more important than the maintenance of a high proton motive force as the driving force for H^+-coupled processes. This is illustrated by observations made by Harold and van Brunt, who showed that fully uncoupled cells of *Streptococcus faecalis* are able to grow, provided the pH of the medium is kept around neutrality, $[K^+]$ is high, $[Na^+]$ is low, and nutrient concentrations are in the millimolar range (119). Recently, Cardoso and Leão (48) reported that the inactivation of cells of *S. cerevisiae* by monocarboxylic acid and ethanol is related to the decrease in intracellular pH. The decrease in intracellular pH was shown to correlate with the accumulation of the compounds in the cytoplasmic membrane. These results emphasize the importance of the control of the intracellular pH in microorganisms, which may be an important parameter that is affected by the disturbance of membrane integrity by cyclic hydrocarbons. Consequently, the pH and (ion) composition of the medium for fermentations in two-liquid-phase systems is essential (273, 350) and may even lead to an increased "apparent" tolerance to lipophilic compounds.

Activity of membrane-bound or -embedded enzymes. Proteins (enzymes) known to be located in the cytoplasmic membrane include ATPases, transport proteins, transferases, various oxidreductases, and signal-transducing enzymes. Many of these enzymes catalyze a vectorial reaction, and their polypeptide chains cross the cytoplasmic membrane several times; other enzymes are located in either the internal or external peripheral regions of the membrane. Large parts of these proteins are bordered by lipid molecules, the so-called boundary lipids or annulus (358). It can be envisaged that accumulation of solutes in the lipid bilayer affects the interactions between the boundary lipids and the protein. Furthermore, interactions of solute molecules with hydrophobic parts of the protein may

occur. Recently, Slater et al. (288) reported that lipid-free protein kinase C is inhibited by alkanols and anesthetics, supporting the protein site model for anesthetic action. However, experiments in the presence of different types of phospholipids indicated that lipids may modulate the effect of lipophilic compounds on the activity of this enzyme (288). It has been known for many years that intrinsic aspects of biological membranes strongly affect the functioning of proteins embedded in that membrane. Parameters such as membrane thickness, head group hydration, fluidity, and fatty acid composition regulate the activity of these enzymes (142, 255, 358). All these parameters are known to be affected by the interaction of lipophilic compounds with membrane lipid bilayers.

The thickness of the membrane is increased by the addition of *n*-alkanes (195, 240), and the effect of anesthetics and drugs on membrane expansion may be related to an increase in membrane thickness (265–267). In't Veld et al. (145) postulated that the activity of transmembrane carrier proteins is strongly affected by the degree of matching between the lipid bilayer and the hydrophobic thickness of the protein and, consequently, that lipophilic compounds will (indirectly) affect these transport processes. Membrane expansion may also affect protein-protein interactions in supramolecular protein complexes such as the electron transport chain, in which electron transfer may be slowed when the protein complexes become dissociated upon excessive accumulation of lipophilic molecules in the cytoplasmic membrane (259).

Hydratation of membrane head groups is difficult to assess, and effects of apolar compounds may not be expected to occur immediately. However, recent data from Shimooka et al. (269) show that the local anesthetic tetracaine interacts with the head groups of the phospholipids, resulting in disturbance of the hydration layer. Results obtained by Yoshida et al. (361) on the effect of the anesthetic halothane showed an enhanced release of water molecules which were bound to the phospholipid head groups. Also, short-chain *n*-alkanols have been shown to affect the hydratation of the lipid bilayer (57), as a result of their preferential binding to the hydrophilic surface of the membrane (248).

Modification of the fluidity or microviscosity of the membrane by the interaction with apolar solutes has frequently been observed (8, 107, 275). Also, under physiologically normal conditions, membranes contain compounds such as cholesterol, hopanoids, and probably carotenoids to maintain optimal fluidity (31). Studies on the effects of membrane fluidity on enzymatic properties show that small changes in fluidity already affect the functioning of various membrane-embedded proteins (107, 362, 366). In contrast to the decrease in electron transfer rates in the electron transport chain as a result of "dilution" of the enzyme components, an increase in membrane fluidity may facilitate an elevated transfer rate of electrons (300).

Changes in the fatty acid composition of cells exposed to lipophilic compounds have been observed. Specific examples of adaptation to lipophilic compounds by changing the lipid composition will be treated in the following section. The requirement for specific types of fatty acids and phospholipids has been observed in several instances. Bolen and Sando (32) reported that the degree of unsaturation of the phospholipid acyl chains has a strong effect on the activity the protein kinase C reconstituted in phospholipid vesicles. Work by In't Veld et al. (144) demonstrated that unsaturation of phospholipid acyl chains has virtually no effect on the activity of the branched-chain amino acid transport system of *Lactococcus lactis* but that it does have a strong effect on the (passive) permeability properties of the membrane. The branched-chain amino acid transport system of *L. lactis*, like many other intrinsic membrane proteins, requires specific phospholipids to retain activity (143). An additional effect of accumulation of lipophilic compounds in the membrane may be the stimulation of redistribution of phospholipids to either side of the bilayer (17, 247, 261).

It can be concluded that interaction of lipophilic compounds with biological membranes results in changes of the membrane structure. These modifications affect the functioning of the membrane, both as a selective barrier and as a matrix for enzymes. The "nonspecific" toxicity effects of lipophilic compounds are likely to be exerted in most cases at the level of lipid-lipid and lipid-protein interactions (329). This hypothesis offers a rationale for observations made by various investigators that the effects of lipophilic compounds on biological systems often correlate with the hydrophobicity of the molecules. The relationship with the hydrophobicity reflects the partitioning of the molecules in the lipid bilayer and is observed for parameters such as membrane permeability (50, 174, 265, 275, 276), enzyme activities (228), membrane swelling (195, 266), antihemolysis (265), luciferase activity (88, 98, 153, 344), and ATP depletion (151).

ADAPTATIONS TO LIPOPHILIC COMPOUNDS

Notwithstanding the clear toxicity of various (cyclic) hydrocarbons to most microorganisms, microbial strains that are able to tolerate high concentrations of such compounds in their environment do exist. A well-known example is the *Pseudomonas* strain that is capable of growing in the presence of 50% toluene (139, 141). The strain, however, is not able to metabolize toluene. Recently, Cruden et al. reported a *Pseudomonas* strain that is able to grow on various aromatic hydrocarbons at concentrations up to 50% (69). Similar results have been obtained in our laboratory with a pseudomonad growing on styrene (341). We also isolated a *Pseudomonas* strain that is able to grow on α-pinene at concentrations up to 90% (276a). The mechanism of this supertolerance is not yet known, although different possibilities such as modification of the outer membrane have been postulated. So far, only strains of the gram-negative genus *Pseudomonas* have been shown to be able to overcome the toxicity of high concentrations of cyclic hydrocarbons (69, 125, 139, 141, 214, 276a, 341). In general, one could say that gram-negative bacteria do tolerate higher concentrations of lipophilic compounds than gram-positive bacteria (120, 140, 331). Below, we discuss the possibilities that may harness microorganisms with an increased tolerance toward hydrocarbons (Fig. 4). Studies in the fields of food microbiology and bacteriology have revealed that microorganisms are extremely inventive in overcoming the threat of chemical agents. Adaptations range from altered cell envelope composition to active transport systems for removal of toxic compounds from the cell (42, 137, 171, 178, 217, 250). In addition to modifications that are directly related to the threat presented by lipophilic compounds, changes in the protein profile of cells treated with solvents have been observed (2, 29). Although many of the proteins induced appeared to be unique for a specific chemical, the reaction per se is a common response to stress in bacteria (1, 215, 217, 223).

Membrane Modification

Cytoplasmic membrane. The plasma membrane of a cell consists of a phospholipid bilayer matrix in which proteins are embedded as indicated above. The membrane bilayer contains lipid-soluble compounds such as cholesterol, hopanoids, or

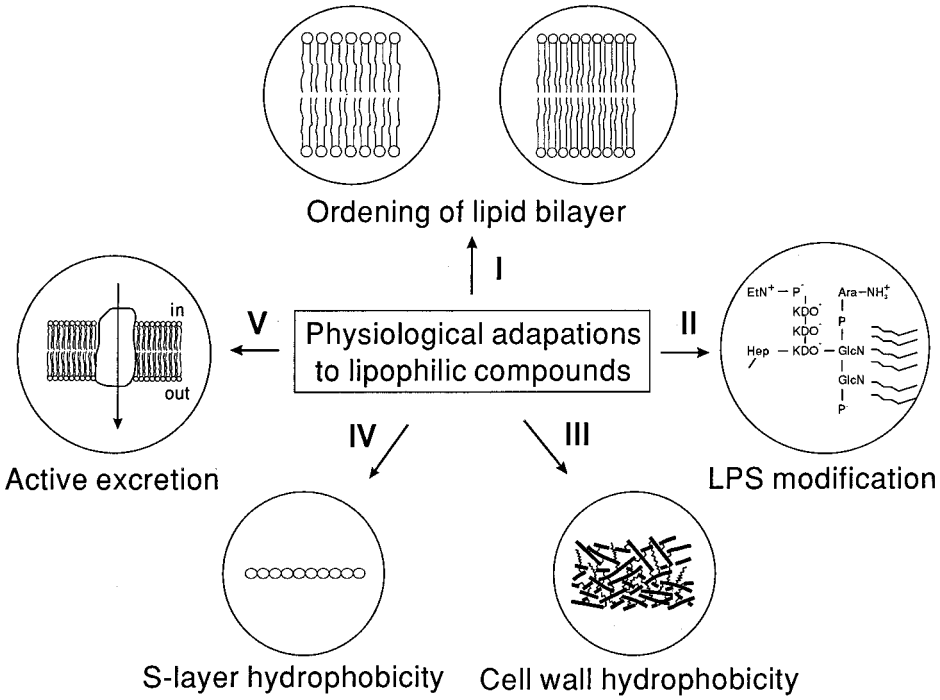

FIG. 4. Schematic presentation of adaptation mechanisms that protect cells against the toxic effects of lipophilic compounds. (I) Increased structuring of the bilayer by changing the fatty acid conformation from *cis* to *trans* or by saturation of the fatty acid acyl chains. (II) Modification of the LPS of the outer membrane (only gram-negative bacteria). (III) Increase in the degree of cross-linking between constituents of the cell wall and modifications of the cell wall hydrophobicity. (IV) Increase in S-layer hydrophilicity. (V) Active excretion by energy-consuming transport systems (e.g., the multidrug resistance system).

carotenoids, which serve as modifiers of membrane fluidity and integrity (3, 30, 169, 244). Although the partitioning of a solute in the membrane is significantly influenced by these lipid-soluble compounds (185), the phospholipid composition of the membrane is the most important determinant of partitioning (4–7). By changing the fatty acid composition of the lipid bilayer from myristoly to stearoyl, the partition coefficient of lindane could be reduced 50-fold (Table 2) (4). Studies with cells of the yeast *S. cerevisiae* showed that strains with an increased tolerance to ethanol were enriched in their content of monounsaturated fatty acids and had a decreased content of saturated fatty acids (21, 202, 203). Similar results have been obtained with *E. coli* (135), *Schizosaccharomyces pombe* (168), and some *Lactobacillus* strains (316, 317). Also the type of phospholipid head group may influence the susceptibility to lipophilic compounds, as was shown for liposomes with different head groups (87) and for strains of *S. cerevisiae* that differed in ethanol tolerance (204). Accordingly, studies on mutants of *E. coli* that are resistant to phenethyl alcohol and ethanol showed that these strains were enriched in the anionic phospholipids phosphatidylglycerol and cardiolipin relative to the phosphatidylethanolamine content (59). In these studies, no changes in other constituents of the cell envelope were observed.

Work by Ingram (136) demonstrated that various lipophilic compounds induce changes in the fatty acid composition of *E. coli* cells. Relatively polar solvents such as acetone, dimethyl sulfoxide, dioxane, and tetrahydrofuran cause an enrichment in unsaturated fatty acids, analogous to changes induced by ethanol (136) or low growth temperatures (190). However, for ethanol this modification is probably not an adaptation mechanism but, rather, a result of the inhibition of enzymes of the biosynthesis of saturated fatty acids (45, 46, 122). Incubation with more-apolar solvents such as benzene, chloroform, amyl acetate, and aniline resulted in an increased synthesis of saturated fatty acids, analogous to adaptation at higher growth temperatures (190). Exposure of the cells to toluene, the most apolar solvent used in this study, had virtually no effect on the fatty acid composition (136). Heipieper et al., who studied the adaptation of a *Pseudomonas* strain to phenol, observed conversion of *cis* fatty acids to their *trans* form (123). The authors suggest that the *cis*-to-*trans* conversion increases membrane ordering and consequently decreases the membrane fluidity (Fig. 5), which is in accordance with physicochemical studies on the behavior of *trans* fatty acids (225). Thus, a decrease in

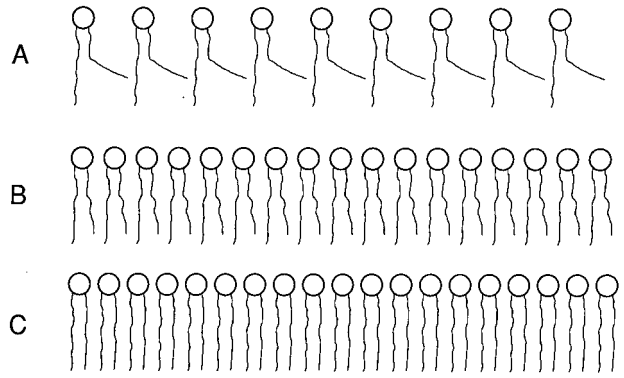

FIG. 5. Different conformations of phospholipids below the beginning of lipid phase transition and their effect on the structuring of the lipid bilayer (38, 68). (A) *cis*-unsaturated fatty acids; (B) *trans*-unsaturated fatty acids; (C) saturated fatty acids.

the ordering of the phospholipid molecules caused by phenol is balanced by changing the configuration of the fatty acids from *cis* to *trans*. Data obtained in our laboratory on a *Pseudomonas* strain tolerant to saturating concentrations of toluene and styrene also revealed that *cis* fatty acids are converted to their *trans* form (340). The alterations in fatty acid composition induced by alcohols and organic solvents resemble modifications as a result of temperature changes (136, 190, 305). Also, observations of cross-effects of solvent-induced heat tolerance and vice versa have been reported (179). The *cis-trans* isomerization has also been reported for bacteria deprived of nutrients, indicating that the *trans*/*cis* ratio may be applicable as a general index of starvation or stress of microorganisms (110).

Although the observations differ significantly depending on the organism studied or the solvent applied, some general features can be deduced. The polar solvents (ethanol, acetone, dimethyl sulfoxide, etc.), which are miscible with water, cause an increase in the content of unsaturated fatty acids (3, 21, 45, 83). The increase in the content of unsaturated fatty acids results in an increased fluidity of the membrane (38, 67), as was indeed shown for artificial membranes prepared from lipid extracts of ethanol-grown cells of *E. coli* (83). The increase in membrane fluidity may result in a lower permeability for these polar solvents, as can be concluded from observations on the reduced permeability of membranes for small molecules at elevated temperatures (216). However, possibly as a result of a decreased lipid-to-protein ratio, Dombek and Ingram (83) observed a lowered membrane fluidity in *E. coli* cells grown in the presence of ethanol. Although the increase in the unsaturated fatty acid content possibly results from the inhibition of the soluble enzymes of saturated fatty acids (45, 46, 122), it also opposes the partitioning of ethanol to the membrane (310). Observations made by Thomas and Rose (310) on the effect of ethanol on cells of *S. cerevisiae* grown in the presence of either oleic acid or linoleic acid showed that the amount of cell-bound ethanol was slightly but consistently smaller in cells grown in linoleic acid-containing medium. Cells cultivated in the presence of linoleic acid also were more tolerant to ethanol than were cells grown in the presence of oleic acid (310), whereas the increase in the content of rigidifying proteins observed by Dombek and Ingram (83) compensated for the fluidizing effects of ethanol and the increased content of unsaturated fatty acids. More-lipophilic solvents that still are soluble in water (benzene, aniline, amyl acetate, etc.) but not completely miscible induce an enrichment of saturated fatty acids and an increase in the total phospholipid concentration. An explanation for this reaction might be that the saturated fatty acids show a higher degree of membrane ordering, which also allows a higher surface density (30). These effects are known to oppose the partitioning of lipophilic solutes to a lipid bilayer (150, 189). Especially for compounds that are rather polar but not water miscible, this adaptation may be advantageous. For more-apolar compounds, the membrane partition coefficients are such (275) that mechanical exclusion does not suffice. To withstand these compounds, cells require additional adaptation mechanisms.

Outer membrane. Gram-negative bacteria appear to be less sensitive to lipophilic compounds than are gram-positive organisms (120, 140, 331). This agrees with the observations that only strains of the gram-negative genus *Pseudomonas* are able to tolerate high concentrations of normally deleterious lipophilic compounds. Our recent studies have indicated that the higher tolerance for lipophilic compounds is related to the resistance of the outer membrane for these molecules. No differences between gram-negative and gram-positive bacteria were observed with regard to the critical concentrations of molecules dissolved in the cytoplasmic membrane (331). Modification of the outer membrane of gram-negative bacteria is often related to the LPS composition of this membrane. As mentioned above, the outer membrane is a highly porous shield that allows small (hydrophilic) solutes to pass via pores (219). However, it has a surprisingly high transfer resistance to hydrophobic compounds (219). This transfer resistance has been shown to be a result of the highly hydrophilic LPS. Mutants that lack certain moieties of the LPS molecules appear to be sensitive to a variety of lipophilic compounds (for review, see references 220 and 221). It can be envisaged accordingly that some strains of gram-negative bacteria are able to further increase the transfer resistance toward lipophilic compounds via either an induction mechanism or a mutagenic event (218).

Cell Wall

Altering the cell wall may also lead to changes in sensitivity to lipophilic compounds. Most of the work has been done in the area of the formation of compounds that increase the rate of transfer of substrates with a low aqueous solubility. These bioemulsifiers are usually modified polysaccharides. However, with respect to the adaptation to toxic effects of lipophilic compounds, these compounds are not relevant. Changes in the hydrophobicity of the cell wall may be of more interest. It has been shown that bacteria with hydrophobic cell walls have a higher affinity for hydrophobic compounds than do bacteria with more-hydrophilic cell walls (150, 327). This implies that modification of cell walls (hydrophobic to hydrophilic) could provide a means of protecting the organism with a shield against these compounds. Park et al. demonstrated that *S. cerevisiae* adapted to the presence of toxic solvents (tributylphosphate and 2-*tert*-butylphenol) by decreasing the hydrophobicity of the cell wall (231).

S-Layer

The function of the S-layer in protection of the cell is still largely unknown (290). Present data indicate that the S-layers serve primarily as molecular sieves. *Bacillus* strains possessing an S-layer showed resistance to lysozyme, suggesting an exclusion limit down to a diameter of 3.5 nm (25, 289). Also, a function as an ion-exchange resin has been demonstrated (290). To our knowledge, no data exist on the exclusion of small hydrophobic molecules by S-layers.

Active Excretion

The concept of multidrug resistance has been known in medicine for quite some time. The discovery that the multidrug resistance phenotype is due to an active (ATP-driven) excretion system (P-glycoprotein or Mdr1) has increased the interest in active transport systems for the removal of toxic compounds from the cell (167, 239). Multidrug resistance-like excretion systems have also been shown to be operative in bacteria (58, 178, 198, 207, 308). For instance, efflux of daunorubicin and doxorubicin by *Streptomyces peucetius* (111), of nalidixic acid and others by *E. coli* (184), of 2′,7′-bis-(2-carboxyethyl)-5(and -6)-carboxyfluorescein by *Lactococcus lactis* (207), and of various P-glycoprotein substrates such as ethidium bromide, daunomycin, chloroquine, rhodamine 6G, gramicidin, and nigericin by *L. lactis* and other bacteria (219) has been shown. These excretion systems are similar in that a variety of structurally unrelated compounds are excreted from the cells. Some of these systems are ATP driven, whereas others exchange the molecules at the expense of one or more protons (cations) (239). Recently, the active excretion of the

cyclic hydrocarbon benzo[*a*]pyrene by P-glycoprotein from human breast cancer MCF-7 cells has been demonstrated (359). Whether similar systems play a role in protecting microbial cells from high concentrations of, for instance, (cyclic) hydrocarbons remains to be established. We believe that there is no precedent why active excretion systems should not play a role in lowering the concentrations in the cytoplasmic membrane (and cytoplasm) of toxic lipophilic molecules like those discussed in this review. It should be noted that active transport systems for the uptake of lipophilic molecules like benzoic acid in *P. putida* (309), 4-chlorobenzoic acid in the coryneform bacterium NTB-1 (109), and 4-toluene sulfonate in *Comamonas testosteroni* T-2 (183) have been demonstrated.

Immobilization

In natural systems, 99% of all microorganisms are growing at surfaces (327). By attaching to surfaces, microorganisms may benefit from the adsorption of hydrophobic molecules to the surfaces, resulting in a lower aqueous concentration. The presence of materials that serve as an adsorption matrix (montmorillonite, activated carbon) has been shown to reduce the toxic effects of benzylamines (303) and phenols (86, 210). Also, for cells entrapped in various polymer matrices, reduction of toxic effects of, e.g., phenol has been observed (24, 124).

CONCLUDING REMARKS

The accumulation of lipophilic compounds in the (cytoplasmic) membrane of microorganisms has considerable effects on the structural and functional properties of these membranes. The numerous observations of toxic effects of terpenes, aromatics, cycloalkanes, alkanes, alcohols, and phenols can be explained largely by the interactions of these compounds with the membrane and with membrane constituents. As a result of accumulated lipophilic molecules, the membrane loses its integrity, and an increase in permeability to protons and ions can be observed. In addition to the long-standing theory that lipophilic compounds disturb exclusively the lipid part of the membrane, it has been found that proteins embedded in the membrane are also affected. It remains to be clarified whether effects on membrane-embedded enzymes result from an altered hydrophobic environment around the membrane-embedded parts of these proteins or from direct interaction of the lipophilic compounds with hydrophobic parts of the proteins.

Work on the interactions of lipophilic compounds with phospholipid membranes has not been restricted to microbiological research but is also being performed in other disciplines, ranging from detailed molecular studies of modified acyl-acyl interactions (physical chemistry), which provide a thermodynamic basis for predicting the membrane-directed action of anesthetics and drugs (anesthesiology and pharmacology), to studies at the organism level on effects of chemical pollutants on insects and fish (ecotoxicology). The aim of such studies is the development of quantitative structure-activity relationships, which can be helpful in designing novel drugs and anesthetics and can be used to define upper limits for concentrations of pollutants. Studies on the toxic effects of lipophilic compounds in microorganisms provide information that will be useful in bridging the gap between mechanisms evaluated at the molecular level and observations at the organism level. Furthermore, knowledge of the mechanism of the inhibitory action of lipophilic compounds is helpful in technical applications of microorganisms (fermentation, environmental biotechnology) and in selection of novel antibiotics and food preservatives.

Recently, a number of reports on microorganisms that are able to tolerate high concentrations of lipophilic compounds such as toluene, xylene, styrene, and phenol have been published (125). Similar reports on the adaptation of *S. cerevisiae* to increased concentrations of ethanol have appeared over a longer period (138). Modifications observed include changes in the lipid composition of the cytoplasmic membrane, modified lipid-to-protein ratios, increased levels of radical scavenging enzymes such as catalase and superoxide dismutase, modified composition of the LPS in the outer membrane of gram-negative bacteria, and altered activities of enzymes involved in energy transduction. However, it remains to be seen whether the modifications in membrane composition and other changes demonstrated in solvent-tolerant cells really lead to an increased resistance or are just part of a general adaptive response. Also, possible secondary effects, such as changes in activities of membrane-embedded enzymes as a result of modified membrane lipid compositions and altered membrane fluidity, should be taken into account. In addition to the compilation of modifications that represent an adaptive response, physiological parameters that trigger this response have to be characterized. Similarities with the heat shock response should also be considered.

The proposed mechanism offers a rationale for toxicity problems encountered by microorganisms, which will be helpful in designing proper incubation conditions. For the isolation of novel strains that can metabolize lipophilic compounds, empirically derived relations between hydrophobicity and substrate toxicity can be used to optimize enrichment conditions. Although such relations can be helpful in estimating order-of-magnitude values, no exact values can be derived, since the distribution of the lipophilic compound can differ significantly depending on membrane composition, compound characteristics, biomass concentration, and presence of other materials that bind lipophilic compounds. In technical applications of microorganisms in the presence of lipophilic compounds, the accumulation of such molecules can be prevented by either selecting resistant strains or changing the conditions (273, 277).

In 1970, Harold (118) concluded his review on antimicrobial agents and membrane function with the following remark: "... antimicrobial agents which act upon membranes offer an amusing instance of compartmentation in science." Unfortunately, this statement is still valid in 1995. Data on the mechanism of the toxic action of lipophilic compounds are available from various scientific disciplines, but as a result of the fragmentation, a great deal of research is performed without taking into account the results of similar studies in other fields. We hope that this overview will provide information for further research within different disciplines.

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