Microbial transformation of tetralin

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Microbial transformation of tetralin

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Foar Anne

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STELLINGEN

1. De remmende werking van cyclische koolwaterstoffen op de groei van bacteriën is met name het gevolg van ophoping van deze verbindingen in het cytoplasmamembraan.

   Dit proefschrift.

2. In het door Sahm *et al.* beschreven zuiveringsproces voor het afvalwater van pulpfabrieken wordt de remming door H_2S ten onrechte buiten beschouwing gelaten.

   Sahm, H., U. Ney, and S. M. Schoberth. 1992. Anaerobic treatment of wastewater from the pulp and paper industry, p. 431-434. *In* M. R. Ladisch, and A. Bose (ed.), Harnessing biotechnology for the 21st century, American Chemical Society, Washington, D.C.

3. Bij het kweken van *Pseudomonas cepacia* ATCC 29351 in hoge celdichtheden in het door White-Stevens en Kamin beschreven medium in combinatie met salpeterzuurtitratie, wordt geen rekening gehouden met het vermogen van deze bacterie uit nitraat het giftige nitriet op te hopen.

   Berg, A.-C., O. Holst, and B. Mattiasson. 1989. Continuous culture with complete cell recycle: cultivation of *Pseudomonas cepacia* ATCC 29351 on salicylate for production of salicylate hydroxylase. Appl. Microbiol. Biotechnol. **30**:1-4.

4. De door Schreiber en Winkler beschreven afbraak van tetralien door een reinkulture van *Pseudomonas stutzeri* AS 39 is een coöxidatief proces.

   Schreiber, A., and U. K. Winkler. 1983. Transformation of tetralin by whole cells of *Pseudomonas stutzeri* AS 39. Eur. J. Appl. Microbiol. Biotechnol. **18**:6-10.

5. Rapportages over de suikersamenstelling van polysachariden zonder duidelijke beschrijving van de gebruikte isolatie- en zuiveringsmethoden kunnen naar het land der fabelen worden verwezen.

6. De omschakeling van de wapenindustrie op civiele produktie wordt bemoeilijkt door de geringe vraag naar ploegscharen.

7. Koppeling van de zuiveringsheffing aan het waterverbruik zal ervoor zorgen dat men pas echt wijs met water wordt.

8. Ten behoeve van de herkenbaarheid van verkeerssituaties verdient het aanbeveling voor de signalering van wegwerkzaamheden een meer werkelijkheidsgetrouwe afbeelding te gebruiken.

9. Het gebruik van het bijvoeglijk naamwoord 'duurzame' bij begrippen zoals landbouw en samenleving betekent dat er meer verandering gewenst wordt dan dit woord doet vermoeden.

10. De hoogte van de adviesprijzen voor laboratoriumapparatuur doet vermoeden dat de leveranciers uitgaan van het motto: "wat een gek ervoor geeft".

Stellingen behorende bij het proefschrift
"Microbial transformation of tetralin"
van Jan Sikkema.

Wageningen, 24 mei 1993

CONTENTS

GENERAL INTRODUCTION

Since the middle of the 19th century many large scale processes for the production of commodity chemicals have been established. The procedures for the production of these commodities have been developed and optimized to highly efficient processes, which are usually performed in specially designed plants. Synthesis of fine chemicals, usually complex molecules, is often performed in laborious multi-step processes. As a result of the small product volumes, fine chemicals are manufactured in multi-purpose plants which do not provide optimal conditions for all of the catalytic steps involved. Classical chemical synthetic processes often lack specificity and selectivity and have to be performed under extreme conditions, thus hindering the efficient application in direct synthesis of fine chemicals. Furthermore, chemical synthetic processes for the production of both commodity and fine chemicals cause serious environmental problems as a result of reagents and solvents necessary for these reactions. Therefore, present developments in organic synthesis are mainly directed at increasing the specificity and selectivity of the synthetic processes (5). This progress is stimulated by the demand for more specific drugs (21) and agrochemicals (22), and the growing awareness of environmental pollution (28).

In addition to chemical synthesis, microorganisms provide highly specific catalysts, which have evolved over many millions of years to perform an enormous variety of chemical reactions (10, 25, 26). Enzymatic reactions are often highly chemo-, regio- and stereoselective, have a high efficiency, and can be performed in an aqueous reaction medium under mild conditions (21). Moreover, in nature an enormous

diversity of biocatalysts, which are involved in a broad spectrum of chemical reactions, is already available (9). As a result of recent developments in molecular biology and protein engineering, the performance of enzymes can be improved and even 'new' enzymes can be made. In addition to the selectivity and specificity, in some instances also the natural origin of biocatalysts will be advantageous. Application of biotransformation reactions for the production of flavorings may help to meet the growing demand for natural products as food additives (7).

Biotechnological production of fine chemicals. Biotechnological methods for the production of fine chemicals comprise either complete biosynthesis or biotransformation. Microbial fermentation processes employing complete biosynthetic routes are widely applied for the production of naturally occurring compounds, e.g., amino acids (1), penicillins (4). Biotransformation processes are usually restricted to a single reaction or a short sequence of reactions in order to transform defined pure compounds into defined final products.

Complete biosynthesis can not be applied for the production of non-natural, synthetic chemicals. Application of enzymes to catalyze transformations of such chemicals, however, is a useful tool to perform specific organic syntheses. The essence of biotransformations can, therefore, be best described as 'chemical reactions by microorganisms or enzymes' (14).

Growing cells, resting cells, permeabilized cells, crude cell extracts, or purified enzymes can be applied as biocatalysts (15). Basically, all types of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases) can be used as biocatalysts (10). Once a suitable biocatalyst has been identified in terms of catalyzing the desired reaction, several other aspects have to be taken into consideration to assess its applicability. This commercial feasibility has to be related to existing chemical and/or biotechnological processes. The present limited application of biocatalysts is to a large extent due to the availability of efficient chemical methods (21). Moreover, biocatalysis has intrinsic drawbacks such as low stability, low tolerance to substrates and/or products, and low aqueous solubility of substrates (21, 24, 32). Furthermore, high investments in new equipment and unfamiliarity with biological processes may also restrain chemical companies from the application of biocatalytic processes (24). Therefore, biocatalysis most likely will be successful in production of high-value fine and specialty chemicals, whereas for the production of commodity chemicals chemical syntheses are likely to prevail (21, 24).

Specific oxidation of non-activated carbon atoms. Direct, specific hydroxylation

is chemically difficult to accomplish, since chemical oxidation processes are unspecific, and are performed under harsh reaction conditions (17). Hydroxylating enzymes, however, are highly specific and thus prevent the formation of undesired by-products (12). Different oxidative reactions, such as hydroxylation of aromatics (11, 24, 31, 33), epoxidation of alkenes (35), and Baeyer-Villiger oxidation of cycloalkanones (2) are known to be catalyzed by highly specific enzymes. And as a result of mild reaction conditions also less stable substrates can be used, whereas easily (auto)oxidizable products (e.g., catechols) are not further oxidized. Despite the potentials of biological hydroxylation, biocatalytic processes are only applied in a few instances, e.g., commercial production of sterols (27), and small scale formation of *cis*-dihydro diols (24). Both the production of sterols and the formation of *cis*-dihydro diols cannot be performed by direct chemical hydroxylation. Alternative chemical synthetic processes are extremely laborious and have very low yields (15, 24, 27). The application of biocatalysis for these processes in particular seems to stem from the lack of suitable chemical methods (21). Present chemical oxidation processes in general are still aspecific and inefficient, which severely hampers the economical feasibility of such methods. Therefore, application of biocatalytic hydroxylation systems may very well be able to compete directly with chemical methods. In addition to the bottlenecks observed for the application of biocatalysis in general, biological hydroxylations also suffer from the requirement of cofactors (NADH or NADPH) (19). Since these cofactors are expensive and, consequently, have to be regenerated, biocatalytic hydroxylations have to be performed with intact cells (37). However, inhibitory effects of cyclic hydrocarbons on microbial cells, as observed for benzene (34), and toluene (13) may severely impair the biocatalytic capacity of the hydroxylating bacteria applied. So far, the cause of the inhibitory action of cyclic hydrocarbons has not received far reaching attention and, consequently, the mechanism of the toxicity is poorly understood.

Oxy-functionalization of tetralin. To evaluate the potentials of biocatalytic oxidation for the oxy-functionalization of cyclic hydrocarbons we have studied some basic aspects of the microbial oxidation of tetralin (1,2,3,4-tetrahydronaphthalene). Oxidized derivatives of tetralin have potential applications as precursors for pharmaceuticals (3, 6, 8, 20, 30, 36) and flavorings (16). Chemical methods for the oxidation of tetralin are very unspecific as a result of auto-oxidation of the activated benzylic carbon atom (18, 29, 38). For our studies we selected the formation of 5,6,7,8-tetrahydro-1-naphthol from tetralin as a model reaction. 5,6,7,8-Tetrahydro-1-naphthol is a fragrance compound with a leather-like smell that can be applied in

e.g., shoe polish. Direct chemical oxidation to produce 5,6,7,8-tetrahydro-1-naphthol is not feasible, therefore, the compound is formed via hydrogenation of α-naphthol (23).

Outline of this thesis

The main aspects that have been addressed in this thesis are the selection and characterization of suitable biocatalysts, and the cause of the toxicity of these substrates. For the application of intact microbial cells as biocatalysts pure strains, that are capable of catalyzing the specific oxygenation reaction(s), have to be obtained. Isolation and cultivation of microorganisms on cyclic hydrocarbons is often hampered by the inhibitory action of these substrates. In Chapter 2 an overview is given of various reports on the toxicity of cyclic hydrocarbons to microbial cells. These examples have been discussed in relation to data on the mechanism of the interaction of lipophilic compounds with biological membranes. The procedure for the selection of tetralin-utilizing microorganisms is presented in Chapter 3. Evaluation of the biocatalytic potential of the selected tetralin-utilizing bacteria indicated that only one strain, *Corynebacterium* sp. strain C125, specifically hydroxylates the aromatic moiety, which may lead to the formation of the desired phenol (Chapter 4). Further investigation of the metabolic pathway of tetralin employed in this strain confirmed that the first intermediate resulted from dioxygenation of the aromatic nucleus (Chapter 5). In Chapter 6 the mechanism of the inhibitory action of tetralin was investigated in liposomes as well as in intact cells. The *Escherichia coli* phospholipid liposomes were also used as a model system to study the action of other cyclic hydrocarbons (Chapter 7). Finally, in Chapter 8 the implications of the membrane interaction of cyclic hydrocarbons have been discussed in relation to the application of microorganisms for the transformation of such compounds.

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Chapter 2

MEMBRANE TOXICITY OF CYCLIC HYDROCARBONS

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Submitted for Publication

INTRODUCTION

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

Terpenes are plant-based organic compounds that consist of isoprene units. In plants they are mainly present 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 due to human activities, e.g., transport and application of mineral oils and products derived thereof. Aromatics are especially abundant due to applications such as fuels, industrial solvents (benzene, toluene), polymer synthesis (styrene), and starting materials for chemical syntheses. A less abundant, though biologically more persistent group of compounds are the cycloalkanes. In particular cyclohexane is becoming increasingly important as an industrial solvent replacing benzene which is carcinogenic.

Many cyclic hydrocarbons are known to be transformed by microorganisms, which may lead to complete mineralization of these compounds. The metabolic pathways via which these compounds are degraded have been elucidated for a great number of compounds in various microorganisms (66, 91, 247, 248). More recently, the genetics of the metabolic routes has received considerable attention (8, 101, 305). During studies on the transformation of cyclic hydrocarbons by microorganisms, many researchers observed that these compounds were toxic to the cells (89, 128, 227, 229). 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. While making a survey of the literature pertaining to the toxic action of cyclic hydrocarbons to microorganisms one finds an enormous amount of reports 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, and molecular volume (152). Biological data on the toxic effects of other lipophilic compounds on microorganisms may be helpful in postulating a mechanism for the toxic action of cyclic hydrocarbons. An excellent review by Harold (102) compiled a wealth of information on various antimicrobial agents that affect membrane functions. A large variety of antibacterial agents were reviewed by Hugo, independent of their primary site of action (112).

In this review we present an overview of the data on the toxicity of cyclic hydrocarbons to microorganisms. Both microorganisms that are able to transform such compounds and microorganisms that do not have the transforming capability will be 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 hand. Microorganisms do exist that withstand extremely high concentrations of cyclic hydrocarbons. These strains will be 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 such compounds with cells and cell constituents will be discussed.

BIOAVAILABILITY OF CYCLIC HYDROCARBONS

Only molecules of cyclic hydrocarbons that are dissolved in water are available for microbial metabolism (298, 299). Furthermore, the involvement of intracellular cyclic hydrocarbon metabolizing enzymes implies that the substrates need to 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 all poorly soluble in water (74, 154, 166). Benzene, the most polar cyclic hydrocarbon is soluble up to 22 mM at 25°C (74). The compounds with a higher molecular weight and/or a higher degree of saturation have a lower solubility. For many of these compounds it is often reported that they are insoluble in water (154), although traces of the hydrocarbon will always dissolve. Nevertheless, it will occur that for biotransformation reactions the availability of such a compound can become limiting (171). Work by Wodzinski and coworkers in the early 1970s showed that microorganisms utilize only those hydrocarbon molecules that are dissolved in the aqueous-phase (298, 299). 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 (251). Recently Volkering and colleagues 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 (281). Thomas et al. (266) showed that a close correlation exists between the dissolution rate, as a function of the solute surface area, and the degradation rate of insoluble compounds such as naphthalene. Also for biphenyl, anthracene, and phenanthrene the dependency of the growth rate of bacteria on the solubilization rate has also been reported (258). Due to their high molecular weight, hydrophobicity and solid state, polycyclic aromatic hydrocarbons are particularly known to adsorb to surfaces (167, 169, 178). Not only microorganisms that live in the suspended state, but also those that are attached to surfaces utilize molecules that are dissolved (158, 185). Therefore, the rate of dissolution of a compound is a critical measure in the bioavailability of such a compound in all environments. Not only for growth, but also for toxic effects the dissolution rate is very critical since the dissolution rate governs the transfer of a compound to the microorganism.

Dissolution of molecules from a distinct organic phase can be described by Fick's first law of diffusion (51):

$$J = \frac{n}{At} = -D \times \left(\frac{\delta c}{\delta x}\right)_t \qquad (mol \cdot m^{-2} \cdot s^{-1})$$

The flux J is the number of moles of solute n that has diffused across the boundary (area A) in a time t. D (m^2 s^{-1}) is the diffusion coeficient of the diffusing substance, and the quantity $(\delta c/\delta x)_t$ is the concentration gradient of the diffusing substance after time t. This equation indicates that the rate of transfer of a compound is dependent upon 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.

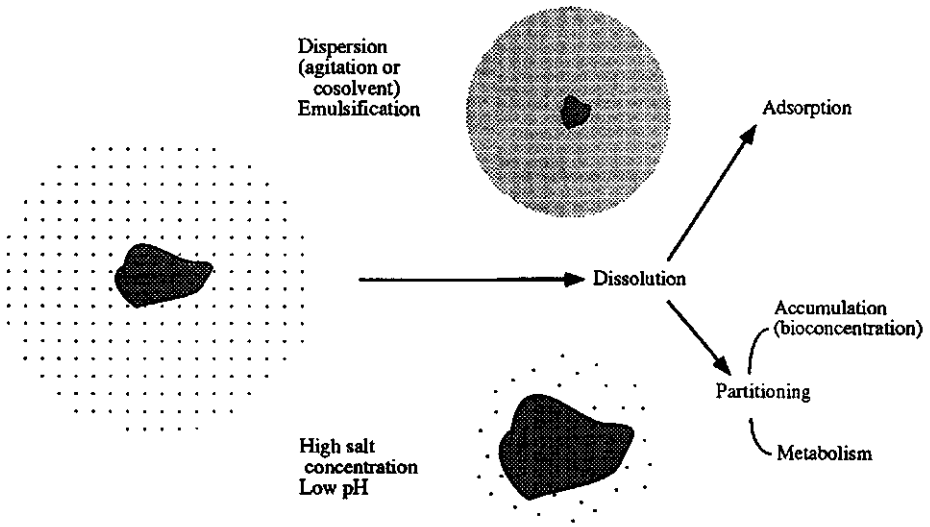

FIG. 1. Dissolution and fate of solid hydrocarbons as affected by some environmental parameters. Increase of dissolution rate as a result of an increase in surafce area of the hydrocarbon bulk, which may result from: i) mechanical dispersion (e.g., stirring, agitation, wave movement), ii) chemical dispersion (cosolvents), or iii) emulsification (surface active compounds). In environments with high salt concentrations or extreme pH values, the solubility may be significantly lowered (280). Dissolved molecules may i) partition to biological systems, resulting in either transformation or in accumulation (bioconcentration), ii) adsorb to surfaces, or iii) remain in the solid/dissolved equilibrium.

Therefore, Volkering and coworkers (281) could decrease mass transfer limitation by selecting smaller naphthalene particles. An alternative method to decrease or even prevent mass transfer limitation is the addition of a cosolvent (Fig. 1). A cosolvent enables the increase of the substrate surface area by dispersion of the particles. Ideally, the cosolvent is both mixable with the aqueous phase and the substrate. Cosolvents that are often used in biochemical studies are amongst others DMF, DMSO, acetone, ethanol, and benzene.

Data obtained by Thomas and coworkers (266) indicated that dissolution rates of solid hydrocarbons were higher in the presence of bacteria as compared to sterile controls. Also for microorganisms growing on long-chain alkanes increase of dissolution rates have been observed, in most instances as a result of surface active compounds produced by the hydrocarbon degrading microorganisms (83, 309). These compounds, usually large amphiphilic molecules, e.g., lipopolysaccharides, are able to form and/or stabilize hydrocarbon emulsions in aqueous systems (111, 308). 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 lipopolysaccharides in the dissolution of hydrocarbons by Gram-negative bacteria may facilitate attachment of the cell to hydrocarbon droplets (297). In technological applications similar methods are applied, resulting in better accessibility of low water soluble polycyclic aromatic hydrocarbons (78, 111).

As a consequence of mass transfer limitation, the amount of hydrocarbon available may not only be limiting for growth but also toxic effects may be minimal. Especially, when microorganisms are studied that can metabolize these compounds, the rate of metabolism may exceed the rate of mass transfer from the environment to the cell. This not only leads to limitation of the growth rate (281), but also to the absence of toxic effects. When compounds with a low dissolution rate, e.g., naphthalene, biphenyl, phenanthrene, are allowed to equilibrate (44) or are added with a cosolvent (230, 231, 249, 275, 276) toxic effects will be observed. Also other physical parameters such as temperature, pH, and salt concentration have a significant effect on dissolution and solubility (280). However, interpretation of these data is difficult since these parameters may also affect other physiological processes.

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 archaebacteria are surrounded by a crystalline surface layer (S-layer) which then forms the outermost component of the cell envelope (245). The cell envelope can differ significantly from one organism to another, and even for one strain it can change as a result of physiological adaptation to the environment.

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, and in addition they posses a thick and rigid cell wall (Fig. 2). Gram-negative bacteria have in addition to the cytoplasmic membrane an outer membrane, that consists of phospholipids and lipopolysaccharides (LPS; 184) (Fig. 2). Between the outer membrane and the cytoplasmic membrane a thin peptidoglycan layer is present. The outer membrane functions as a molecular sieve through which molecules with a molecular mass > 600-1000 daltons can not penetrate. Despite the presence of porins with low specificity, the outer membrane shows a very low permeability towards hydrophobic compounds which has been ascribed to the presence of the lipophilic LPS (183). However, recently it has been demonstrated that highly lipophilic compounds such as steroids penetrate relatively easily through the outer membrane of several bacteria (197).

The cell wall consists of a variety of sugar polymers, the most common group of polymers 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 in order to attach to surfaces (158). 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 (244). Furthermore, a role in the protection of cells against antimicrobial agents can be envisaged.

The cytoplasmic membrane of cells consists of a phospholipid bilayer (95) and forms a matrix in which enzymes and transport proteins are embedded (62, 239). The carrier (transport protein) molecules allow the selective uptake and excretion of solutes (200). In addition to its role in solute transport, the cytoplasmic membrane plays a crucial role in the maintenance of the energy status of the cell (107), the regulation of the intracellular environment (33), the

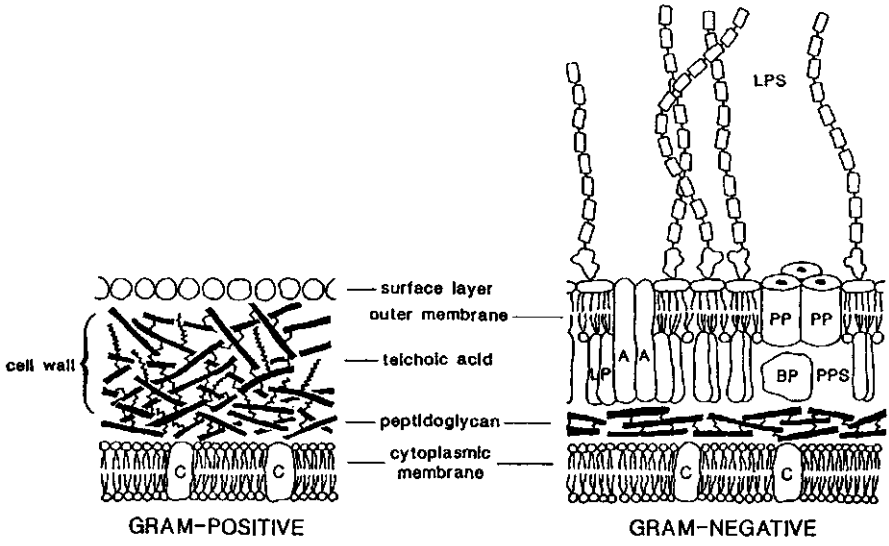

FIG. 2. Schematic presentation of the cell envelope of Gram-positive and Gram-negative bacteria. PP, porine; C, cytoplasmic membrane embedded protein (e.g., carrier); BP, binding protein; PPS, periplasmic space; A, outer membrane protein; LP, lipoprotein; LPS, lipopolysaccharide.

turgor pressure (110), signal transduction (253), and other energy transducing processes. Under physiological conditions the enzyme-containing bilayer can be best described as a liquid crystal (238, 239). Although the lipid molecules only constitute a part of the total membrane mass, it forms the matrix in which the other components are embedded. The physical properties of the cytoplasmic membrane thus not only reflect the lipid bilayer but also influence the structure and functioning of the other components (29). 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 (29, 237). This aspect will be discussed below in more detail when the effects of lipophilic compounds on biological membranes are treated.

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. The permeability of the membrane is dependent upon the hydrophobicity of the solutes that have to cross the membrane (55, 73, 155). In addition, Lieb and Stein (155) demonstrated that also the size of solute molecules plays a role in the permeability. Bateman et al.

(16) who studied the uptake of naphthalene by a *Pseudomonas* species, showed that neither ATP nor an electrical potential were 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 preceeds the metabolism of these compounds. Although the uptake of hydrocarbons could essentially be a passive transport process, different adaptations have evolved in order to increase the uptake rates. For the uptake of alkanes, Witholt et al. (297) postulated that lipopolysaccharides related to outer membrane lipopolysaccharides are released and encapsulate hydrocarbon droplets, thus increasing the efficiency of mass transfer (297). Studies of bacteria and yeasts, mainly growing on aliphatic hydrocarbons, show that these cells contain inclusions of unmodified hydrocarbon substrate (218). This has also been observed for a *Pseudomonas* strain growing on naphthalene (218). 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 (219, 220). These observations show 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 its availability to the cell, but may also cause toxicity problems (230, 231). The partitioning of hydrocarbons has been studied by some groups in membrane-buffer systems (3-6, 69, 70, 71, 160, 230, 231). 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 obtained partition coefficients were used to predict effects of the compounds on intact cells: e.g., bioconcentration (52), biodegradation, toxicity (189), anesthesia (221). Although these methods, especially the octanol-water partition coefficient (151), showed good correlations with biological effects (221, 147, 189, 231), quantitative estimations were impossible as a result of differences in membrane composition. As shown by the results of Antunes-Madeira and

TABLE 1. Partition coefficients of lipophilic compounds between membrane and aqueous phases, depending on the composition of the membrane. DMPC: dimyristoylphosphatidylcholine, DPPC: dipalmitoylphosphatidylcholine, and DSPC: distearoylphosphatidylcholine. Data were taken from Antunes-Madeira and Madeira (3-6).

	DMPC	DPPC	DSPC	references
Malathion	225	135	48	6
Parathion	1,950	650	270	3
Lindane	2,450	600	50	4
DDT	336,000	180,000	88,000	5

Madeira (3-6), the specific lipid composition of a lipid bilayer can strongly influence the partitioning behaviour of a compound (Table 1), and, consequently, its biological effect.

De Young and Dill studied the partitioning of benzene and hexane in dilauroylphospatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), and dipalmitoylphosphatidylcholine (DPPC) membrane vesicles as a function of the surface density (69, 70). Their results showed that partition coefficients of these compounds between the membrane and the aqueous phases not only depended on simple partioning behaviour as observed for oil/aqueous systems,

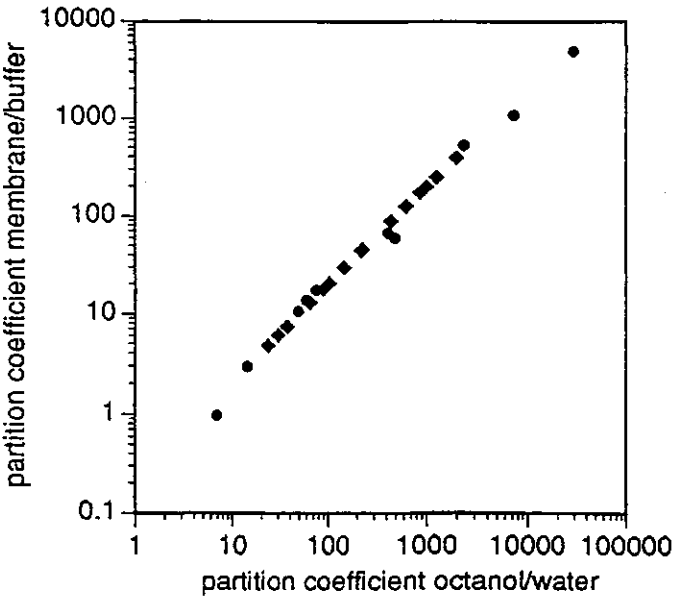

FIG. 3. Correlation between partition coefficients of lipophilic compounds in membrane/buffer systems and the partition coefficients in a standard octanol/water system. (●) Data obtained for cyclic hydrocarbons in *E. coli* phospholipid liposomes (231). (♦) Data obtained for substituted phenols in erythrocyte membranes (160).

but also on ordering constraints of the lipid bilayer. Different methods to alter the surface density were applied, with no significant differences in the outcome of 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 (163). The high degree of ordering of solutes in a lipid bilayer as compared to a bulk liquid phase also changes the thermodynamics of the partitioning significantly (235). Nevertheless, a good correlation between the partition coefficient of various lipophilic compounds in membrane/buffer and octanol/water two-phase systems has been observed (160, 231) (Fig. 3).

MEMBRANE TOXICITY OF LIPOPHILIC COMPOUNDS

Cyclic Hydrocarbons

As long ago as 1921 Jentsch reported that cyclic hydrocarbons impaired the growth of bacteria and fungi (129). Cyclic hydrocarbons, e.g., turpentine-constituents and also tetralin, were consequently applied in cleansers as antibacterial agents (204). Baier (10) 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 (285), which indicates that volatile hydrocarbons (e.g., benzene, 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 (283). 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 (59, 90). 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 on the inhibitory action of purified terpene hydrocarbons is restricted. The most common representative of cyclic terpenes, α-pinene, together with β-pinene, limonene, and terpinolene were shown to inhibit bacterial growth in agar plate diffusion tests (127). Andrews et al. (2) studied the toxic effects of α-pinene and some other terpenes produced by the Douglas fir on some *Bacillus* strains and on *Saccharomyces cerevisiae* (2). 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 *B. 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 (275). β-Pinene inhibited the respiration of both intact cells of *Saccharomyces cerevisiae* and mitochondria isolated from this yeast. The extent of the inhibitory effect strongly depended on the ratio between the β-pinene concentration and the amount of 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 de-energization 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 could be detected on the activity of the mitochondrial ATPase. Other observations made by Uribe and coworkers in rat liver

mitochondria showed that β-pinene stimulated the passive efflux of potassium ions, and decreased the transmembrane electrical potential (273). Furthermore, a strong increase in ATPase activity was observed which is indicative of an elevated proton leakage through the membrane (216). 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 a decrease in the potassium permeability (273). 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 (275). Similar effects on energy transduction in mitochondria were observed with limonene but not with other hydrophobic molecules (275). The toxic effects of β-pinene and limonene on *Saccharomyces cerevisiae* were proportional to the size of the monoterpene droplets in suspension (274). Experiments with β-pinene and limonene dissolved in different cosolvents, added to yeast cell cultures, decrease the droplet size and enhanced the toxic effect 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 (231).

Aromatics. Toxicity of benzene to a strain of *Pseudomonas putida* was observed by Gibson and coworkers (89). These authors reported that addition of benzene to the culture medium prevented growth of this bacterium, whereas addition of the substrate via 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 (90). 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 and coworkers (270) 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. (302), 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 strain of *Pseudomonas* (227). An inhibitory effect of toluene under comparative conditions was observed for a strain of *Pseudomonas putida* (128). In addition to the 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, and not by an increase of extracellular levels of adenine nucleotides, the lower energy status of the cell was most probably a result of 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 (42), and the effects of toluene on the ultrastructure and physiology of cells have been observed. It was shown by Jackson and DeMoss (125) that toluene impaired growth of *E. coli*, caused leakage of macromolecules (e.g., RNA, and proteins), and altered the ultrastructure of the cells (300). Electron micrographs showed that the cells are not completely lysed. The galactose permease system became totally inactive, which may have been the result of impaired energy transduction (231, 233). De Smet et al. (246) showed that treatment of cells of *E. coli* with toluene resulted in an increased permeability of the cytoplasmic membrane. Electron microscopical studies confirmed that the cytoplasmic membrane is considerably damaged, whereas the outer membrane is still intact. Their studies also indicate that magnesium ions protect the membrane against deleterious effects of toluene.

Hartmans and coworkers (104) were able to isolate 16 different strains of styrene-utilizing microorganisms from 12 soil samples by supplying styrene to the vapor phase (concentration in aqueous phase approximately 1.5 mM). Omori and coworkers, who added styrene directly to the aqueous medium in a concentration of approximately 175 mM obtained no styrene-utilizers from 101 soil samples (187). These results suggest that the amount of styrene added was critical in isolating new strains of styrene-utilizers.

Attempts made by us (229) to obtain microorganisms capable of growing on tetralin were only succesful when tetralin was applied in low (sub-saturating) concentrations. It appeared that tetralin was toxic to microorganisms when present in concentrations of $\geq$ 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, the passive flux of protons across the membranes was increased thereby causing dissipation of the components of the proton motive force (230). It was also observed that tetralin accumulated in the membrane ($P_{membrane/buffer}$ = 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 experiment 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 $P_{m/b}$ value may explain the effectiveness of tetralin as a biocide against moths (57), and bacteria (202, 254) In addition, tetralin was also shown to be toxic to *Salmonella typhimurium* in an Ames' test that was performed to screen mutagenic and toxic effects of tobacco smoke constituents (82).

Cerniglia and coworkers (48) 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 contrast, the phenolic and quinonic naphthalene derivatives inhibited the growth of the cells (48). A possible explanation for the absence of an 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 (281, 298, 299), 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 (DMF) was instantaneously toxic (231). This could also offer an alternative explanation for the observed 'synergistic' toxicity of ethylbenzene and biphenyl to a *Pseudomonas* strain (249).

Toxic effects of polycyclic aromatic hydrocarbons 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 derivates thereof (191, 293). A careful set of experiments on the toxicity of polycyclic aromatic hydrocarbons on microorganisms was reported by Calder and Lader (44), 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 other studies, the compounds were allowed to equilibrate prior to inocculation. It has also been demonstrated that an increase in cultivation temperature results in an increased inhibition of growth of the pyrene-utilizing *Rhodococcus* strain (286). 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 PAH-contaminated soil were observed in bioluminescence assays (290). The toxcicity greatly depended on the adsorption characteristics of the eluted soil, and toxicity was prevented by the addition of activated carbon. Mahaffey et al. (161) 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 cell surface. 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 (40). However, in addition to some polycyclic aromatic hydrocarbons significant quantities of other oil components were present. The observed toxicity, therefore, might have been the result of the presence of either the unidentified oil components, the polycyclic aromatic hydrocarbons, or the simultaneous presence of both (40).

In general, polycyclic aromatic hydrocarbons are expected to have aspecific toxic effects on cells, though this effect may be negligible in comparison with the enormous toxic potential of oxidized derivatives of these compounds (47, 49, 50, 260). 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 (231).

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 bacterium capable of utilizing cyclohexane as sole source of carbon and energy (263). However, this claim and other claims of pure cultures growing on alicyclic compounds, have been disputed (268). Meanwhile, transformation of cycloalkanes was observed by mixed cultures (17, 139) or by pure cultures in the presence of cosubstrates (188, 203). The first undisputed isolation of a pure culture growing on cyclohexane was reported in 1977. Stirling et al. obtained a *Nocardia* strain that could grow on cyclohexane as sole source of carbon and energy from mud flats near Sittingbourne (England) by selective enrichment on methylcyclohexane that was applied to the vapor phase (252). Ooyama and Foster (188), on the other hand were not able to isolate or cultivate microorganisms in the presence of alicyclic compounds which were

added to the aqueous medium (cyclohexane concentrations of 31 and 140 mM for isolation and cultivation, respectively). Recently, Uribe and coworkers reported the toxic effects of cyclohexane on the energy transduction in yeast (276). Cyclohexane inhibited oxygen uptake in intact cells and isolated mitochondria. Studies on isolated mitochondria showed that ATP synthesis was impaired, whereas the hydrolysis of ATP was slightly increased. Uptake of potassium ions was impaired and dissipation of the mitochondrial membrane potential was observed (276).

Pelz and Rehm tried to isolate microorganisms capable of utilizing decalin (decahydronaphthalene) as sole source of carbon and energy, but none of their 250 enrichment cultures containing decalin in a concentration of 100 mM lead to the isolation of a decalin-utilizing strain (193). 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 against microbial attack, the removal of this compound from mixtures of hydrocarbons by different microorganisms has been reported (250, 255). In 1960 Colla and Treccani reported the isolation of a *Flavobacterium* capable of growing on decalin as sole source of carbon and energy (56).

Other Lipophilic Compounds

Toxic effects of various lipophilic compounds have been observed. Compounds that are applied as food preservatives, disinfectants, and also a wide variety of drugs have been shown to affect membrane functions. Food preservatives such as lactic acid, benzoic acids (287, 288), and other lipophilic acids (88) act predominantly by dissipating the pH gradient (ΔpH) across the cytoplasmic membrane (211). Since the undissociated acids act as uncouplers, the inhibitory effect of the weak acids highly depends on pH (156). The essential oils that are applied as flavor compounds in food are also studied for their antimicrobial activities. This group comprises a wide range of mainly terpenoid compounds, e.g., limonene, carvone, pinene, ocimene, verbenol. Present studies are mainly directed at obtaining qualitative data on the toxicity of such compounds, isolated from various plants (68, 72, 109, 137, 138, 140).

Applications of lipophilic compounds as disinfectants range from soaps and detergents to antiseptics applied in drinking water facilities (296), and agents used to prevent biodeterioration. Since the beginning of this century compounds

with a wide anti-microbial spectrum are applied as disinfectants (18, 262), e.g., coal tar liquids (e.g., tetralin, and decalin), lysol-type disinfectants (cresols), chlorinated compounds, and quaternary ammonium compounds (269). As a result of their lipophilic character these compounds most probably act on the cytoplasmic membrane (84, 132).

Observations that various non-antibiotic drugs exhibit an antimicrobial activity have been reported for over 60 years (145). Since side-effects of drugs are common to physicians very little attention was paid to these antimicrobial effects. At the conference, "Antimicrobial Activity of Non-Antibiotics", held in Copenhagen (May 1990), contributions from bacteriologists and physicians revealed that a wide variety of drugs do show antimicrobial activities (145). Local and general anesthetics, antimalarial agents, probenecid, antihistaminic drugs, barbiturates, acetylsalicylic acids, procaine, diuretics, steroids, etc. were found to be active against microorganisms. The microorganisms that were studied were mainly pathogenic strains, e.g., *Listeria monocytogenes*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Vibrio cholerae*, and *Mycobacterium tuberculosis* (145). Effects on microorganisms include changes in morphology, impaired growth, reduced metabolic activities, and inhibition of DNA synthesis. Silva and coworkers, who studied the effect of local anesthetics on bacterial cells (232), 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 microscopical studies) were also observed. The extent of the effects depended on the amount of anesthetic added, and on their hydrophobicity.

In the last decade, enormous efforts have been made by bioprocess technologists to find solvents that are suitable for continuous extraction during fermentation processes (39, 41, 148, 301). These studies comprise information on the biodegradability, biocompatibility, and applicability of the solvents as extraction medium of 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 10 % (vol/vol) of a specific solvent is added, resulting in the formation of a distinct second phase. As a result of the presence of a distinct second phase also effects of the phase transition may occur. Therefore, Bar discerned molecular toxicity and phase toxicity (12). In order 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 as a measure for the hydrophobicity of the compound (147, 148). Recently, Osborne and coworkers studied the effects of a great number of organic solvents, applied in sub-saturating and saturating amounts, on the activity of a membrane bound dehydrogenase (189). From the data obtained on the dependency 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 related to a critical membrane concentration by an estimated partition coefficient (221). 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. (189) enables the prediction of the biocompatibility of an organic solvent with the specific enzyme used in their investigations.

Also in the field of ecotoxicology, the effects on microorganisms of lipophilic compounds that are present in the environment as pollutants has 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 (20, 21). 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 (240, 241, 285), polycyclic aromatic hydrocarbons, industrial solvents (135, 196), agrochemicals (27, 28, 65, 194), and surface active compounds that are applied in soaps and detergents (132, 269). Parameters that were affected included growth, active transport systems, CO_2-fixation in cyanobacteria, and gas production by anaerobic bacteria (196, 228). Not only inhibition of growth but also effects on bioluminescence, respiration, and methanogenesis, have been used to assess the toxicity of various environmental pollutants on microorganisms (38, 99, 133, 257, 284). Below a few groups of lipophilic compounds, non-cyclic hydrocarbons, are treated in more detail. The examples provide additional information on the mechanism of the membrane toxicity.

Alkanes. Similar to cyclic hydrocarbons, also aliphatic hydrocarbons may be toxic to microorganisms (9). As was demonstrated by Gill and Ratledge (92) the toxicity of these compounds is related to their chain length, which correlates perfectly with their solubility. 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. 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* was sensitive to a larger range of *n*-alkanes than the *Candida* strains. The authors suggested that this might be a result of the capability 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 the inhibition of the glucose transport system of the cell (93). Studies on the application of the alkane hydroxylating system of *Pseudomonas oleovorans* showed that aliphatic substrates, i.e., *n*-octane, cause loss of biocatalyst stability, but did not fully inactivate the cells (297). 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 (31).

Alcohols. Ethanol is probably the most often 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. 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 toxcity of ethanol, as well as on the adaptation mechanisms has been treated in an excellent review by Ingram and Buttke (120). 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 (46, 149). 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 (45). Studies on the inhibitory action of butanol on cells of the butanol producing bacterium *Clostridium acetobutylicum* confirmed the close relation between effective concentration and lipid solubility (36). For this reason, solvent production in a ABE-fermentation depends on the concentration of butanol and not on the amounts of ethanol or acetone present (131). In addition to the increase in proton flux, a partial inhibition of ATPase activity was observed (36). 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 (199).

In general, the observations made for cyclic hydrocarbons mimmick those made for alcohols and other lipophilic compounds. Phenethyl alcohol which is a bacteriostatic agent that not only impairs growth, but also inhibits the DNA synthesis in *Escherichia coli* (23, 206, 267). Further studies on the mechanism of these effects showed that phenethyl alcohol affected the cytoplasmic membrane resulting in the loss of intracellular potassium (233). Parallel experiments with toluene, conducted by these researchers, showed that both compounds act via a similar mechanism (125, 233). In conclusion, the degree of toxicity of the alcohols is directly proportional to the molecular chain length (118, 120), and the degree of toxicity parallels the partitioning behaviour of the alcohols in *n*-octanol/water (155).

Phenols. Since the 19th century phenol has been used as a biocide (100). Recently, the effects of phenol and its substituted derivatives have regained attention, due to the wide spread presence of these compounds in the environment. Phenolic compounds are commonly found in waste waters of industrial origin (34). Due to 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 low (24, 80, 291). Also phenolic food additives such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), used as antioxidants, are known to have antimicrobial effects (67). Phenolic compounds probably exert their toxic effects at the level of the membrane as can be deduced from the good correlation that exists between the toxicity and the hydrophobicity of different phenolic compounds (22, 228). This conclusion is supported by observations that phenol changes membrane functioning and influences protein-to-lipid ratios in the membrane (134). In addition, Heipieper et al. demonstrated that addition of phenol and 4-chlorophenol to suspensions of *E. coli*, induced efflux of potassium ions and ATP (106).

In conclusion, in the studies where 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 (2, 106, 108, 189, 230, 231, 275, 276, 296). Furthermore, the inhibitory action is in most cases directly related to the partitioning behaviour 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 basis of their lipophilic nature due to the specific properties of the molecules. In the next paragraph an outline of studies on the mechanism of the toxic action of lipophilic compounds is presented.

SITE(S) OF 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 and Overton approximately one century ago (170, 190). 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 anesthetic efficacy of a compound showed an excellent correlation with the partioning of that compound in olive oil. The theory that developed from this hypothesis was the so called 'Lipid-Theory of Anesthesia' and still this theory is applied in anesthesiology. However, the mechanism of the inhibitory action of anesthetics and also other lipophilic compounds has not been elucidated. In addition to observed changes in membrane structure and function, alterations in enzyme activities have been observed (85, 130, 189). Since the late 1970s, Franks and Lieb have advocated proteins as the primary site of the action of lipophilic compounds (85-87, 165). And today it is a well recognized alternative theory, although full acceptance seems to lack. Since the protein targets of lipophilic compounds are often membrane-embedded enzymes we have recently postulated that the toxic effects could be at the level of lipid-protein as well as lipid-lipid interactions which are likely to be disturbed when a molecule accumulates in the lipid bilayer (231).

In this chapter the knowledge on the action of lipophilic compounds on lipid bilayers and/or membrane-embedded proteins, and relate the effects to observations made for microorganisms is evaluated.

Changes in Membrane Structure

As a result of partitioning of lipophilic compounds to lipid bilayer membranes one can 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 polarity of the compounds, and consequently in differences in location in the bilayer. As already mentioned above, membranes do not behave as bulk liquid phases with respect to partitioning. A concentration gradient of lipophilic compounds exists (163), due to variations in polarity of the lipid bilayer, i.e., when looking at cross-sections of the membranes (213). Also lateral heterogeneity and cluster forming of membrane lipids can be affected by accumulation of lipophilic compounds in the lipid bilayer (64). Therefore, solutes that interact with the membrane will cause different perturbations of the bilayer depending on their preferential site of accumulation (124). This effect may be accentuated by an asymmetric distribution of 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 (224). However, intrinsic elasticity of the bilayer may largely compensate for the changes induced by the amphipaths (79). 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 (60). 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 (224) discerned crenating (anionic) and cup-forming (cationic) drugs. Further work by the group of Singer showed that these amphipathic drugs caused a marked redistribution of protein and lipid components in the plane of the membrane (162). These effects may include restructuring of domains of specific constituents, such as supramolecular protein complexes and lipid patches (29), that exist in biological membranes. Also vesiculation of the membrane may occur as result of the intercalation of lipophilic molecules with the bilayer (98).

Studies by the group of Seeman on the interaction of various drugs, and local

and general anesthetics with erythrocyte membranes revealed that these compounds have significant impact on the volume of the membrane bilayer (221). 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 (242) in lipid monolayers 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 all pertain to compounds that posses a polar function (e.g., 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 (224). 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 (168, 295). The major change in membrane dimension as a result of interaction with lipid soluble compounds, e.g., hexane, is an increase in the area occupied per phospholipid molecule rather than the bilayer thickness (58).

Interaction of the terpene hydrocarbon squalene has been studied in connection with the applicability of the compound for the modification of membrane (bilayer) thickness. Squalene ($C_{30}H_{50}$) is a highly apolar liquid hydrocarbon that is insoluble in dipalmitoyl dispersions (234) and does not dissolve in glyceryl monooleate bilayers (294). It was shown that squalene, applied in molar ratios of squalene to dipalmitoyl phosphatidylcholine up to 9 to 1, has no effects on the thermodynamic phase transition parameters and fluidity of membrane bilayers (234). These authors concluded that squalene does not reside in the the dipalmitoyl phosphatidylcholine phase (calorimetric, Raman spectroscopy studies), and probably forms a distinct phase in the aqueous medium. This is surprising since other highly apolar compounds, e.g., pyrene, DPH, penetrated into the hydrophobic interior of the bilayer (13, 142, 226). Furthermore, predictions on the basis of extrapolations of partitioning data would suggest that compounds like squalene preferentially reside in the lipid bilayer. Some researchers have postulated the inner core of the bilayer, between the two monolayers as the location for these apolar compounds, e.g., pyrene, DPH (13, 150). Also studies on the interaction of *n*-alkanes with lipid bilayers (168) show that *n*-alkanes, hexane to hexadecane, enter the hydrocarbon region of the lipid bilayer. It was demonstrated for the longer alkanes (dodecane to

hexadecane) that accumulation causes an increase in bilayer width (X-ray diffraction). Also an increase in transition temperature of DPPC-membranes was observed, probably as a result of an increase in hydrocarbon chain interactions. The increase in hydrocarbon interaction is probably due to a parallel alignment of the longer alkanes and the lipid acyl chains. In contrast, shorter alkanes which also partition in the central part of the lipid bilayer (295), lower and broaden the transition temperaure. The observation that the presence, in the bilayer, of approximately one hexane molecule per phospholipid does not cause any increase in membrane volume (136, 295) suggests that the hexane molecules perfectly align with the fatty acid acyl chains. The interaction between the hexane molecules and the acyl chains disturbs the interactions between the acyl chains and reduces the ordering of the lipid bilayer. Long-chain alkanes probably also disturb the interactions between the acyl chains, 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 (277, 278). Cell lysis is observed, and changes in lipid-protein interactions in the annulus or 'boundary lipids' surrounding membrane embedded proteins have been postulated. The authors suggest that the observed cell lysis is caused by the combined effect of the δ-hexachloro-cyclohexane 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 (7).

The interaction of cyclic hydrocarbons with *E. coli* phospholipid liposomes, as assessed by studies on liposomes labeled with fluorescent probes, showed that these hydrocarbons primarily interacted with the bilayer interior. Addition of the lipophilic cyclic hydrocarbons caused a decrease in fluorescence polarization of DPH, which is located in the lipophilic interior of the bilayer (150). On the other hand no effect on TMA-DPH, that is located in the headgroup region of the membrane, could be observed (231). The decrease in DPH polarization parallelled 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 (58, 231).

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 affects 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) the 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 (141, 198). The chemical proton potential (ΔpH) and the electrical potential ($\Delta\psi$) together form the proton motive force (Δp). In the chemiosmotic theory, which was established by Mitchell (174, 175), the proton motive force forms an energy intermediate that can be used to drive various endergonic processes such as solute accumulation, ATP synthesis, and others (107). More recently it has been shown that not only a proton but also sodium motive force plays a role in the energy transduction across the cytoplasmic membrane. The proton- and sodium motive force are interlinked with each other via special transport proteins, but also interlinked with the phosphate potential (ΔG_P) via $H^+(Na^+)$ ATPases (200).

An increase in the passive flux of protons or ions across the membrane may lead to lowering of Δp (or $\Delta p_{Na}+$) which will impair proper functioning of the membrane in energy transduction. 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 by an increase in temperature, mechanical stress, interaction with lipophilic molecules, and others. Studies on the proton leakage in membrane vesicles from mesophilic and thermophilic bacilli showed that the passive flux increased with increasing temperature but that leakage in vesicles from the thermophilic *Bacillus* strain was affected at higher temperatures (282). Apparently, the phospholipid composition of the membrane of the thermophilic *Bacillus* strain has been adapted to life at higher temperatures. Bangham and coworkers showed that various anesthetics stimulated the permeability of a phospholipid bilayer to potassium ions (11). The concentrations at which an increased permeability was observed perfectly correlated with chemical activities inducing narcosis in biological systems. Stimulation of the leakage of protons and

in particular potassium ions have been observed in various membranes upon addition of lipophilic compounds such as ethanol (46, 149), butanol (36), fatty acids (35, 209), β-pinene (275), dolichol (177), and also cyclohexane (276), tetralin (230), and other cyclic hydrocarbons (231). For some fragrance compounds decreases of the transmembrane electrical potential have been shown (76). The changes in membrane potential parallelled the fluidity changes of the membrane, as was also observed for the effects of cyclic hydrocarbons in proteoliposomes (231). Furthermore, Enomoto et al. (76) showed that modification of the phospholipid head group composition of the liposomes altered the sensitivity to the fragrance compounds.

Dissipation of the proton motive force also affects other gradients across the cytoplasmic membrane (113). 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 (128, 133). The observation that lipophilic compounds stimulate the activity of proton and/or ion translocating ATPases (94) 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 also impairs pH homeostasis (33). This results in altered (transport) enzyme activities (199), which may also lead to impairment of cellular viability.

Activity of membrane bound/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 (303). 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. It has been known for many years that intrinsic aspects of biological membranes strongly affect functioning of proteins embedded in that membrane. Parameters such as membrane thickness, headgroup hydration, fluidity, and fatty acid composition regulate the activity of these enzymes (114, 212, 303). 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 (168, 201) and also the effect of anesthetics and drugs on membrane expansion may be related to an increase in membrane thickness (221-223). In 't Veld et al (115) 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, 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, where electron transfer may be slowed down when the protein complexes become dissociated upon excessive accumulation of lipophilic molecules in the cytoplasmic membrane (215).

Hydration of membrane head groups is difficult to assess, and effects of apolar compounds may not immediately be expected. However, recent data from Shimooka et al. (225) show that the local anesthetic tetracaine interacts with the headgroups of the phospholipids, resulting in the disturbance of the hydration layer. Results obtained by Yoshida et al. (306) 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 hydration of the lipid bilayer (53), as a result of their preferential binding to the hydrophilic surface of the membrane (208).

Modification of the fluidity or microviscosity of the membrane by the interaction with apolar solutes has frequently been observed (7, 94, 231). Also under physiologically normal conditions, membranes contain compounds such as cholesterol, hopanoids, and probably carotenoids to maintain optimal fluidity (30). 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 (94, 307, 310). Opposite 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 (256).

Changes in the fatty acid composition have been observed in cells exposed to lipophilic compounds, (237). 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. (116) 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 unsaturation 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 in order to retain activity (117). 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 (15, 207).

It can be concluded that interaction of lipophilic compounds with biological membranes results in changes of the membrane structure. These changes include: increase in bilayer thickness, increase in membrane surface area, modification of bilayer fluidity, alteration of the phospholipid distribution, and changes in hydratation characteristics of the membrane surface. These modifications affect the functioning of the membrane, both as a selective barrier and as a matrix for enzymes. The "unspecific" toxicity effects of lipophilic compounds are likely to be exerted in most cases at the level of lipid-lipid and lipid-protein interactions (277). This hypothesis offers a rationale for observations made by various investigators, that 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 (46, 149, 221, 230, 231), enzyme activities (189), membrane swelling (168, 222), antihaemolysis (221), luciferase actvity (77, 85, 130), and ATP depletion (128).

ADAPTATIONS TO LIPOPHILIC COMPOUNDS

Notwithstanding the clear toxicity of various (cyclic) hydrocarbons to most microorganisms, microbial strains do exist that are able to tolerate high concentrations of such compounds in their environment. A clear example is the *Pseudomonas* strain that is capable of growing in the presence of 50 % toluene (121, 123). The strain, however, is not able to metabolize toluene. Recently, Gibson and coworkers reported a strain of *Pseudomonas* that is able to grow on various aromatic hydrocarbons, present in concentrations up to 50 % (63).

Similar results have been obtained in our laboratory with a pseudomonad growing on styrene (289). We also isolated a strain of *Pseudomonas* that is able to grow on α-pinene at concentrations up to 90 % (Sikkema et al., unpublished results). The mechanism of this super-tolerance is not known yet, 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. In general, one could say that Gram-negative bacteria do tolerate higher concentrations of lipophilic compounds than Gram-positive bacteria (103, 122). Below we discuss the possibilities that may harness microorganisms with an increased tolerance towards (cyclic) hydrocarbons (Fig. 5). 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 to remove toxic compounds from the cell (146, 210).

Membrane Modification

Cytoplasmic membrane. The plasma membrane of a cell consists of a phospholipid bilayer matrix in which proteins are embedded as indicated above. In the membrane bilayer lipid soluble compounds such as cholesterol or carotenoids can be found which serve as modifiers of membrane fluidity and integrity (29, 144, 205). Although, the partitioning of a solute in the membrane is significantly influenced by these lipid soluble compounds (159) the phospholipid composition of the membrane is the most important determinant of partitioning (3-6). By modifying the fatty acid composition of the lipid bilayer from myristoyl to stearoyl the partition coefficient of lindane could be reduced 50-fold (Table 1) (4). Studies with cells of the yeast *Saccharomyces cerevisiae* showed that strains with an increased tolerance to ethanol were enriched in their content of mono-unsaturated fatty acids which was accompanied by a decrease in saturated fatty acids (19, 172, 173). Similar results have been obtained with *Escherichia coli* (118), and some *Lactobacillus* strains (271, 272). Additional tolerance to ethanol can be acquired through addition of calcium ions, as was shown by Nabais et al. (181). These results seem to agree with observations made by de Smet et al. (246), that addition of magnesium ions to *Escherichia coli* cells decreases the

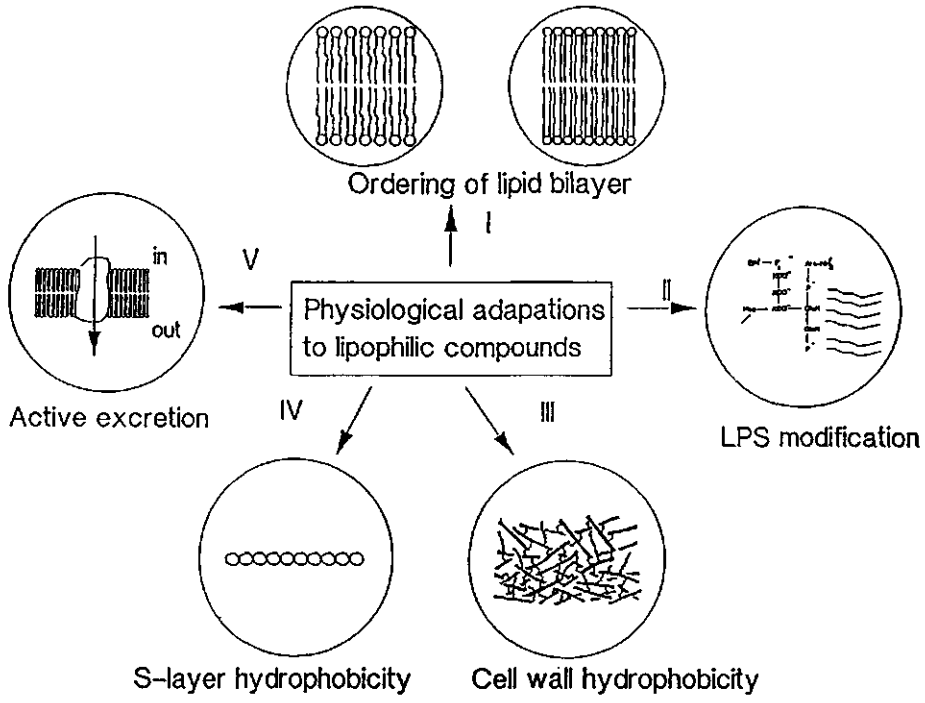

FIG. 4. Schematic presentation of adaptation mechanisms that protect the cells against lipophilic compounds. I. Increase in ordering of the bilayer by changing the fatty acid conformation from *cis* to *trans*, or by saturation of fatty acid acyl chains. II. Modification of the KDO-region of lipopolysaccharides of the outer membrane (only Gram-negative bacteria). III. Increase in the degree of cross-linking between constituents of the cell wall, and modification of the cell wall hydrophobicity. IV. Increase in S-layer hydrophilicity. V. Active excretion.

deteriorating effect of toluene (246). Studies on mutants of *Escherichia 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 (54). In these studies no changes in other constituents of the cell envelope were observed. Work by Ingram (119) demonstrated that also other lipophilic compounds induce changes in the fatty acid composition of *Escherichia coli* cells. Relatively polar solvents such as acetone, dimethylsulfoxide, dioxane and tetrahydrofuran cause an enrichment in unsaturated fatty acids, analogous to changes induced by ethanol (118) or low growth temperatures (164). 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 (164). Exposing the cells to toluene, the most apolar solvent used in this study, had virtually no effect on the fatty acid composition (119). Heipieper and coworkers, who studied the adaptation of a strain of *Pseudomonas* to phenol, observed conversion of *cis* fatty acids to their *trans* form (105). 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 behaviour of *trans* fatty acids (186). Thus decreases in the ordering of the phospholipid molecules caused by phenol are 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 indicate that *cis* fatty acids are converted to their *trans* form (289).

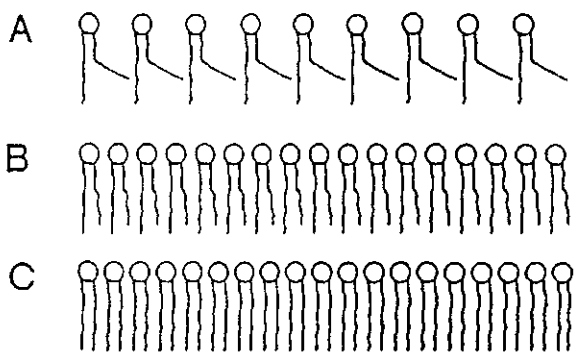

FIG. 5. Different conformations of phospholipids below the beginning of lipid phase transition, and their effect on the ordering of the lipid bilayer (37, 61).

Observations that alcohols and organic solvents induce alterations in the fatty acid composition show resemblances with modifications as a result of temperature changes (119, 164, 261). Also observations of cross-effects of solvent induced heat tolerance and vice versa have been reported (153).

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, dimethylsulfoxide, etc.), which are miscible with water, cause an increase in unsaturated fatty acids resulting in an increased fluidity of the membrane (37, 61). This 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 (182). Observations made by Thomas and Rose (265) on the effect of ethanol on cells of *Saccharomyces cerevisiae* grown in the presence of either oleic acid or linoleic acid showed that the amount of cell-bound ethanol was slightly but consistentently lower in cells grown in linoleic acid containing medium. Cells cultivated in the presence of linoleic acid also were more tolerant to ethanol than cells grown in the presence of oleic acid (265). More lipophilic solvents that are soluble in water (benzene, aniline, amyl acetate, etc.), but not completely miscible, induce an enrichment in saturated fatty acids and an increase in total phospholipid. 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 (29). These effects are known to oppose partitioning of lipophilic solutes to a lipid bilayer (163). This is especially true 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 (231) that 'mechanical' exclusion does not suffice. In order to withstand these compounds cells need other, additional, adaptation mechanisms.

Outer membrane. Gram-negative bacteria appear to be less sensitive to lipophilic compounds than Gram-positive organisms (103, 122). 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 (279). Modification of the outer membrane of Gram-negative bacteria is always related to the lipopolysaccharide composition of this membrane. As mentioned above the outer membrane is a highly porous shield that allows small (hydrophilic) solutes to pass via pores (183). However, it has a surprisingly high transfer resistance to hydrophobic compounds (183). This transfer resistance has been shown to be a result of the highly hydrophilic lipopolysaccharides (LPS). Mutants that lack certain moieties of the LPS-molecules appear to be sensitive to a variety of lipophilic compounds, see for a review Nikaido and Vaara (183). It can be envisaged accordingly that some strains of Gram-negative bacteria are able to further increase the transfer resistance towards lipophilic compounds either via an induction mechanism or a mutagenic event.

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 bacteria with more hydrophilic cell walls (158). This implies that modification of cell walls (hydrophobic to hydrophilic) could provide a means to protect the organism with a shield against these compounds. Park and coworkers demonstrated that *Saccharomyces cerevisiae* adapted to the presence of toxic solvents (tributylphosphate and 2-*tert*-butylphenol) by decreasing the hydrophobicity of the cell wall (192).

S-layer

The function of the S-layer in protection of the cell is still largely unknown (245). Present data indicate that the S-layers primarily serve as molecular sieves. *Bacillus* strains possessing a S-layer showed resistance to lysozyme, suggesting an exclusion limit down to a diameter of 3.5 nm (25, 244). Also functioning as an ion exchange resin has been demonstrated (245). To our knowledge no data exist on the exclusion of small hydrophobic molecules by S-layers.

Active Excretion

The concept of multidrug resistance (MDR) has been known in medicine for quite some time. The discovery that the multi drug 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 (141, 200). MDR-like excretion systems have also been shown to be operative in bacteria. For instance, efflux of daunorubicin and

doxorubicin by *Streptomyces peucetius* (97), nalidixic acid and others by *E. coli* (157), efflux of 2',7'-bis-(2-carboxyethyl)-6(and6)-carboxyfluorescein (BCECF) by *Lactococcus lactis* (176), various P-glycoprotein substrates such as ethidium bromide, daunomycin, chloroquine, rhodamine 6G, gramicidin, and nigericin by *L. lactis* and others have been shown (200). These excretion systems have in common 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) (200). Recently, the active excretion of the cyclic hydrocarbon benzo[*a*]pyrene by P-glycoprotein from human breast cancer MCF-7 cells has been demonstrated (304). Whether similar systems play a role in protecting microbial cells from high concentrations of, for instance, (cyclic) hydrocarbons remains to be established. We feel 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 *Pseudomonas putida* (264), and 4-chlorobenzoic acid in coryneform bacterium NTB-1 (96) have been demonstrated.

Immobilization

In natural systems 99 % of all microorganisms is growing at surfaces (158). 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 (259) and phenols (75, 179). Also for cells entrapped in various polymer matrices, reduction of toxic effects of, e.g., phenol, has been observed (24, 106).

Cooxidation and Commensalism

Although solid data is not available regarding the background of the transformation of hydrocarbons by microorganisms (especially fungi) to alcohols, phenols, or ketones without further metabolism (195), it may very well serve to

detoxify these compounds. Many researchers studying cooxidation processes have included this as rationale for cooxidation processes.

Also for the relative ease with which mixed cultures can be obtained growing at the expense of cyclic hydrocarbons at concentrations that are toxic to pure cultures no definite explanation can be given (17, 229). Most probably, a differentiation exists between the initial attack and the further metabolism (1, 243). Also, the initial oxidation step can be catalyzed by an extracellular oxidase, thereby circumventing the necessity for uptake of the hydrocarbon. As a result of the oxidation, the compound becomes less hydrophobic and will accumulate to a lesser extent in the cytoplasmic membrane, which lowers the toxicity burden. Other strains may be involved in further metabolizing the oxidized intermediates.

CONCLUDING REMARKS

The accumulation of cyclic hydrocarbons 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 cyclic hydrocarbons can be largely explained by the interactions of these compounds with the membrane and membrane constituents. As a result of accumulated hydrocarbon molecules, the membrane looses its integrity and an increase in permeability to protons and ions can be observed. In addition to the long standing theory that lipophilic compounds exclusively disturb the lipid part of the membrane, also proteins embedded in the membrane are affected. The effects on the membrane embedded proteins probably result to a large extent from changes in the lipid environment. Direct effects of lipophilic compounds on proteins have also been observed, but in most instances these occur at higher concentrations. The data presented here support the view that the cytoplasmic membrane is the primary site of action. The resulting changes in the membrane structure affect the functioning of membrane embedded proteins, as well as the barrier function of the membrane. This barrier function, which allows specific molecules to be taken up and others to be excreted by metabolic energy requiring processes, discriminates the cytoplasmic membrane from the outer membrane (*in* Gram-negative bacteria). Consequently, cells will be much more vulnerable to changes in the cytoplasmic membrane than in the outer membrane. The outer membrane, and especially the LPS, does however play a role in

protecting the cell against highly lipophilic compounds by forming a resistance barrier for these molecules.

In 1970, Harold (102) 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 1993. Data on the mechanism of the toxic action of lipophilic compounds are available from various scientific disciplins, but as a result of the fragmentation a great deal of research is performed in many-fold without taking into account the results of similar research in other fields. We hope that this overview will provide information for further research within different disciplines.

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ISOLATION AND INITIAL CHARACTERIZATION OF BACTERIA GROWING ON TETRALIN

SUMMARY

Eight strains of bacteria utilizing tetralin as sole source of carbon and energy have been obtained. Four strains have been selected from culture collections. The others were isolated from hydrocarbon-polluted areas. The newly isolated strains belong to the genera *Acinetobacter*, *Arthrobacter* and *Moraxella*. Most of the selected strains were able to grow on other aromatic hydrocarbons, but none of them grew on cyclohexane. Tetralin-utilizing organisms were difficult to isolate and cultivate, because tetralin was toxic to the cells at concentrations above 15 μl/liter. Consequently tetralin was supplied either via the vapor phase or an organic solvent/water two-phase system was employed.

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INTRODUCTION

The production of fine chemicals is an area with many opportunities for biotechnology. The initial step in the design of such a biotechnological process is the selection of a suitable biocatalyst for the production of the desired compound. Bioproduction of 5,6,7,8-tetrahydro-1-naphthol (Fig. 1) for the fragrance industry from 1,2,3,4-tetrahydronaphthalene (tetralin) may eventually lead to an interesting process. Detection and selection of a suitable biocatalyst for the tetrahydro-1-naphthol reaction seems most feasible by isolating tetralin-degrading microorganisms. The rationale for selecting tetralin-degrading organisms is based on several known biodegradative routes in the metabolism of aromatics involving the formation of *cis*-dihydro diols through a dioxygenase type of reaction. *cis*-Dihydro diols may chemically rearrange to hydroxy-aromatics.

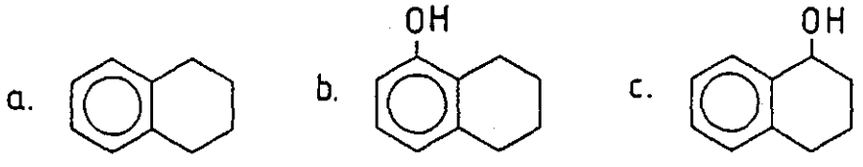

FIG. 1. Structural formulas of (a) 1,2,3,4-tetrahydronaphthalene (Tetralin), (b) 5,6,7,8-tetrahydro-1-naphthol, and (c) 1,2,3,4-tetrahydro-1-naphthol.

Many microorganisms have been isolated that can grow on various aromatic and alicyclic compounds, and a great deal of knowledge exists on the microbial metabolism of such hydrocarbons (7, 30). Tetralin is a bicyclic molecule, that consists of an aromatic and an alicyclic moiety (Fig. 1). It may be attacked either at the aromatic or at the alicyclic ring. So far, organisms studied degrade or transform tetralin by an initial hydroxylation of the alicyclic ring (9, 12, 14).

Isolation of microorganisms that can utilize tetralin as sole source of carbon and energy apparently is a difficult task. Several pure cultures of hydrocarbon utilizers were unable to grow on tetralin (17, 31). Tetralin utilization, however, was observed with mixed cultures (28), and with pure cultures when mixed substrates were supplied (15, 25, 26). Only in one instance has the isolation of a bacterium been reported that can grow, though poorly, on tetralin as sole source of carbon and energy (23). This bacterium, a *Pseudomonas* sp., starts with an initial hydroxylation of the alicyclic ring and accumulates 1,2,3,4-tetrahydro-1-

naphthol and 1,2,3,4-tetrahydronaphthalenone.

We now report the isolation of eight bacteria that can grow on tetralin and show that the toxicity of tetralin for these organisms explains the difficulties met by others in isolating tetralin-degrading organisms.

MATERIALS AND METHODS

Microorganisms. Microorganisms tested for tetralin utilization were from different collections. Bacteria from our culture collection were: *Xanthobacter* 124X (33), *Nocardia* S3 and *Rhodococcus* S5 (11) isolated on styrene; *Pseudomonas* strain 50 isolated on benzene (34); *Mycobacterium* E3 isolated on ethene (10); *Alcaligenes* OBB65 isolated on 1,3-dichlorobenzene (5); *Pseudomonas putida* LW4 isolated on D-phenylglycine (32). In addition unidentified strains isolated from soil on benzene (EM1, EM3, EM4, EM6); on toluene (KZ4 and RA15); on ethylbenzene (EB1 and EB2); on naphthalene (N1 and N3); and on cyclohexane (C2) were also tested.

Microorganisms obtained from the culture collection of the Department of Microbiology of the Agricultural University were: *Arthrobacter* A177 and *Corynebacterium* C125 isolated on *o*-xylene (21); *Pseudomonas* A2 isolated on mesitylene (1,3,5-trimethylbenzene); *Pseudomonas* P47 isolated on D-phenylglycine; *Pseudomonas* P53 isolated on *o*-cresol; *Pseudomonas putida* P60 isolated on phenol; the yeast *Trichosporon cutaneum* isolated on phenol; and an unidentified bacterium, strain 102, isolated on lindane.

A laboratory strain of *Aspergillus nidulans* able to grow on various substituted aromatic compounds was obtained from the collection of the Department of Genetics (Agricultural University Wageningen). *Nocardia corallina* (*Rhodococcus* sp.) V49, ATCC 19070, was obtained from the American Type Culture Collection (Rockville, Md.) (14).

Organisms isolated on tetralin during the present investigation were *Arthrobacter* T2, *Acinetobacter* T5, *Arthrobacter* T6 and *Moraxella* T7.

Maintenance of microorganisms. Microorganisms were kept on slants of 5 g/liter glucose and 3.5 g/liter yeast extract medium to which Oxoid no. 3 agar (15 g/liter) had been added. Tetralin-utilizing strains were also kept on slants of a mineral salts medium, containing in 1 l of demineralized water: K_2HPO_4, 1.55 g; $NaH_2PO_4.2H_2O$, 0.85 g; NH_4Cl, 2.0 g; $(NH_4)_2SO_4$, 0.1 g; $MgCl_2.6H_2O$, 0.075 g and 0.2 ml of a trace elements solution (35). Slants were placed in a 5-liter desiccator containing a flask with 50 μl tetralin.

Selection and isolation of tetralin-utilizing strains. Pure cultures were tested for tetralin utilization by supplying 10 ml mineral salts medium in 100-ml serum bottles with 5 μl tetralin. Alternatively, tetralin was supplied *via* the vapor phase (6) using 300-ml Erlenmeyer flasks containing 50 ml mineral salts medium. A test tube was placed in the flask. After sterilization and inoculation (0.1 mg wet weight/ml), tetralin (10 μl) was pipetted into the tube and flasks were sealed with Teflon Mininert valves (Pierce Europe, Oud Beijerland, The Netherlands). The tetralin concentration in the water under the applied conditions was 16 μl/liter, as determined spectrophotometrically (22) using standard but sterile incubations. In a third approach, tetralin was supplied by adding tetralin-containing fluorocompound 40 (FC 40) to the mineral salts medium, resulting in a solvent/water (10:90) two-phase system with approximately 15 μl/liter tetralin in the water phase. The partitioning coefficient for tetralin over the FC 40/water system was 8. Enrichment of tetralin-degrading microorganisms was in 300-ml Erlenmeyer flasks that were fitted with Teflon Mininert valves. Inocula of 1 gram of soil were introduced in the flasks with 50 ml of mineral salts medium, and tetralin was added using the three techniques described above. Control flasks did not contain tetralin. The flasks were incubated at 30°C on a rotary incubator (200 rpm).

Carbon dioxide production was measured daily and once growth was observed, 0.1 ml of a suitable dilution was plated onto agar plates with mineral salts medium. The agar plates were incubated in a 5-liter desiccator containing a flask with 50 μl tetralin. Control plates were incubated in a 5-liter desiccator in the absence of tetralin. Colonies that developed on tetralin plates were isolated and checked for purity by plating on yeast extract/glucose agar plates. These pure cultures were subsequently tested for tetralin utilization by plating on mineral salts medium and incubation in desiccators in the presence and absence of tetralin.

Identification of bacteria. Bacteria isolated during this investigation were characterized according to Bergey's Manual of Determinative Bacteriology (eighth edition). Additional information was obtained from identification kits, API 20 NE and API 20 B (Analytical Profile Index, Montalieu Vercieu, France).

Culture conditions. In growth experiments, volatile hydrocarbons were provided indirectly to the mineral salts medium via the vapor phase as described for tetralin. Growth was assessed by monitoring the culture fluid turbidity together with the production of carbon dioxide from the supplied substrates. Cultivation of cells to be used in incubation experiments was in 5-liter Erlenmeyer flasks, containing 1 liter mineral medium supplied with 10 g/liter of sodium-acetate and 0.5 g/liter yeast extract or with 50 μl/liter hydrocarbon supplied in a separate reservoir.

Preparation of washed cell suspensions. The cells were harvested in the mid-exponential growth phase by centrifugation (16,000 $\times$ *g* for 10 min at 15°C), washed twice with potassium phosphate buffer pH 7.0 (50 mM) and resuspended in the same buffer (2 times the cell volume).

Whole cell incubations. Incubation experiments with washed cells were performed with suspensions of freshly harvested cells. Toxicity of tetralin was monitored in incubations in 1-liter serum bottles. Cells (0.1 g wet weight) were resuspended in 100 ml phosphate buffer (50 mM; pH 7.0) with various amounts of tetralin in the absence or presence of an organic solvent. Carbon dioxide production was determined after 7 days of incubation.

Cells for incubations with organic solvents were resuspended in 9 ml mineral medium supplemented with acetate (1.0 % wt/vol), and yeast extract (0.1 % wt/vol), and 1 ml organic solvent was added. Incubations were in 100-ml serum bottles for two weeks at 30°C in a rotary incubator (200 rpm). Carbon dioxide production was then determined by head space analysis.

Analytical techniques. Carbon dioxide concentrations were determined by injecting 0.1 ml head space samples on a Packard 427 gas chromatograph (Packard/Becker, Delft, The Netherlands) fitted with a Porapack Q column (Chrompack B.V., Middelburg, The Netherlands).

Tetralin concentrations in both aqueous and organic phases were determined spectrophotometrically at 274 nm (22) using a UV-Vis spectrophotometer (The Perkin-Elmer corp., Norwalk, Conn.).

Chemicals. 1,2,3,4-tetrahydronaphthalene was purchased from Janssen Chimica (Beerse, Belgium). Fluorocompound 40 was obtained from 3M (St. Paul, Minn.). All other chemicals were of commercially available analytical grade.

RESULTS

Utilization of tetralin by microorganisms from culture collections. Twenty eight strains (see Materials and Methods) tested for tetralin utilization were selected on the basis of their ability to utilize hydrocarbons. Tetralin (500 μl/liter) was added to the water phase, and turbidity of the medium, as well as production of carbon dioxide from tetralin, was monitored. None of the

organisms utilized tetralin supplied in this manner. Subsequently, the substrate was supplied indirectly to the water phase via the vapor phase and under these conditions four strains utilized tetralin (Table 1). In another attempt to lower the substrate concentration in the direct environment of the microorganism, a two-phase system with the inert fluorocompound 40 (FC 40) was used. In this system three organisms used tetralin as carbon and energy source (Table 1).

TABLE 1. Utilization of tetralin as sole source of carbon and energy by bacteria selected from culture collections.

Organism[a]	Isolation substrate	Growth[b] I	II	III	Reference
Arthrobacter A177	*o*-Xylene	—	+	+	21
Corynebacterium C125	*o*-Xylene	—	+	+	21
Nocardia S3	Styrene	—	+	+	11
Pseudomonas A2	Mesitylene	—	+	—	

[a] Twenty four other organisms tested did not utilize tetralin as sole source of carbon and energy.

[b] Incubations with tetralin added in the water phase (I), via the vapor phase (II) or in a two-phase system with FC 40 (III).

Isolation of microorganisms. Initial attempts to isolate tetralin-utilizing microorganisms were not successful. Various soil samples, sludge from industrial waste water treatment facilities, and mud from the river Rhine were used as inoculum, and tetralin was added to the enrichment medium both at 250 μl/liter and 100 μl/liter. Growth was not observed in incubations containing 250 μl/liter but did occur in several instances at 100 μl/liter. Purification of these mixed cultures, however, was unsuccessful.

In a new attempt, cultures were set up with tetralin added via the vapor phase. Growth occurred in several incubations and two organisms were isolated by plating on solid mineral medium and by supplying tetralin via the vapor phase. From an enrichment set up with soil from a petrol station a Gram-positive, strictly aerobic non-motile coccoid organism was obtained (strain T2). During growth in complex media, the cells changed from predominantely rod-shaped to coccoid. Based on these characteristics and further tests on the utilization of various substrates for growth (24), this bacterium was classified as an *Arthrobacter* sp. From an enrichment set up with activated sludge from an

industrial waste water treatment facility a strictly aerobic, non-motile Gram-negative organism was obtained (strain T5). Cells changed from rod-shaped during the logarithmic phase to coccoid in the stationary phase. The organism hydrolyzed gelatin and esculin; it was oxidase and urease negative. On the basis of these characteristics and on the results from the API 20 NE test the organism was identified as an *Acinetobacter* sp. (3).

TABLE 2. Growth of tetralin-utilizing bacteria on aromatic hydrocarbons.

Organism	Doubling time on tetralin	Benzene	1,2-Diethyl-benzene	Ethyl-benzene	Mesity-lene	Naph-thalene	Styrene	Toluene	o-Xylene
Arthrobacter A177	20	—	—	+	—	—	—	—	+
Corynebacterium C125	20	+	+	+	—	+	—	+	+
Nocardia S3	56	+	—	+	—	+	+	+	—
Pseudomonas A2	52	—	—	—	+	—	—	—	+
Acinetobacter T5	24	—	—	—	—	—	—	—	—
Arthrobacter T2	18	—	—	+	—	+	+	—	+
Arthrobacter T6	>72	+	—	+	—	+	+	—	—
Moraxella T7	23	+	—	—	—	—	+	+	—

The tested compounds were applied via the vapor phase. + indicates growth, — indicates no growth relative to a control incubation without substrate added.

The isolation of microorganisms was also attempted using an organic solvent/water two-phase system with an inoculum taken from a Diesel spillage at a farmyard. Different tetralin concentrations up to 150 μl/liter were supplied in the presence and absence of FC 40. After two weeks of incubation, growth was observed in enrichments without solvent at tetralin concentrations up to 7 μl/liter. However, growth occurred at 70 μl/liter tetralin in the presence of solvent (10% vol/vol). After purification, two bacteria were obtained which grew on tetralin. One bacterium, referred to as strain T6, was purified from an enrichment culture with 15 μl/liter tetralin in the presence of 10% FC 40. This Gram-positive organism that changed from rod-shaped in fresh cultures to coccoid in older cultures was tentatively classified as an *Arthrobacter* sp. based on the results from an API 20 B test. From an enrichment culture with 35 μl/liter tetralin in the presence of 10% FC 40, a Gram-negative, non-motile, coccoid organism was isolated. This organism was referred to as strain T7. The results from the API 20 NE test indicated that this organism belonged to the *Moraxella*

group. On the basis of the positive oxidase reaction this bacterium was tentatively classified as a member of the genus *Moraxella* (2).

Growth characteristics. The eight tetralin-utilizing organisms obtained were further characterized. Mean generation times for the selected bacteria for growth on tetralin ranged from approximately 20 hours to more than 70 hours (Table 2). The organisms were tested for their ability to grow on various aromatic hydrocarbons. Strain T5 did not grow on any other aromatic compound tested but all other bacteria were able to use various other aromatic compounds (Table 2). None of the organisms grew on cyclohexane.

Toxicity of tetralin in strain T2. Maximal tetralin concentrations allowing growth were determined and it appeared that for all strains the substrate was toxic at concentrations of 15 μl/liter and higher. *Arthrobacter* T2 was studied in more detail, since it had a relatively short doubling time. It is clear that the tetralin concentration in the aqueous phase should be minimized to prevent

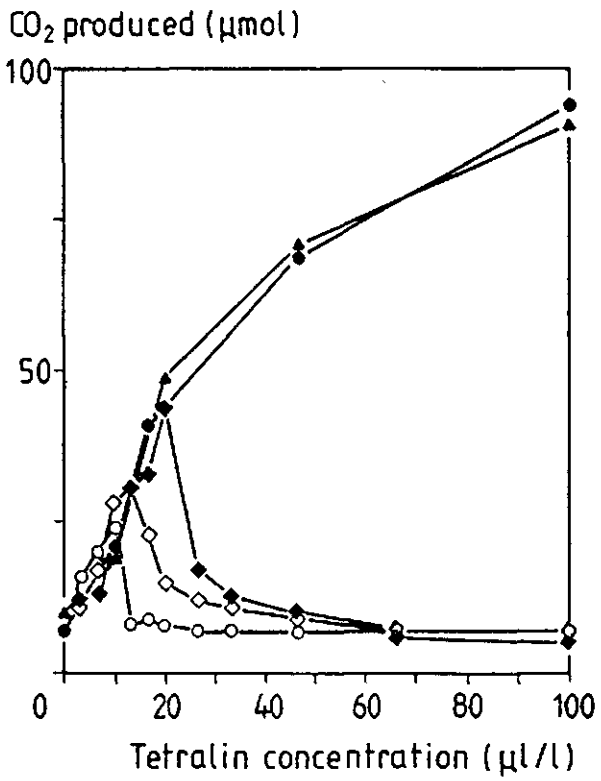

FIG. 2. Effects of organic solvents on carbon dioxide production from various tetralin concentrations by *Arthrobacter* T 2. CO_2 production was monitored after 7 days of incubation. Mineral salts medium in the absence of a solvent (○), and in the presence of 10 % FC 40 (◇), 20 % FC 40 (◆), 10 % DBP (●), and 20 % DBP (▲).

damaging effects on the cells. One method to lower the substrate concentration in the aqueous phase is the addition of an water-immiscible organic solvent. In Fig. 2 the beneficial effect of organic solvents is shown for dibutylphthalate (partition coefficient for tetralin 60) and the inert solvent fluorocompound 40 (partition coefficient for tetralin 8).

TABLE 3. Effect of various organic solvents (10% vol/vol) on the growth of pure cultures in yeast extract/glucose medium.

Organism	Solvent						
	FC 40	DOP	Hexa-decane	DBP	Tetra-lin	Hexane	DEP
logP	11.4	9.6	7.8	5.6	3.9	3.5	2.3
Arthrobacter A177	+	+	+	+	−	+	−
Corynebacterium C125	+	+	+	+	−	+	−
Nocardia S3	+	+	+	+	−	+	−
Pseudomonas A2	+	+	+	+	−	+	+
Acinetobacter T5	+	+	+	+	−	+	+
Arthrobacter T2	+	+	+	+	−	+	−
Arthrobacter T6	+	+	+	+	−	+	−
Moraxella T7	+	+	+	+	−	+	+
Mycobacterium E3	+	+	+	−	−	−	−
Nocardia corallina V49	+	+	+	+	−	+	−
Pseudomonas P58	+	+	+	+	−	+	+
Xanthobacter 124X	+	+	+	+	−	−	−

Serum bottles containing 8.5 ml of yeast extract/glucose medium and 1 ml of an organic solvent were inoculated with 0.5 ml of a pre-culture (1 mg wet weight/ ml) of the microorganism to be tested. Growth was assessed by monitoring the culture fluid turbidity and by measuring the amount of carbon dioxide produced.

General aspects of tetralin toxicity. It was also tested whether tetralin is toxic for bacteria that are not able to metabolize this compound. Following the procedure described by Inoue and Horikoshi (13) for toluene resistant organisms, it was attempted to obtain tetralin resistant organisms growing in a yeast extract/glucose medium. Incubating soil samples in yeast extract/glucose medium in the presence of tetralin (1% and 10% vol/vol) did not result in any growth after a two week incubation period, and it was concluded that no such tetralin resistant organisms were present in these samples. Also, pure cultures that were tolerant of various organic solvents (Table 3) were tested for their

resistance to tetralin. As shown in Table 3, none of the organisms tested was able to grow in the presence of 10 % (vol/vol) tetralin, although they were resistant to various other organic solvents (10% vol/vol).

DISCUSSION

Tetralin degradation by microorganisms has mainly been reported for mixed cultures or for pure cultures not growing on tetralin, but carrying out a cooxidative reaction. Hydrocarbon utilization by mixed cultures is not uncommon as demonstrated for instance for cyclohexane utilization (4). Cyclohexane, however, is also utilized by pure cultures (1, 27, 29). Degradation of hydrocarbons by pure cultures through cooxidation (18) has been reported for several compounds including cyclohexane, and methyl-substituted aromatic compounds. For tetralin, both mixed cultures growing on the compound as well as cooxidative systems have been described. Strawinski and Stone (28) established a mixed culture of motile Gram-negative rods growing on, amongst others, tetralin. Cooxidative degradation of tetralin has been observed in complex substrate mixtures (15, 26), and under defined conditions with one cosubstrate available (14, 23). Microorganisms isolated on hydrocarbons were not able to use tetralin as sole source of carbon and energy (17, 31). Only one report is available on tetralin utilization by a pure culture. Schreiber and Winkler (23) isolated *Pseudomonas stutzeri* AS 39 which grew poorly on tetralin-vapor after precultivating it on naphthalene.

During this investigation, eight bacteria were obtained that grow on tetralin. Both Gram-negative and Gram-positive strains were encountered, indicating tetralin utilization is not restricted to specific bacteria. However, growth rates on tetralin were low in all cases (Table 2), also when compared with growth rates of these organisms on other hydrocarbons. The observed low growth rates may be one of the reasons for the problems encountered in isolating microorganisms on tetralin. But toxicity of tetralin is also very important in the isolation and cultivation of the bacteria. Our results show that it is quite well possible to establish mixed cultures growing on tetralin. Purification of these enrichment cultures caused problems since tetralin inhibition occurs at concentrations above 15 μl/liter. Attempts to obtain pure cultures from the enrichments were only successful when tetralin was supplied in low concentrations. As shown in Fig. 3,

the beneficial effect of a two-phase system is directly related to the partition coefficient of the substrate over the aqueous phase and the organic solvent applied as well as to the relative amount of solvent in the system.

In expressing toxicity of solvents, the logP-value is a very useful parameter (16, 20). This logP-value is defined as the logarithm of the partition coefficient of a certain solvent in a standard octanol/water two-phase system (19). Laane et al. (16) and Rezessy-Szabo et al. (20) both found that solvents with a logP-value higher than 4 were generally not harmful to growing bacterial cells, and solvents with a logP-value higher than 3 did not fully inhibit biological activity. Tetralin has a logP-value of 3.86 (19) and therefore was not expected to be as toxic as observed. This toxicity is not directly coupled to metabolism of tetralin since bacteria not able to metabolize the compound were also affected by it. When tetralin was applied as a second phase (10% vol/vol) to cultures growing on glucose, bacteria were no longer metabolically active whereas other organic solvents, with a lower logP-value, did not affect these organisms. Similar observations have been made by Furuhashi et al. (8) when applying tetralin in an organic solvent/water two-phase system for the biocatalytic production of epoxyoctane. Moreover, it was not possible to isolate bacteria with an increased tetralin resistance following the protocol described for toluene (logP-value 2.5) by Inoue and Horikoshi (13). These observations indicate that tetralin is very toxic to microbial cells, and that the basis of this toxicity can not solely be explained on basis of the logP theory.

The catabolic pathways of the different organisms isolated will be studied subsequently to determine if one or more of the isolates initially attack tetralin at the aromatic ring. Two of the selected bacteria are known to attack substituted aromatics on the phenyl group and not on the more activated benzylic atom(s) of the substituent (11, 21). It therefore seems reasonable to expect that the aim, production of 5,6,7,8-tetrahydro-1-naphthol using one of these organisms eventually may be met.

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BIOCATALYTIC PRODUCTION OF HYDROXYLATED AROMATIC AND ALICYCLIC COMPOUNDS: PRODUCTS DERIVED FROM TETRALIN

SUMMARY

Biological hydroxylation is an area with many opportunities for the production of fine chemicals. Especially for the production of hydroxylated aromatic and alicyclic compounds, the specificity and mildness of biocatalysis can be beneficial. 1,2,3,4-Tetrahydronaphthalene (Tetralin) is a bicyclic aromatic compound, consisting of an aromatic and an alicyclic moiety. The biological oxidation of this compound provides an interesting model reaction, because different sites of initial attack are possible. Eight strains of bacteria that utilize tetralin as sole source of carbon and energy have been tested for the formation and accumulation of interesting oxidation products. Accumulation of oxidation products characteristic of both the oxidation of the aromatic and the alicyclic moiety has been demonstrated. Two strains, a *Corynebacterium* and an *Acinetobacter* have been studied in more detail. *Corynebacterium* C125 starts with initial attack on the aromatic ring by a dioxygenase type of enzyme resulting in the formation of a *cis*-dihydro diol that is chemically dehydrated to a phenol in an acid environment. *Acinetobacter* T5 starts with oxidation of the benzylic carbon atom, resulting in the formation of an alcohol, that is further oxidized via a ketone.

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INTRODUCTION

Hydroxylated aromatic and alicyclic compounds are important in the agrochemical, pharmaceutical, petrochemical and flavour industries (17, 4). In addition to bulk compounds such as phenol and cresols, many different specialty hydroxylated cyclic compounds are needed. However, these specialty compounds cannot be isolated in sufficient amounts from natural resources (e.g., mineral oil, plants) and, consequently, must be synthesized. Chemically, this can be achieved either by direct hydroxylation reactions or by indirect syntheses. Biologically, it is also possible to produce hydroxylated specialty compounds. In this article, we discuss opportunities to perform such direct biological hydroxylations and compare them to direct chemical hydroxylations. Biological oxidation of tetralin has been studied and serves to illustrate the biocatalytic potential of microbial enzymes in the production of hydroxylated compounds.

Chemical oxidation. Due to its aromatic ring structure, direct hydroxylation of the benzene nucleus is a difficult task in organic chemistry. Several catalysts have been investigated, each with specific advantages but usually with more disadvantages (22, 41, 44, 50, 64, 68, 75, 76). A general major problem is that introduction of the OH group activates the ring to further attack and, under these rather harsh hydroxylation conditions, most phenols are further oxidized to catechols and quinones (44, 76).

In alicyclic compounds, primary and secondary bonds especially resist hydroxylation and, consequently, preferentially tertiary bonds are hydroxylated (6, 15, 20, 29). Only in special situations are secondary bonds hydroxylated (44, 50, 65, 68, 75). For instance, cyclohexanol is produced from cyclohexane but yields are low (21). Furthermore, chemical oxidation reactions result mostly in racemic mixtures (58).

Hydroxylation of aliphatic side chains of aromatic compounds is also preferentially at tertiary CH bonds (46). The resulting tertiary alcohols are less susceptible to further oxidation than primary or secondary alcohols (33).

Biological oxidation. Microbial oxidation of aromatic and alicyclic compounds is common and the enzymes involved are either peroxidases (47), oxidases (42), or oxygenases (48, 57). Peroxidases and oxidases form activated oxygen derivatives (e.g., singlet oxygen, hydroxyl radicals and H_2O_2), and as with most chemical oxidations, are rather unspecific (3, 43). High yields are obtained only under special conditions (13, 42, 43).

Oxygenases, on the other hand, mainly oxidize only a limited number of substrates and, most important, are highly regiospecific. Monooxygenases catalyze the incorporation of one oxygen atom from molecular oxygen into the substrate, whereas the other atom is reduced to water (30). The electron donor required is reduced NAD(P) or ascorbic acid (49). Dioxygenases incorporate both atoms of molecular oxygen in a substrate and also require a reductant, such as NADH (49).

Aerobic degradation of aromatic compounds in yeasts and fungi occurs via monooxygenases, yielding epoxides which are further degraded via *trans*-dihydro diols to catechols (Fig. 1) or to glutathion adducts (79). In bacteria, monooxygenases in isolated cases, such as in the oxidation of toluene (18), may result in the formation of a phenol which in turn is further oxidized by another monooxygenase. In most bacteria, however, aromatic compounds are oxidized by a dioxygenase, yielding *cis*-dihydro diols which are further oxidized to catechols (11).

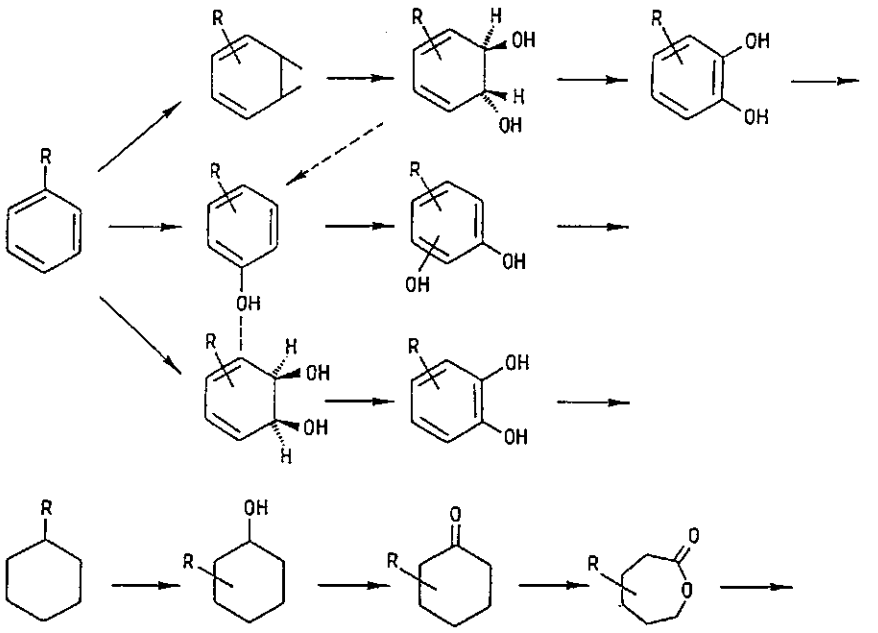

FIG. 1. Schematic presentation of oxygenase-catalyzed reactions of aromatic and alicyclic compounds. I: arene monooxygenase; II: epoxide hydrolase; III: *trans*-dihydrodiol dehydrogenase; IV: arene hydroxylase; V: phenol hydroxylase; VI: arene dioxygenase; VII: *cis*-dihydrodiol dehydrogenase; VIII: cycloalkane hydroxylase; IX: cycloalkanol dehydrogenase; X: cycloalkanone monooxygenase. Dotted lines indicate chemical rearrangement reactions from diols to phenols.

Catechols thus obtained in the various routes are cleaved by other dioxygenases. This fission can proceed by different routes, depending on the substrate and the available ring-cleaving enzymes (10).

Aerobic degradation of cycloalkanes occurs by an initial hydroxylation to an alcohol which may be optically active. The alcohol is then dehydrogenated to a ketone and, subsequently, an oxygen atom is introduced into the ring by a biological Bayer-Villiger oxidation (66, 71). The lactone formed is opened either enzymatically or hydrolytically (Fig. 1) (72).

In many instances, microorganisms prefer oxidation of an aliphatic substituent of either aromatic or alicyclic compounds rather than initial oxidation of the ring. The presence of a substituent thus introduces an extra site of attack (32, 37).

Biological oxidations in the production of fine chemicals from aromatic and alicyclic hydrocarbons. Biological oxidations are attractive in commercial processes in view of their regio- and enantioselectivity. However, when biocatalysis must compete with chemical synthesis, other aspects are important, such as stability, efficiency, recycling of the catalyst, substrate and product concentrations, and product recovery from aqueous solutions. Consequently, for most products, chemical synthesis is the method of choice. Biocatalysis is preferred only when the chemical synthesis takes too many steps, or when the reactions are difficult to perform. Presently, industrial production of hydroxylated aromatics and alicyclic compounds by direct biological oxidation is restricted to the hydroxylation of steroids (61). Some other biological processes are operated commercially on a very limited scale or are under consideration as illustrated below with a few examples.

Phenols: Phenols can be formed directly by the action of a monooxygenase or indirectly by acid hydrolysis of dihydro diols. A process based on direct hydroxylation has been considered for the formation of L-tyrosine from L-phenylalanine (31).

***cis*-Dihydro diols:** *cis*-Dihydro diols are formed by dioxygenase catalyzed oxidation of the aromatic ring (26, 27, 38). At present, ICI applies a fed-batch process for the commercial production of *cis*-dihydro diols from benzene, toluene and some other alkylsubstituted aromatic compounds (69). A small British biotech company, Enzymatix Ltd, produces halogen-substituted *cis*-dihydro diols (82).

Catechols: Catechols (benzene diols) are formed from dihydro diols by dehydrogenases (41, 54) or from phenols by monooxygenases. A process for the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) (63, 83, 84) has been studied.

Cycloalkanols: Cycloalkanols are formed from cycloalkanes by monooxygenases and are further oxidized to cycloalkanones. Apart from the transformation of steroids (61), apparently no other biological transformations of alicyclic compounds have been applied.

Alkyl substituents: Several monooxygenases regio- and enantioselectively oxidize alkyl groups of aromatic compounds (36, 37, 81). The introduction of an asymmetric carbon atom can be advantageous. Some pharmaceutical companies are especially interested in oxidized propyl and isopropyl derivatives, such as naproxen (28).

Tetralin. Tetralin (1,2,3,4-tetrahydronaphthalene, **1**) is a bicyclic molecule with an aromatic and a saturated ring. As a consequence, it has features of aromatic and alicyclic compounds, as well as of aliphatic substituents of aromatics. It is, therefore, very suitable as a model compound in studying microbial hydroxylation of hydrocarbons. Tetralin is mainly used as a solvent for oils, fats, waxes, resins, asphalt, and rubber, and as a substitute for turpentine in shoe polish, oil paints and lacquers. It has also been used as a larvicide against mosquitoes (8, 24).

Presently, only limited information is available on the biological transformation of tetralin. Inhalation studies performed with dogs, rabbits, rats (16, 53, 56), and humans (14, 56) indicated tetralin is oxygenated preferentially at the benzylic carbon atom (16), C1 or C4.

Microbial transformation of tetralin has received even less attention, probably because the compound is very toxic to microbes (62, 67). Some conversion studies performed under cooxidation conditions (23, 36, 39, 60), indicated microorganisms prefer initial attack at the benzylic carbon atom. In this way, an asymmetric carbon atom is formed. It has been demonstrated (36) that the fungus *Cunninghamella elegans* produced α-tetralol (1,2,3,4-tetrahydro-1-naphthol, **2**) with an enantiomeric excess of 60% *R*. Tetralol may be further oxidized to yield α-tetralone (1,2,3,4-tetrahydro-1-naphthalenone, **3**) (23, 36, 39, 60) or α-hydroxybenzenebutanoic acid as a result of alicyclic ring cleavage (39).

However, apart from initial attack directed at the benzylic carbon atoms, it may also be envisaged that a microorganism would rather oxidize the aromatic ring. Most likely, *cis*-dihydro diols (bacteria) or *trans*-dihydro diols (yeasts and fungi) would be formed, which in the organisms would be further degraded via catechols. Chemically, the intermediairy dihydro diols may undergo acid rearrangement to phenols. It is even possible to obtain phenols via direct hydroxylation (18). Figure 2 gives an overview of intermediates and products that may possibly accumulate from tetralin by microbial action. Some of these intermediates can be applied in

different industries:

* α-Tetralol (2) and α-tetralone (3) are used as building blocks in the synthesis of insecticides (7), tranquilizers (80), and hormone analogs (5). These compounds are presently produced chemically (12, 35, 70).
* 5,6,7,8-Tetrahydro-1-naphthol (5) is an interesting fragrance compound with a leather-like smell, which presently is chemically synthesized by a catalytic hydrogenation of 1-naphthol (52).

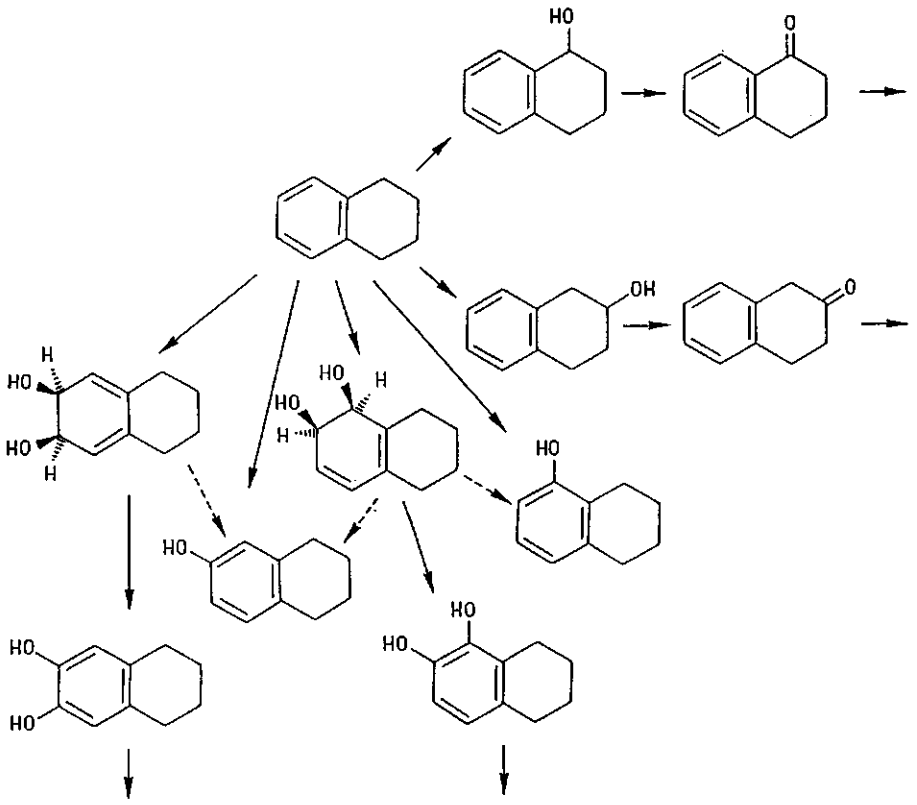

FIG. 2. Potential sites for initial oxidation of tetralin. I and II: dioxygenases analogous to bacterial benzene dioxygenase (2) which oxidizes benzene to *cis*-3,5-cyclohexadiene-1,2-diol which subsequently is dehydrogenated to catechol. Similar enzymes exist for toluene (26), ethylbenzene (25) and naphthalene (40); III and IV: hydroxylases analogous to bacterial cyclohexane hydroxylases (66, 71) which introduce one oxygen atom into the ring of a wide range of cycloalkanes (78); V and VI: hydroxylases analogous to bacterial toluene hydroxylase (18). Dotted lines indicate chemical rearrangement reactions from diols to phenols.

MATERIALS AND METHODS

Microorganisms. The bacteria were obtained from the culture collections of the Department of Microbiology (*Arthrobacter* A177 (59), *Corynebacterium* C125 (59) and *Pseudomonas* A2) and the Division of Industrial Microbiology (*Nocardia* S3 (34)) of the Agricultural University Wageningen, The Netherlands. Newly isolated strains were obtained by setting up enrichment cultures with tetralin as sole source of carbon and energy (62).

Maintenance and cultivation of microorganisms. Bacteria were kept on slants of 5 g/liter glucose and 3.5 g/liter yeast extract medium to which Oxoid no. 3 agar (15 g/liter) had been added, and also on slants of a mineral salts medium, containing in 1 liter of demineralized water: K_2HPO_4, 1.55 g; $NaH_2PO_4.2H_2O$, 0.85 g; NH_4Cl, 2.0 g; $(NH_4)_2SO_4$, 0.1 g; $MgCl_2.6H_2O$, 0.075 g and 0.2 ml of a trace elements solution (74). The mineral medium slants were placed in a 5-liter desiccator containing a flask with 50 μl tetralin.

Cells were grown in 5-liter Erlenmeyer flasks containing 1 liter of mineral salts medium, with tetralin supplied by the vapor phase (9).

Incubations. Cells were harvested in the late-exponential growth phase by centrifugation (16,000 × *g* for 10 min at 15°C), washed twice with potassium phosphate buffer, pH 7.0 (50 mM) and resuspended in the same buffer (2 times the cell volume). Incubation experiments with washed cells were performed at 30°C with suspensions of freshly harvested cells. The cells were incubated in 100 ml serum bottles with 10 ml phosphate buffer (50 mM; pH 7.0) with 10 μl tetralin.

Extraction procedure. Cells were removed by centrifugation, the supernatants were acidified to pH 2.0 with 5.0 M HCl and extracted three times with a half-volume of ethyl acetate. The solvent was removed in a rotary evaporator after drying over anhydrous Na_2SO_4 and the residue was dissolved in hexane.

Analytical techniques. TLC analysis of the extracts was performed on silica gel plates (Merck, Darmstadt, Germany) as described by Elliott and Hanam (16), the solvent system used was hexane/ethyl acetate (1:1, vol/vol).

GLC analysis was performed using a CP-9000 gas chromatograph with on-column injector (Chrompack BV, Middelburg, The Netherlands) fitted with a 25 m × 0.32 mm fused silica WCOT CP-Sil 8 CB column (Chrompack). Gas-flow rates were: $He/H_2/air$ = 30/20/300 (ml min^{-1} each); temperature of the FID detector was 300°C. The column oven was programmed from 80°C initial temperature to 200°C at a rate of 10°C/min.

Mass spectra were recorded on a MAT 6H7A mass spectrometer (Finnigan-MAT), inlet temperature 100°C, electron impact 70 eV by Mr. A. de Boer, Quest International, Naarden, The Netherlands.

Determination of the enantiomeric composition of α-tetralol. The enantiomeric composition of the accumulated α-tetralol was determined by capillary gas chromatography (77) after derivatizing the alcohol with acetyl chloride to α-tetralol acetate. The column, a Chrompack-Cyclodextrin β-236-M19 (50 m by 0.32 mm) was fitted into a CP 9000 gaschromatograph (Chrompack) with split injector (100 to 1). The injector temperature was 200°C, the temperature of the FID was 300°C and He was used as the carrier gas. The analyses were performed at 140°C. The α value for the two enantiomers was 1.03.

Chemicals. 1,2,3,4-Tetrahydronaphthalene (**1**), 1,2,3,4-tetrahydro-1-naphthol (**2**), 5,6,7,8-tetrahydro-1-naphthol (**5**), 5,6,7,8-tetrahydro-2-naphthol (**6**), and *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol were purchased from Janssen Chimica (Beerse, Belgium). 1,2,3,4-Tetrahydro-1-naphthalenone (**3**), (*R*)-(−)-1,2,3,4-tetrahydro-1-naphthol and (*S*)-(+)-1,2,3,4-tetrahydro-1-naphthol were obtained from Aldrich (Brussels, Belgium). Fluorocompound 40 was obtained from 3M (St. Paul, Minn.). All other chemicals were of commercially available analytical grade.

RESULTS AND DISCUSSION

Selection of tetralin-utilizing microorganisms. Two approaches were followed to obtain organisms with the ability to regioselectively oxidize tetralin. Firstly, it was attempted to isolate tetralin degraders from soil and water samples. To achieve this, enrichment cultures were set up with tetralin added to the water phase in quantities of 250 and 500 μl/liter. This method, however, did not result in the isolation of microorganisms, probably as a consequence of the toxicity of tetralin (19, 60, 67).

This toxicity was overcome by supplying tetralin via the vapor phase (9), resulting in less than 15 μl tetralin per liter in the water phase. From such enrichment cultures, two strains were isolated which grew on tetralin. In another attempt to lower the substrate concentration in the water phase, an organic solvent/water two-phase system was applied. A biologically inert organic solvent, fluorocompound 40, was used as organic phase, which functions as a reservoir for the toxic substrate (55). From these enrichments, two other strains were obtained that also grew on tetralin.

TABLE 1. Tetralin-utilizing bacteria and accumulation of products[a].

Organism	Isolation substrate	Doubling time on tetralin (h)	Rf-values[b] TLC	Retention times[c] GLC (min)
Acinetobacter T5	Tetralin	24	0.73	7.4; 7.9
Arthrobacter T2	Tetralin	18	0.75	7.5; 7.9
Arthrobacter T6	Tetralin	>72	0.76	7.8
Moraxella T7	Tetralin	23	0.76	7.9
Nocardia S3	Styrene	56	0.75	7.8
Arthrobacter A177	*o*-Xylene	20	0.73; 0.82	7.5; 8.9
Pseudomonas A2	Mesitylene	50	0.73; 0.82	7.5; 7.9; 8.9
Corynebacterium C125	*o*-Xylene	20	0.83	8.6; 8.9

[a] Products accumulated in cultures growing on tetralin were analyzed by TLC and GLC.

[b] R_f-values for the standard chemicals chromatographed were: 1,2,3,4-tetrahydro-1-naphthol **(2)** 0.74; 1,2,3,4-tetrahydro-1-naphthalenone **(3)** 0.75; 5,6,7,8-tetrahydro-1-naphthol **(5)** 0.83; 5,6,7,8-tetrahydro-2-naphthol **(6)** 0.82.

[c] The retention times for the standard chemicals chromatographed were: tetralin **(1)** 5.2; 1,2,3,4-tetrahydro-1-naphthol **(2)** 7.5; 1,2,3,4-tetrahydro-1-naphthalenone **(3)** 7.9; 5,6,7,8-tetrahydro-1-naphthol **(5)** 8.6; 5,6,7,8-tetrahydro-2-naphthol **(6)** 8.9 (minutes).

In a second approach, pure cultures from culture collections were tested for tetralin degradation. Cultures tested were selected on the basis of their ability to grow on substituted aromatic compounds. Using this method, four strains were obtained which had originally been isolated on *o*-xylene (59), styrene (34), and mesitylene.

The eight bacteria thus obtained were classified and culture-doubling times on tetralin were assessed (Table 1). Tetralin is very toxic to these strains since no organism could grow at tetralin concentrations higher than 15 μl/liter. Previously, other researchers found it difficult to obtain microorganisms that can utilize tetralin as sole source of carbon and energy (45, 60, 73). From our results, it appears that the toxicity of tetralin, in combination with the observed low growth rates, may explain the limited success of others in obtaining tetralin utilizers.

Accumulation of various intermediates. The eight tetralin-utilizing bacteria were tested for accumulation of intermediates in tetralin metabolism. Identification of the compounds accumulated by resting cells was carried out by TLC and GLC analysis of ethyl acetate extracts. Interestingly, these analyses revealed the presence of different compounds characteristic of the different possibilities of initial attack on tetralin (Fig. 2). Most strains (T5, T2, T6, T7, S3) favoured initial attack on the benzylic carbon atom since they accumulated α-tetralol (2) and α-tetralone (3) (T2, T5), or α-tetralone (S3, T6, T7). Accumulation of α-tetralol and α-tetralone has previously been reported for strains of *Aspergillus niger* (23), *Nocardia corallina* (4), *Pseudomonas stutzeri* (60), and *Cunninghamella elegans*, *Helminthosporium sp.* and *Mortierella isabellina* (36). Two strains produced α-tetralol and 5,6,7,8-tetrahydro-2-naphthol (**6**) (A177 and A2) with strain A2 also producing α-tetralone. The formation of these mixed products is indicative of both aromatic and alicyclic attack. This is probably due to an unspecific action by the hydroxylase. Only one organism, *Corynebacterium* sp. C125, produced predominantly phenolic compounds. The observed accumulation of 5,6,7,8-tetrahydro-1-naphthol (**5**) and 5,6,7,8-tetrahydro-2-naphthol (**6**) in acidified supernatants of incubation media of *Corynebacterium* sp. C125 indicates initial attack on the aromatic moiety. This is in agreement with the observation by Schraa et al. (59) that *Corynebacterium* strain C125 starts with an oxidation of the ring, and not with hydroxylation of the methyl groups. These results show the biological potential available in microorganisms and that regioselectivity can differ from one strain to another.

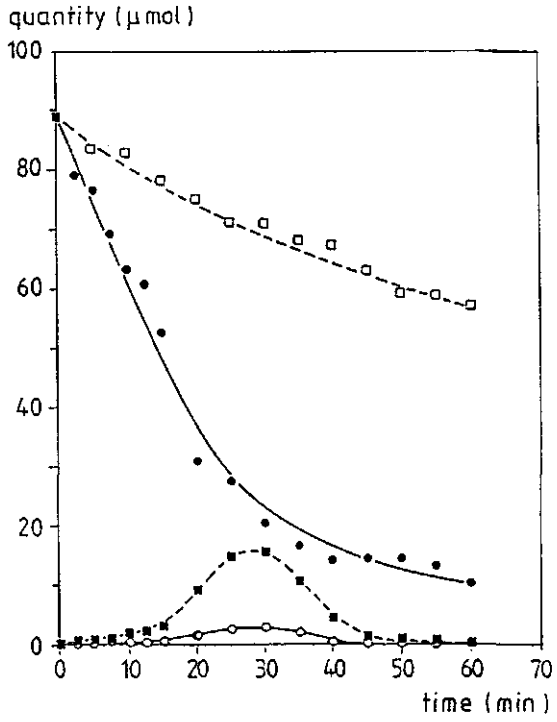

FIG. 3. Consumption of tetralin and accumulation of products by *Corynebacterium* C125. Tetralin (1) consumption (●) and accumulation of 5,6,7,8-tetrahydro-1-naphthol (5) (○) and 5,6,7,8-tetrahydro-2-naphthol (6) (■) by cell suspensions of *Corynebacterium* C125 cultivated on *o*-xylene. The incubation mixture (10 ml) contained 6.2 mg protein, 90 μmol tetralin and 100 μmol *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol. In 60 minutes approximately 50% of the initially added tetralin was consumed. This resulted in an accumulation of 25% of oxygenated products at maximum. (□) Gives the depletion of tetralin due to evaporation and to adhesion to bottles and stoppers, in the absence of cells.

Product accumulation by *Corynebacterium* sp. C125 and *Acinetobacter* sp. T5. Strains C125 and T5 were taken for studying accumulation of intermediates in more detail. *Corynebacterium* C125 cells were incubated with tetralin and with an equimolar amount of *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol. This dihydro diol probably serves as a competitive inhibitor of the dehydrogenation for any *cis*-dihydro diol formed. Upon acidification of the reaction mixture, 5,6,7,8-tetrahydro-2-naphthol (**6**) and traces of 5,6,7,8-tetrahydro-1-naphthol (**5**) were observed (Fig. 3). The identity of these products was confirmed by GC/MS analysis of the extracts (Fig. 4). These compounds were probably formed in the acid environment from *cis*-1,2,5,6,7,8-hexahydroxy-1,2-naphthalene diol (**4**). Formation of tetralin *cis*-dihydro diol on a preparative scale would, therefore, be feasible by selecting a mutant that lacks the *cis*-dihydro diol dehydrogenase, as reported for the production of *cis*-dihydro diols of amongst others naphthalene (40), toluene (26), and benzene (74). However, the hydroxylation of tetralin by *Corynebacterium* C125 is not applicable for the production of 5,6,7,8-tetrahydro-1-naphthol (**5**), due to the chemically preferred rearrangement of the *cis*-dihydro diol to 5,6,7,8-tetrahydro-2-naphthol (**6**).

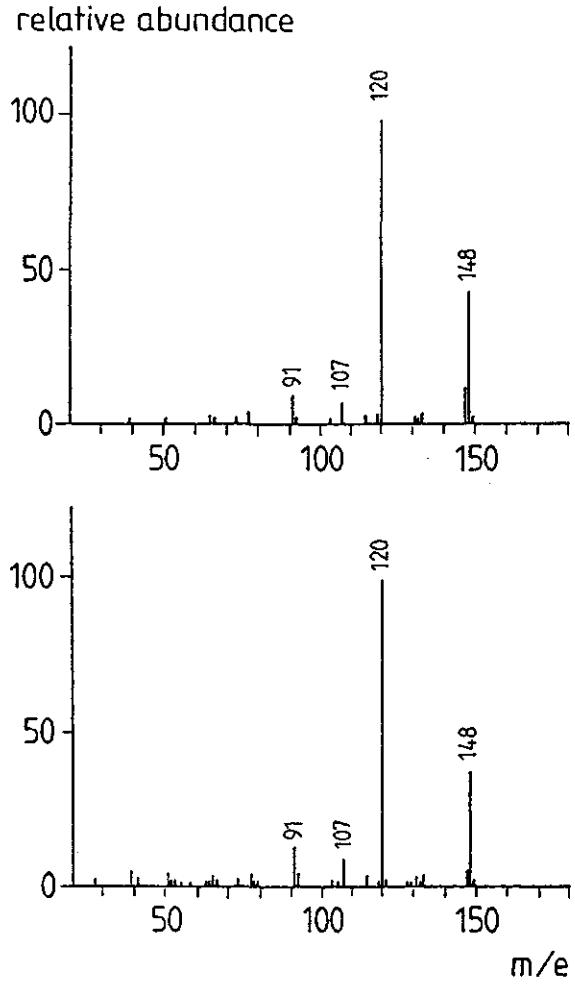

FIG. 4. Mass spectra from products derived from tetralin. Relative abundance of mass fragments in the spectra of 5,6,7,8-tetrahydro-1-naphthol (**5**, top) and 5,6,7,8-tetrahydro-2-naphthol (**6**, bottom).

During growth on tetralin, *Acinetobacter* T5 showed accumulation of 1,2,3,4-tetrahydro-1-naphthalenone (**3**) from tetralin, while also trace amounts of 1,2,3,4-tetrahydro-1-naphthol (**2**) were detected (Table 1). This observation suggests that the degradation of tetralin in strain T5 is by initial oxidation to 1,2,3,4-tetrahydro-1-naphthol, which is further oxidized to 1,2,3,4-tetrahydro-1-naphthalenone. Partial inhibition of the dehydrogenation of 1,2,3,4-tetrahydro-1-naphthol was achieved by adding cyclohexanol to the reaction mixture (Fig. 5). Cyclohexanol probably acts as a competitive inhibitor of the alcohol dehydrogenase. The enantiomeric composition of the accumulated alcohol was determined and it appeared that *Acinetobacter* T5 produced the *S* form with an enantiomeric excess of 20 %.

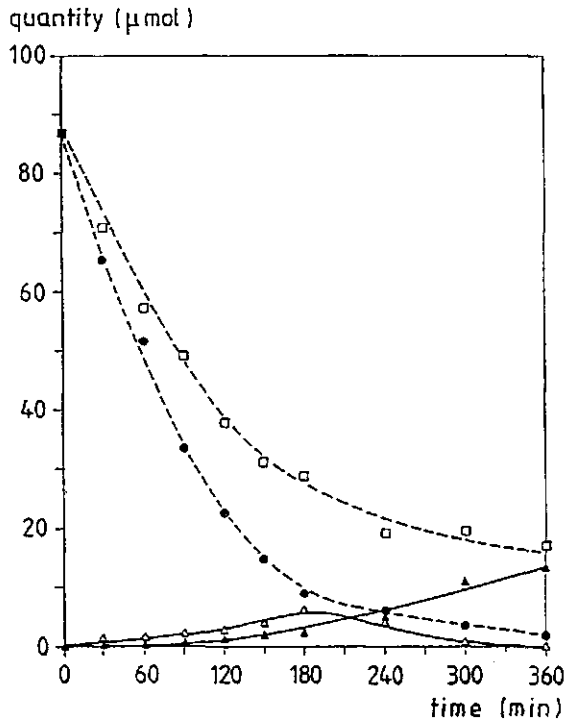

FIG. 5. Consumption of tetralin and accumulation of products by *Acinetobacter* T5. Tetralin (1) consumption (●) and accumulation of 1,2,3,4-tetrahydro-1-naphthol (2) (△) and 1,2,3,4-tetrahydro-1-naphthalenone (3) (▲) by cell suspensions of *Acinetobacter* T5 cultivated on tetralin. The incubation mixture (10 ml) contained 1.4 mg of protein, 90 µmol tetralin and 100 µmol cyclohexanol. In 6 h, 20% of the initially added tetralin was oxidized; during this period no further consumption of the oxygenated products seemed to occur. (□) Gives the depletion of tetralin due to evaporation and to adhesion to bottles and stoppers, in the absence of cells.

CONCLUSIONS

Bio-oxidation of tetralin provides an interesting model reaction, because different initial sites of oxidative attack are possible (Fig. 2). From our results, it appears that microorganisms exist that can selectively oxidize tetralin either at the aromatic moiety or at the alicyclic substituent (Table 1). However, at present, conversion rates and tolerance towards tetralin are too low to establish any economically interesting hydroxylation process. Therefore, the physiological basis of these factors must be studied in more detail.

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Chapter 5

METABOLISM OF TETRALIN (1,2,3,4-TETRAHYDRONAPHTHALENE) IN CORYNEBACTERIUM SP. STRAIN C125

SUMMARY

Corynebacterium sp. strain C125, originally isolated on *o*-xylene, was selected for its ability to grow on tetralin (1,2,3,4-tetrahydronaphthalene) as the sole source of carbon and energy. The catabolism of tetralin in *Corynebacterium* sp. strain C125 was shown to proceed via initial hydroxylation of the benzene nucleus at positions C-5 and C-6, resulting in the formation of the corresponding *cis*-dihydro diol. Subsequently, the dihydro diol was dehydrogenated by a NAD-dependent dehydrogenase to 5,6,7,8-tetrahydro-1,2-naphthalene diol. The aromatic ring was cleaved in the extradiol position by a catechol-2,3-dioxygenase. The ring fission product was subject to a hydrolytic attack, resulting in the formation of a carboxylic acid-substituted cyclohexanone. This is the first report of the catabolism of tetralin via degradation of the aromatic moiety.

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INTRODUCTION

Tetralin (1,2,3,4-tetrahydronaphthalene) consists of an aromatic and an alicyclic moiety. The compound occurs in coal tar and petroleum and is produced for industrial purposes either from naphthalene by catalytic hydrogenation or from anthracene by cracking. Tetralin is widely applied as a solvent in the petrochemical industry, in which it is particularly used in connection with coal liquefaction. It is also used in paints and waxes as a substitute for turpentine (11).

Tetralin was slowly degraded by mixed cultures of microorganisms (41) or in the presence of cosubstrates (19, 40), but it was persistent as a single substrate in experiments with pure cultures (33). Until recently, its metabolism had only been studied in strains that transformed tetralin under cooxidative conditions (10, 17, 19, 33).

In a previous paper, we reported eight bacteria that were able to utilize tetralin as the sole source of carbon and energy (35). It was shown subsequently that tetralin is extremely toxic to microbial cells as a result of its selective partitioning into cell membranes (36). Four of the eight tetralin-utilizing bacteria were isolated by selective enrichment on tetralin, while the other organisms had been isolated previously by others on other substrates (*o*-xylene, styrene, and mesitylene). In this paper, we report on the metabolism of tetralin in the *o*-xylene-isolated *Corynebacterium* sp. strain C125, which grew relatively well on tetralin (32).

MATERIALS AND METHODS

Microorganism and cultivation conditions. *Corynebacterium* sp. strain C125 was isolated previously from an enrichment culture with *o*-xylene as the sole source of carbon and energy (32). The strain was kept on slants of a mineral salts medium to which 15 g of Oxoid no. 3 agar liter^{-1} was added. The mineral salts medium contained the following (per liter of demineralized water): 1.55 g of K_2HPO_4, 0.85 g of $NaH_2PO_4 \cdot 2H_2O$, 2.0 g of $(NH_4)_2SO_4$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 10 mg of EDTA, 2 mg of $ZnSO_4 \cdot 7H_2O$, 1 mg of $CaCl_2 \cdot 2H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 0.2 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg of $CuSO_4 \cdot 5H_2O$, 0.4 mg of $CoCl_2 \cdot 6H_2O$, and 1 mg of $MnCl_2 \cdot 2H_2O$ (14). The organism was routinely grown in a chemostat on mineral medium with *o*-xylene added by the vapor phase (43), tetralin added with a micropump (Braun, Melsingen, Germany), or succinate (0.5% [wt/vol]) added directly to the medium.

Growth studies were performed in 100-ml serum bottles containing 10 ml of mineral medium. The hydrocarbon substrates were added in the vapor phase via small tubes placed in the bottle. Growth was assessed by monitoring the culture fluid turbidity together with the production of carbon dioxide from the supplied substrates (35).

Suspensions of washed cells and cell extracts. Cells were harvested by centrifugation in a Sorvall 5-B centrifuge at 4°C and 16,000 × *g*, washed twice with potassium phosphate buffer (pH 7.0, 50 mM),

and suspended in the same buffer. Cell extracts were prepared by ultrasonication of a washed cell suspension (probe type sonicator; Branson, Danbury, Conn.) 10 times for 30 s each time at 4°C. Debris was removed by centrifugation at 27,000 × *g* for 30 min (4°C); the supernatant, containing 10 to 15 mg of protein ml^{-1}, was designated the crude cell extract. Protein was determined by the method of Bradford (4), using bovine serum albumin as a standard.

Oxygen consumption experiments. Oxygen consumption by washed suspensions of intact cells in 50 mM potassium phosphate buffer (total volume, 3 ml) was measured polarographically with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C. Endogenous oxygen uptake was measured for 5 min at 30°C; subsequently, 0.05 ml of a mixture containing 10 mM substrate in *N*-dimethylformamide was added, and oxygen uptake was monitored for at least another 5 min. *N*-dimethylformamide neither induced oxygen uptake nor inhibited respiratory activity of the cells at the concentration applied.

Enzyme assays. All enzyme assays were performed at 30°C. The aryl dioxygenase was assayed polarographically with a Clark type oxygen electrode (12) in 50 mM potassium phosphate buffer (pH 7.0) in the presence of NAD(P)H (0.1 mM), and the substrate was dissolved in *N*-dimethylformamide (final assay concentration, 0.1 mM). Results were corrected for endogenous oxygen consumption in the absence of the aromatic substrate.

The activity of *cis*-1,2-dihydro diol dehydrogenase was determined by monitoring the rate of reduction of NAD^+ at 340 nm in potassium phosphate buffer (pH 7.0). The reaction was started by adding the *cis*-1,2-dihydro diol to a final concentration of 1 mM (3).

The activities of the *ortho* ring fission dioxygenase with the various catechols were measured polarographically with an oxygen electrode by the method of Hayaishi et al. (15). The *meta* cleavage dioxygenase was assayed with various catechols by measuring the formation of ring fission products spectrophotometrically (22). The molar extinction coefficients, if not known, were determined by the method of Duggleby and Williams (8).

The ring fission products hydrolase and dehydrogenase were assayed by monitoring the disappearance of the substrates prepared by the method of Sala-Trepat et al. (31), except that heat-treated (55°C for 15 min) cell extracts of *Corynebacterium* sp. strain C125 (prepared from *o*-xylene-grown cells) were used. The assays were performed with dialyzed cell extracts; in the dehydrogenase assay, NAD^+ (final concentration, 1 mM) was included (30).

Incubation experiments. Incubations with whole cells were performed at 30°C in 100-ml serum bottles containing 50 mM potassium phosphate buffer, 75 μmol of tetralin, and freshly harvested cells of *Corynebacterium* sp. strain C125 (10 mg of protein) in a total volume of 10 ml.

Inhibition of the *cis*-dihydro diol dehydrogenase was achieved by adding 100 μmol of *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol (*cis*-toluene glycol) as a competitive inhibitor (34). After 30 min, the cells were removed by centrifugation and the supernatant was extracted two times with 0.5 volume of ethyl acetate. The catechol-cleaving dioxygenase was inhibited by 0.05 mg of pyrogallol (1,2,3-trihydroxybenzene) ml^{-1} (16, 38). After various times of incubation, cells were removed by centrifugation, and supernatants were acidified to pH 2.5 with 5.0 N HCl and extracted three times with 0.5 volume of ethyl acetate. The solvent was removed in a rotary evaporator after drying over anhydrous Na_2SO_4, and the residue was dissolved in hexane. The hexane phase was washed twice with an equal volume of water to remove excess pyrogallol and oxidation products.

Chemical analyses. Dihydro diols were determined by gas chromatography (GC) of acidified extracts, as described previously (34). Catechols were detected by the method of Nair and Vaidyanathan (26). The presence of a free aldehyde group was assessed by the Tollens test (9). Enols were assayed by the $FeCl_3$ test, as outlined in Mann and Saunders (24). Picolinate derivatives were prepared by the method of Canonica et al. (5). Pyruvate was determined as described by Chakrabarty (6) by measuring the oxidation of NADH at 340 nm in the presence of an excess of lactic acid dehydrogenase.

Analytical techniques. Carbon dioxide production was determined by injecting 0.1-ml headspace samples on a Packard 427 GC (Packard/Becker, Delft, The Netherlands) fitted with a Porapack Q column (Chrompack B.V., Middelburg, The Netherlands).

GC of the incubation extracts was performed on a Chrompack CP 9000 GC with on-column injector (Chrompack) fitted with a fused silica WCOT CP-Sil 8 CB column (Chrompack) (25 m by 0.32 mm). Gas flow rates were as follows: $He/H_2/air$ = 30/20/300 ml/min. The temperature of the flame ionization detector was 300°C. The column oven was programmed from 80°C initial temperature to 280°C at a rate of 10°C/min.

Mass spectra of the accumulated intermediates were recorded on a MAT 6H7A mass spectrometer (MS; Finnigan-MAT, San Jose, Calif.), with an inlet temperature of 100°C, and an electron impact of 70 eV. Accumulated incubation products were identified by GC/MS, using an HP 5890 GC (Hewlett-Packard, Palo Alto, Calif.) coupled to an HP 5970 mass selective detector.

Spectral analyses were performed with a Perkin-Elmer λ2 spectrophotometer (Perkin-Elmer, Ueberlingen, Germany).

Chemicals. 1,2,3,4-Tetrahydronaphthalene, catechol, *cis*-3,5-cyclohexadiene-1,2-diol, and *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol were obtained from Janssen Chimica (Beerse, Belgium). 3-Methylcatechol was purchased from Lancaster (Morecamb, United Kingdom), 4-methylcatechol was obtained from Merck (Darmstadt, Germany), and 1,2-dihydroxynaphthalene and 2,3-dihydroxynaphthalene were obtained from Aldrich (Brussels, Belgium). Lactic acid dehydrogenase and all other biochemicals were purchased from Boehringer (Mannheim, Germany).

RESULTS

Growth characteristics. *Corynebacterium* sp. strain C125 is able to use tetralin and several other aromatic hydrocarbons as the sole source of carbon and energy. Toluene, ethylbenzene, and *o*-xylene were good substrates (specific growth rate [μ] = approximately 0.17 h^{-1}), while moderate growth was observed with benzene, tetralin, naphthalene, *o*-diethylbenzene, biphenyl, and indane (μ = approximately 0.05 h^{-1}). No growth occurred with cyclohexane, cyclohexene, and decalin (decahydronaphthalene).

Oxygen consumption experiments. Oxygen consumption rates of washed cell suspensions of *Corynebacterium* sp. strain C125 grown on *o*-xylene, tetralin, or succinate were monitored in the presence of various substrates (Table 1). Cells grown on either *o*-xylene or tetralin readily oxidized related aromatic hydrocarbons and catechols. Succinate-grown cells were not adapted to the aromatic compounds.

Accumulation and identification of intermediates. Under specific conditions, the activities of enzymes involved in the metabolism of tetralin could be inhibited. Accumulation of the first intermediate in the degradative pathway of tetralin was achieved by the addition of 0.5 mM *cis*-3-methyl-3,5-cyclohexadiene-1,2-diol (*cis*-toluene glycol) as a competitive inhibitor for the *cis*-dihydro diol dehydrogenase. Whole-cell incubations in the presence of *cis*-toluene glycol resulted in the accumulation of an intermediate which disappeared upon the addition of 5 N HCl. The addition of the acid also resulted in the disappearance of *cis*-toluene glycol.

TABLE 1. Rates of oxygen consumption by washed cell suspensions of *Corynebacterium* sp. strain C125 grown on *o*-xylene, tetralin, or succinate

Assay substrate	Oxygen consumption[a] (nmol of $O_2 \cdot min^{-1} \cdot mg$ of cell protein^{-1})		
	o-Xylene	Tetralin	Succinate
o-Xylene	280	155	<5
Benzene	100	35	<5
Toluene	300	165	<5
Ethylbenzene	230	110	<5
o-Diethylbenzene	215	85	<5
Naphthalene	145	65	<5
Tetralin	180	80	<5
Biphenyl	135	ND[b]	<5
Catechol	305	180	<5
3-Methylcatechol	760	525	5
4-Methylcatechol	265	150	5
1,2-Dihydroxynaphthalene	2,800	785	5
2,3-Dihydroxynaphthalene	10	5	<5

[a] Corrected for endogenous oxygen uptake.
[b] ND, not determined.

GC of the acidified mixture revealed four new peaks that were identified by MS as 5,6,7,8-tetrahydro-1-naphthol, 5,6,7,8-tetrahydro-2-naphthol, *o*-cresol, and *m*-cresol. These data are consistent with 1,2,5,6,7,8-hexahydro-*cis*-1,2-naphthalene diol and *cis*-toluene glycol being the original compounds which had been acid dehydrated to the respective phenols. The ratio of 5,6,7,8-tetrahydro-1-naphthol to 2-naphthol formed by acid-catalyzed dehydration was 1:6.

Addition of pyrogallol to washed cells of *Corynebacterium* sp. strain C125 incubated with tetralin resulted in the accumulation of a compound which gave a positive reaction in the catechol assay. GC/MS analysis of ethyl acetate extracts revealed a compound with a molecular ion peak at *m/e* 164 [M^+] and with other prominant peaks at *m/e* 136 [M-28]$^+$, *m/e* 119 [M-28-17]$^+$, *m/e* 105 [M-28-17-14]$^+$ (Fig. 1). Comparison with mass spectra of related catechols showed that this structure is consistent with a dihydroxylated tetralin.

During growth of *Corynebacterium* sp. strain C125 on aromatic compounds, coloring of the medium was observed. To obtain more insight into the nature of these yellow compounds, spectra of the incubation mixtures were recorded at pH 2.5, 7.0, and 12.0. The absorption maxima observed for the ring fission products of

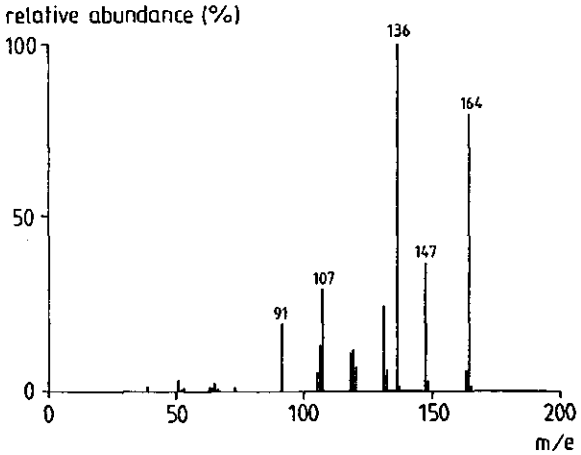

FIG. 1. Relative abundances of mass fragments in the spectrum of 5,6,7,8-tetrahydro-1,2-naphthalene diol.

benzene, toluene, ethylbenzene, *o*-xylene, and naphthalene were identical to values reported in the literature (2, 7, 8). In Fig. 2 the absorption spectra of the incubation mixture of *Corynebacterium* sp. strain C125 with tetralin at different pH values are presented. The shifts in absorption maxima upon changing the pH suggest that the compounds accumulated in the incubation broth were capable of keto-enol tautomerism. Extraction of incubation mixtures yielded only small quantities of a brown oil, which gave a positive $FeCl_3$ test, indicating the presence of an enol function. The isolated product showed a negative reaction in the Tollens test (9), indicating the absence of a free aldehyde group. Lyophilization of the cultivation broth of *Corynebacterium* sp. strain C125 grown on tetralin yielded a yellow powder.

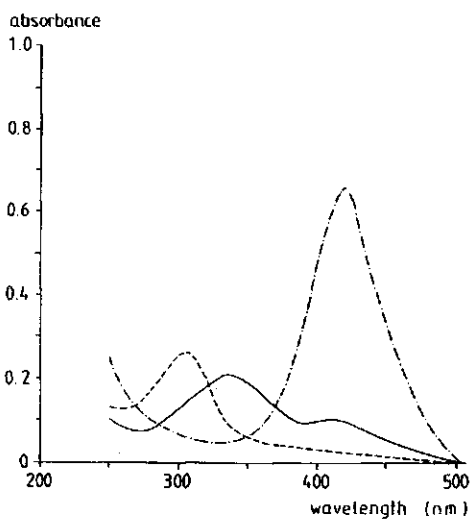

FIG. 2. Absorption spectra of the ring fission product of tetralin at different pHs. The molar extinction coefficients at the respective absorption maxima are as follows: pH 2.5, ϵ_{306} = 19,520 $M^{-1} \cdot cm^{-1}$; pH 7.0, ϵ_{336} = 16,400 $M^{-1} \cdot cm^{-1}$; pH 12.0, ϵ_{418} = 51,200 $M^{-1} \cdot cm^{-1}$.

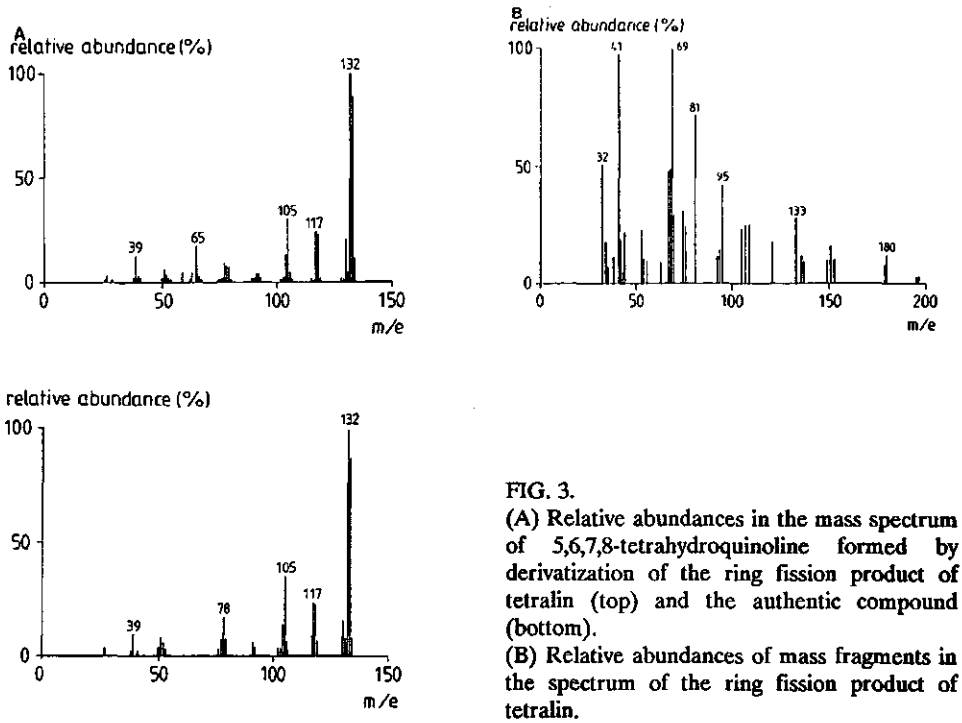

FIG. 3.
(A) Relative abundances in the mass spectrum of 5,6,7,8-tetrahydroquinoline formed by derivatization of the ring fission product of tetralin (top) and the authentic compound (bottom).
(B) Relative abundances of mass fragments in the spectrum of the ring fission product of tetralin.

Dissolution of the powder in diethyl ether and subsequent GC/MS analysis revealed the presence of a number of structures consistent with carboxylic acid-substituted cyclohexanone derivatives. Only the presence of 2-oxocyclohexane propionic acid could be confirmed by comparison with the authentic compound.

Reaction of the powder with ammonia, after standing at room temperature for 5 days, yielded a new compound which showed an absorption maximum at 284 nm. The position of this maximum was not affected by changes in pH. Further analysis by GC/MS of an ether extract of the reaction solution revealed a quinoline derivative with a molecular weight of 133, which was identified as 5,6,7,8-tetrahydroquinolin (Fig. 3A).

The absence of an aldehyde and the presence of an enol, the observed formation of a quinoline derivative, and the identification of a cyclohexanone moiety suggest that fission of the catechol occurred between positions C-5 and C-9 (Fig. 4). A major peak in the GC of the lyophilized cultivation broth of *Corynebacterium* sp. strain C125 showed a mass spectrum with a small molecular ion peak at *m/e* 196 [M^+] (Fig. 3B). Other peaks in the mass spectrum were at *m/e* 96, *m/e* 81, *m/e* 68,

and *m/e* 41. These data indicate that 4-(2'-oxocyclohexane)-2-hydroxy-buta-2,4-dienoic acid is the ring fission product, analogous to the product observed in the metabolism of naphthalene (7).

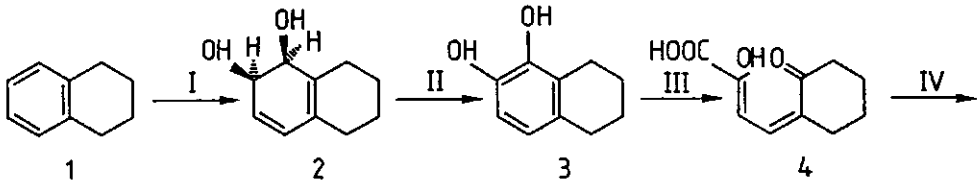

FIG. 4. Proposed pathway for the degradation of tetralin by *Corynebacterium* sp. strain C125. (1) Tetralin; (2) 1,2,5,6,7,8-hexahydro-*cis*-1,2-naphthalene diol; (3) 5,6,7,8-tetrahydro-1,2-naphthalene diol; (4) 4-(2'-oxocyclohexane)-2-hydroxy-buta-2,4-dienoic acid. I, dioxygenase; II, *cis*-dihydro diol dehydrogenase; III, catechol-2,3-dioxygenase; IV, ring fission product hydrolase.

Enzyme activities in cell extracts. Extracts from cells of *Corynebacterium* sp. strain C125 grown on *o*-xylene, tetralin, or succinate were assayed for activities of enzymes involved in the initial steps in the degradation of aromatic compounds in general and tetralin in particular (Table 2). The intermediates of the degradative pathway of tetralin compounds used in these assays were produced in incubations with *Corynebacterium* sp. strain C125. The *cis*-dihydro diol of tetralin was produced by a mutant strain of *Corynebacterium* sp. strain C125 lacking the *cis*-dihydro diol dehydrogenase (unpublished results).

The dioxygenase type of enzyme was assayed by monitoring the consumption of oxygen with seven different assay substrates. The results presented in Table 2 were obtained with NADPH as the electron donor, although activities were comparable when NADH was used (data not shown). Extracts of cells grown on *o*-xylene had higher specific activities than extracts of tetralin-grown cells, but in both cases activities were highest with toluene and low with benzene.

The *cis*-dihydro diol dehydrogenase was assayed with the commercially available *cis*-dihydro diols of benzene and toluene and the *cis*-dihydro diol of tetralin. All three *cis*-dihydro diols were good substrates for the NAD^+-dependent dehydrogenase. The *trans*-dihydro diol of benzene was not a substrate for the dehydrogenase.

TABLE 2. Activities of enzymes involved in the catabolism of tetralin in cell extracts of *Corynebacterium* sp. strain C125 grown on tetralin, *o*-xylene, or succinate

Enzyme	Assay substrate	Enzyme activity ($nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$)		
		o-Xylene	Tetralin	Succinate
Dioxygenase				
	o-Xylene	22	8	0
	Tetralin	14	6	0
	Naphthalene	9	5	0
	Toluene	30	11	0
	Benzene	2	0	0
	Ethylbenzene	24	8	0
	o-Diethylbenzene	17	6	0
Dehydrogenase				
	cis-Benzene glycol	41	36	7
	trans-Benzene glycol	0	0	0
	cis-Toluene glycol	96	103	12
	1,2,5,6,7,8-hexahydro-*cis*-1,2-naphthalene diol	84	67	8
2,3-Dioxygenase				
	Catechol	84	52	10
	3-Methylcatechol	202	178	28
	4-Methylcatechol	47	35	8
	1,2-Dihydroxynaphthalene	532	487	47
	2,3-Dihydroxynaphthalene	22	23	6
	5,6,7,8-Tetrahydro-1,2-naphthalene diol	371	346	27
Hydrolase				
	RFP[a] of catechol	12	9	0
	RFP of 3-methylcatechol	53	37	3
	RFP of 4-methylcatechol	4	3	0
	RFP of 1,2-dihydroxy-naphthalene	107	89	4
	RFP of 5,6,7,8-tetrahydro-1,2-naphthalene diol	71	58	6

[a] RFP, ring fission product.

The catechol fission dioxygenase was a *meta* fission enzyme (Table 2). Yellow intermediates, which showed keto-enol tautomerism similar to the compounds observed during growth experiments (Fig. 2), were accumulated from both aromatic hydrocarbons and catechols. The highest activities of the ring fission dioxygenase were observed with 3-methylcatechol and 1,2-dihydroxynaphthalene, both catechols

with the substituent proximal to the hydroxyl groups. No *ortho*-cleaving activity could be detected in any of the extracts. The products of the catechol-2,3-dioxygenase-catalyzed ring opening were further catabolized by a hydrolytic reaction catalyzed by a hydrolase. There was no observance of a dehydrogenase acting on the ring fission product. In the enzyme assays a transient accumulation of the yellow intermediates in the catabolism of 3-methylcatechol, 1,2-dihydroxynaphthalene, and 5,6,7,8-tetrahydro-1,2-naphthalene diol was observed. Interestingly, incubation of 4-methylcatechol with cell extracts resulted in a continuous increase in A_{381}. The resulting ring fission product was apparently not further degraded, even when NAD^+ was added to the incubation mixture.

DISCUSSION

The results obtained in this study show that *Corynebacterium* sp. strain C125 attacks tetralin by an inital oxidation of the aromatic nucleus at positions C-5 and C-6 (Fig. 4). Metabolism of *o*-xylene in this organism (32), and in strains of *Pseudomonas stutzeri* (2) and *Nocardia* sp. (13), also proceeded via initial hydroxylation of the benzene nucleus proximal to a substituent methyl group. Metabolic routes reported in organisms cooxidizing tetralin involved an initial attack of the benzylic carbon (17, 19, 33), leading to cleavage of the alicyclic ring (19).

In cell extracts, the *cis*-dihydro diol dehydrogenase was only active with NAD^+ as the electron acceptor, which is in accordance with observations made with *cis*-dihydro diol dehydrogenases in other bacteria (1, 27). However, exceptions to this hypothesis do exist (29).

The accumulation of yellow intermediates by cells of *Corynebacterium* sp. strain C125 growing on various aromatic compounds, and especially the shift in absorption maxima of these compounds upon a change in pH, is indicative of an extradiol cleavage of catechols. This was supported by the high activity of the *meta* cleavage dioxygenase in cell extracts and the absence of activity of the *ortho* cleavage enzymes. The catechol cleaving enzyme showed a marked preference for catechols that posessed a hydrocarbon substituent proximal to the hydroxyl groups. Similar observations have been made for *meta* cleavage enzymes in other bacteria growing on hydrocarbon-substituted aromatic compounds, e.g., biphenyl and alkylbenzenes (18, 38, 39). However, unlike the catechol dioxygenases in *Pseudomonas cruciviae* described by Ishigooka et al. (18) and *Pseudomonas* sp. strain NCIB 10643 described

by Smith and Ratledge (39), the *meta* cleavage enzyme of *Corynebacterium* sp. strain C125 is also able to cleave 3,4-dihydroxy compounds (4-substituted catechols).

The consecutive metabolism of the compounds after fission of the aromatic ring was by a hydrolase. Tetralin-grown cells of *Corynebacterium* sp. strain C125 apparently did not possess a ring fission product dehydrogenase. This was supported by the observed inability of cell extracts of *Corynebacterium* sp. strain C125 to catabolize the ring fission product of 4-methylcatechol, which is believed to be exclusively catabolized by the dehydrogenase type of enzyme (31, 44). Also, in the metabolism of *o*-xylene by *Corynebacterium* sp. strain C125, a hydrolase was involved (32). However, the substituted cyclohexane compounds derived from 4-(2'-oxocyclohexane)-2-hydroxy-buta-2,4-dienoic acid by hydrolysis could not be identified. Therefore, the products of the hydrolase-catalyzed reaction are not known. The inability to demonstrate the formation of pyruvic acid indicated that the metabolism of the ring fission product of tetralin differed from the routes observed for naphthalene (7) and *o*-xylene (13). The observation that *Corynebacterium* sp. strain C125 formed acetate in the hydrolase-catalyzed metabolism of the *o*-xylene ring fission product indicated that this organism possesses a different type of hydrolase (32).

The enzymes that catalyze the initial steps of the metabolic pathway are induced by the presence of aromatic substrates. This can be concluded from the absence of oxygen consumption (Table 1) and the activities of these enzymes (Table 2) in cells grown on succinate.

On the basis of the above results, we propose an inducible degradation pathway (Fig. 4) for tetralin (step 1) that starts with hydroxylation of the aromatic moiety at the C-5 and C-6 positions. The resulting *cis*-dihydro diol (step 2) is oxidized by a *cis*-dihydro diol dehydrogenase, which yields the 5,6,7,8-tetrahydro-1,2-naphthalene diol (step 3). This catechol is subsequently cleaved by a *meta*-cleaving catechol dioxygenase, resulting in the formation of 4-(2'-oxo-cyclohexane)-2-hydroxy-buta-2,4-dienoic acid (step 4). This compound was further metabolized by a hydrolytic enzyme. The reaction products of this step were not identified nor were the subsequent reactions investigated. These results show that the metabolism of tetralin initially proceeds via a pathway analogous to the route described for naphthalene in pseudomonads (7).

The metabolism of tetralin in *Corynebacterium* sp. strain C125 as presented here is the first report on the catabolism of tetralin by an initial attack at the aromatic moiety (37). The presence of a specifically dioxygenating enzyme system which, moreover, is able to attack a broad range of aromatic substrates makes

Corynebacterium sp. strain C125 an excellent catalyst for the specific production of special *cis*-dihydro diols (28). Compounds attacked by dioxygenases include alkylbenzenes, haloaromatics, and benzoic acids, though to our knowledge the dioxygenation of tetralin has not been reported (21, 28). Since tetralin derivatives have an enormous potential in the pharmaceutical (25, 42) and also flavoring (23) industries, methods to optimize the oxy-functionalization of this compound are of interest.

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Chapter 6

EFFECTS OF THE MEMBRANE ACTION OF TETRALIN ON THE FUNCTIONAL AND STRUCTURAL PROPERTIES OF ARTIFICIAL AND BACTERIAL MEMBRANES

SUMMARY

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

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INTRODUCTION

Interest in the application of water-immiscible organic compounds in fermentations has increased in the last decade. Many lipophilic compounds are harmful to microorganisms, impair growth, and even inhibit other biological reactions. Knowledge of the toxic action of lipophilic compounds on bacterial cells is mainly restricted to the relation between the hydrophobicity (12) of a compound and its effect on a specific enzyme (18). In most studies, the cytoplasmic membrane is mentioned as a possible target, but information about the nature of the toxic action is not presented (1, 18).

A correlation between the hydrophobicity of a compound and its effects on cells was first observed for anesthetics (26-28), which provided a basis for calculating a dose-effect relationship. In addition, the uncoupling effects of lipophilic compounds on energy transduction have been studied in animal cells (22). For microorganisms, only a few studies on the toxic effects of lipophilic compounds on various membrane functions have been performed (3, 31, 32). These studies showed that hydrocarbons, e.g., β-pinene (31) and cyclohexane (32), impaired energy transduction in both mitochondrial and plasma membranes of yeast cells. Studies on the toxic effects of ethanol on yeast cells indicated that the Δp across the plasma membrane was dissipated in the presence of ethanol (3), probably due to an increased influx of protons (3, 11).

In this investigation, the toxic action of the lipophilic compound tetralin on bacteria was studied. Tetralin was found to be toxic to bacterial cells at concentrations below 100 μmol/liter (29). Tetralin, 1,2,3,4-tetrahydronaphthalene, is a bicyclic molecule that consists of an aromatic and an alicyclic moiety. The compound is widely applied as an industrial solvent and as a substitute for turpentine (6). In the early 1920s, it was already known that tetralin was toxic to bacteria (20, 30) and bacteriophages (20), and it was therefore applied as a biocide. Since detailed information about the mode of interaction of lipophilic compounds with membranes is difficult to obtain with intact cells, the effects of tetralin were first studied in (proteo)liposomes. The inhibitory action of tetralin in intact cells was addressed in both tetralin-utilizing and nonutilizing bacterial strains.

Chapter 6

MATERIALS AND METHODS

Organisms. *Acinetobacter* strain T5, and *Arthrobacter* strain T2 were isolated from the environment by selective enrichment on tetralin as described previously (29). *Corynebacterium* strain C125 was kindly provided by G. Schraa, Department of Microbiology, Wageningen Agricultural University, The Netherlands (24). *Escherichia coli* K-12 (ATCC 25404) and *Bacillus subtilis* ATCC 6633 were obtained from the American Type Culture Collection (Rockville, Md.).

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

Preparation of liposomes. Lipids dissolved in $CHCl_3$-methanol (9:1;vol/vol) were mixed together in appropriate quantities and dried under a stream of N_2 gas. Traces of solvent were then removed under vacuum for 1 h. Dried lipid was suspended in 50 mM potassium phosphate (pH 7.0) at a concentration of 20 mg of lipid per ml and dispersed by ultrasonic irradiation, using a bath sonicator (Sonicor; Sonicor Instruments, New York, N.Y.). Liposomes were obtained by sonication (probe-type sonicator; MSE, West Sussex, United Kingdom) for 300 s at maximal amplitude, using intervals of 15 s of sonication and 45 s of rest, at 4°C under a constant stream of N_2 gas.

Reconstitution of cytochrome *c* oxidase into proteoliposomes. Forty milligrams of aceton-ether-washed *E. coli* lipid and 18 mg *n*-octyl-β-D-glucopyranoside in 2 ml of 50 mM potassium phosphate (pH 7.0) were cosonicated till clarity under a constant stream of N_2 gas at 4°C with a probe sonicator. Cytochrome *c* oxidase (9 nmol of heme *a*) was added, and the suspension was dialyzed at 4°C for 4 h against a 500-fold volume of 50 mM potassium phosphate (pH 7.0). Dialysis was repeated for another 4 h and continued overnight at 4°C (7).

Partitioning of tetralin. The partitioning of tetralin over membrane and buffer phases was determined in a liposome-buffer system (10). Increasing amounts of tetralin were added to 50 mM potassium phosphate (pH 7.0) containing liposomes (5.0 mg phospholipid per ml)(final volume, 0.5 ml). After equilibration (30 min), the liposomes were spun down in an Airfuge (Beckman Instruments, Inc., San Ramon, Calif.) for 30 min at 135,000 x *g*. The supernatant was removed with a Pasteur pipette and subsequently extracted twice with an equal volume diethyl ether (containing 0.1 % *n*-decane as an internal standard). The pellet was dried and resuspended in 100 μl diethyl ether (containing 0.1 % *n*-decane as internal standard). Both the pellet and the supernatant were analyzed quantitatively by gas chromatography.

Gas chromatography. Gas-liquid chromatographic analysis was performed on a CP-9000 gas chromatograph with an on-column injector (Chrompack BV, Middelburg, The Netherlands) fitted with a fused silica WCOT CP-Sil 8 CB column (25 m by 0.32 mm) (Chrompack). Gas flow rates were: He-H_2-air, 30:20:300 ml/min, each; temperature of the flame ionization detector was 300°C. The column oven was programmed from 80°C initial temperature to 200°C at a rate of 10°C/min.

Membrane expansion and extraction of phospholipids. The expansion of liposomal membranes and extraction of phospholipids from the liposomes resulting from the addition of tetralin was monitored in liposomes labeled with the fluorescent fatty acid octadecyl rhodamine β-chloride (R_{18}; Molecular Probes Inc., Junction City, Oreg.) or the fluorescent phospholipid analog, *N*-(lissamine rhodamine β-sulfonyl)phosphatidylethanolamine (*N*-Rh-PE; Avanti Polar Lipids Inc., Alabaster, Ala.). The method is based on the relief of fluorescence self-quenching (8) of rhodamine β-chloride as a result of expansion of the membrane and/or extraction of the probe from the membrane. The fatty acid probe was incorporated into liposomal membranes at a concentration of 4 mol% phospholipid phosphorus. Maximum R_{18} fluorescence was determined upon the addition of 1% (vol/vol) Triton X-100. Fluorescent changes were measured in a spectrofluorometer by using the excitation-emission pair 560 and 590 nm. To discriminate between fluorescence increase due to expansion of the membrane or due to extraction of membrane constituents, we centrifuged incubation mixtures with different concentrations of tetralin at 135,000 x *g* (Beckman Airfuge, 30 min). Subsequently, the fluorescence of the supernatant was determined

relative to that of the supernatant of an incubation without added tetralin.

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

Cytoplasmic pH of intact cells. The internal pH of cells was measured by monitoring the pH-dependent fluorescence of BCECF (2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein) (Molecular Probes Inc., Junction City, Oreg.). Cells were loaded with BCECF as described by Molenaar et al. (16) and stored on ice. Fluorescent changes were measured using the excitation-emission pair 502 and 525 nm with a Perkin-Elmer spectrofluorometer. The values obtained were corrected for efflux of BCECF as described previously (16).

Electrical potential across membranes of proteoliposomes and intact cells. The transmembrane electrical potential ($\Delta\psi$) generated in cytochrome *c* oxidase-containing proteoliposomes in the presence of the electron donor system ascorbate-TMPD(N, N, N',N'-tetramethyl-p-phenylene diamine)-cytochrome *c* was determined by monitoring the distribution of tetraphenylphosphonium ion (TPP^+) across the membrane with a TPP^+-sensitive electrode, as previously described (13). To estimate $\Delta\psi$ in *E. coli* and *Acinetobacter* sp., cells were treated with EDTA before TPP^+ distribution was monitored as described by Sarkar et al. (23). A correction for concentration dependent probe binding was applied according to the model of Lolkema et al. (13). Specific reaction conditions are specified in the figure legends.

Proton fluxes through liposomal membranes. $\Delta\psi$-induced proton flux measurements in cytochrome *c* oxidase-containing proteoliposomes were performed in the presence of increasing concentrations of tetralin in a well-stirred thermostat-equipped 2-ml cuvette, using phenol red (20 μg/ml, final concentration) as the indicator of the external pH. The rate of change of external pH, monitored by A_{560} -A_{610} (33), was converted into H^+ flux by using pulses of calibrated amounts of oxalic acid or KOH.

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

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

Miscellaneous. *E. coli* phospholipids, obtained from Sigma Chemical Co. (St.Louis, Mo.), were washed with acetone-ether as described by Kagawa and Racker (9).

The concentration of tetralin in water, and water-DMF mixtures was determined either spectrophotometrically at 270 nm using ϵ_{270} = 740 M^{-1} cm^{-1} (25) or by gas chromatography of

ether extracts. Tetralin was prepared as a solution in DMF. In all cases, the amount of DMF added was 2% (vol/vol) of the total volume. DMF had no effect on any parameter studied except for the binding of TPP^+ to membranes (binding of TPP^+ was less in the presence of DMF); $\Delta\psi$ values were corrected accordingly (13).

Cytochrome *c* oxidase activity was measured spectrophotometrically by monitoring the decrease in the absorbance of the alpha peak of cytochrome *c*, using an extinction coefficient (reduced minus oxidized) of $\epsilon_{550\text{-}540} = 19.5\ mM^{-1}\ cm^{-1}$ (33).

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

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

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

RESULTS

Partitioning of tetralin between membrane and aqueous phase. The solubility of tetralin in the buffer used was estimated spectrophotometrically by the method of Schreiber (25). Up to 130 μmol/liter added, the concentration of tetralin in the aqueous phase increased linearly, but then slowly curved to a

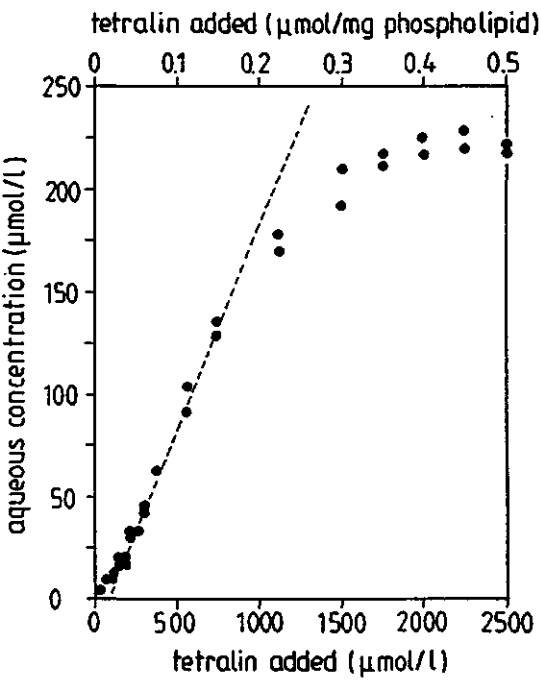

FIG. 1. Concentration of tetralin in the water phase as determined in the presence of liposomes (5.0 mg of phospholipid per ml). The partition coefficient of 1,100 was determined in the linear part of the curve.

maximum of approximately 200 μmol/liter. Above 200 μmol of tetralin per liter, a distinct second phase was formed.

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

Expansion of liposomal membrane in the presence of tetralin. Accumulation of tetralin in the phospholipid bilayer of liposomes could cause an expansion of the membrane. To analyze the effect of tetralin on the swelling of the membrane, we labeled liposomes prepared from *E. coli* phospholipid with R_{18} or *N*-Rh-PE. The rationale is that expansion of the membrane should lead to a dilution of the probes in the membrane which can be observed as a relief in fluorescence self-quenching. Since the fluorescence signal is related to the lipid concentration (8), a change in fluorescence will be proportional to a change in surface area. Alternatively, relief of self-quenching might be observed as a consequence of extraction of the fluorescent probe from the membrane by tetralin. Addition of tetralin to a liposome suspension resulted in a partial relief of self-quenching and reached a maximum when 2 to 3 μmol of tetralin per mg of phospholipid was added (Fig. 2). Further addition of tetralin did not result in an extra increase of fluorescence. However, a discrete tetralin phase was not observed at concentrations up to 10 μmol of tetralin per mg of phospholipid.

Ultracentrifugation of liposomes equilibrated with various amounts of tetralin (0.1, 0.3, 0.6, 3.0 and 7.5 μmol/mg of phospholipid) did not reveal substantial rhodamine fluorescence in the supernatant. At 0.1, 0.3 and 0.6 μmol/mg of phospholipid, extraction of fluorescent probe could maximally account for 1% of the observed increase in R_{18} fluorescence. At 3.0 μmol/mg, 5.6%, and at 7.5 μmol/mg, 9.7% of the fluorescence increase could be attributed to extraction of the fluorescent compounds. Experiments performed with liposomes labeled with

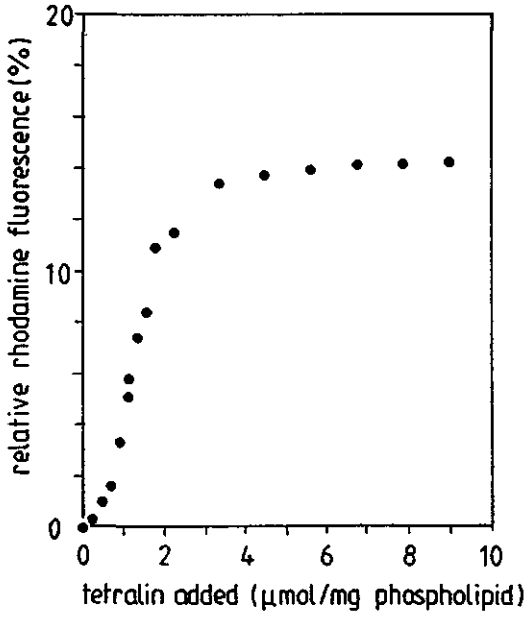

FIG. 2. Effect of tetralin on the relief of fluorescence self-quenching of R_{18}-labeled liposomes. The reaction mixture contained liposomes (0.33 mg of *E. coli* phospholipid per ml) labeled with fluorescent probe (4 mol%) in potassium phosphate buffer (50 mM, pH 7.0). The temperature of the solution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

the fluorescent phospholipid *N*-Rh-PE yielded results similar to those with R_{18} (fatty acid)-labeled liposomes. The results indicate that the observed increase in fluorescence was primarily due to swelling of the membrane and not to extraction of probe from the membrane.

Effect of tetralin on pH gradient and electrical potential in proteoliposomes. To analyze the effect of tetralin on the generation of the pH gradient and the electrical potential in artificial membranes, we reconstituted beef heart cytochrome *c* oxidase into liposomes as proton motive force-generating mechanism. At pH 7.0, cytochrome *c* oxidase-containing proteoliposomes generated a $Z\Delta pH$ and $\Delta\psi$ of -54 and -60 mV, respectively, in the presence of the electron donor system ascorbate-TMPD-cytochrome *c*. Incubation of the proteoliposomes with increasing concentrations of tetralin resulted in a decrease of both ΔpH and $\Delta\psi$, the effect being more pronounced on the ΔpH than on the $\Delta\psi$ (Fig. 3). ΔpH and $\Delta\psi$ were measured in the presence of valinomycin and nigericin, respectively, to estimate the maximal gradients; the effects of tetralin were similar in the absence of the ionophores (data not shown). Because of the high partition coefficient, the extent of the inhibition depended on the liposome (phospholipid) concentration in the incubation mixture, i.e., when the tetralin concentration was expressed as micromoles added per liter (data not shown).

However, when the amount of tetralin added is expressed as micromoles per milligram of phospholipid, the effects are (almost) independent of the phospholipid concentration (Fig. 3).

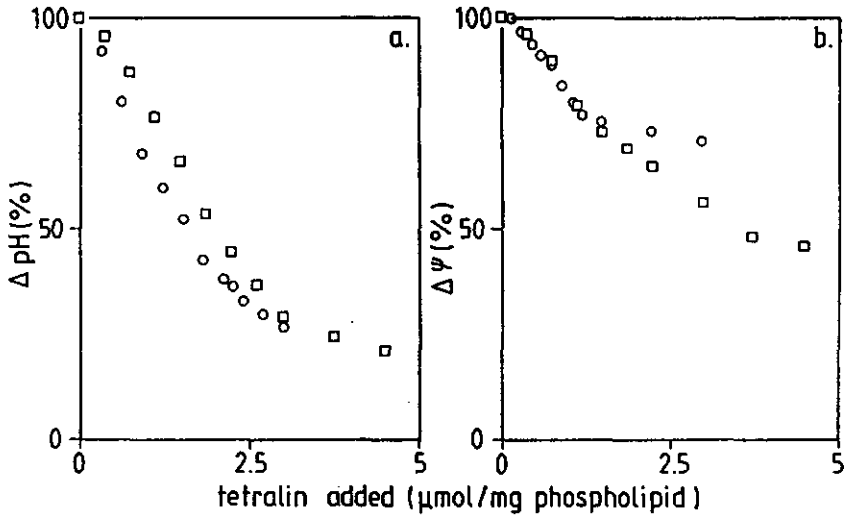

FIG. 3. Effect of tetralin on ΔpH (a) and $\Delta\psi$ (b) generated by cytochrome *c* oxidase-containing proteoliposomes. The effect of tetralin on the ΔpH was measured by monitoring the change in intensity of pyranine fluorescence in the presence of the potassium ionophore valinomycin (1 μM, final concentration). The effect of tetralin on the $\Delta\psi$ was measured by observing the distribution of TPP^+ across the membrane by using a TPP^+- sensitive electrode in the presence of the ionophore nigericin (20 nM, final concentration). The electron donor system to energize the proteoliposomes was composed of cytochrome *c* (20 μM), potassium ascorbate (10 mM, pH 7.0) and TMPD (400 μM). (□) 0.1 mg phospholipid per ml, (○) 0.25 mg phospholipid per ml.

Site(s) of action of tetralin. The decrease in ΔpH and $\Delta\psi$ could be due to inhibition of cytochrome *c* oxidase activity or to increased ion permeability of the membrane in the presence of tetralin or both. Addition of tetralin to cytochrome *c* oxidase-containing proteoliposomes led to a partial inhibition of enzyme activity, i.e., a maximum of 50% inhibition at 15 μmol/mg of phospholipid. The effect of a lowered cytochrome *c* oxidase activity on the generation of the pH gradient and electrical potential in proteoliposomes was assessed by incubating cytochrome *c* oxidase-containing liposomes with different concentrations of sodium azide. At 0.12 mM NaN_3, cytochrome *c* oxidase was 50% inhibited; the enzyme, however, was able to generate a ΔpH and $\Delta\psi$ that were reduced by 17 and 12%, respectively, relative to incubations in the absence of NaN_3.

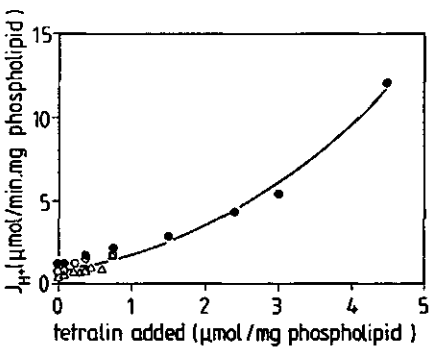

FIG. 4. Influence of tetralin on the proton permeability of liposomal membranes. Liposomes (0.25 mg of phospholipid per ml) were washed and resuspended in a medium in which sodium ions were substituted for potassium ions and additional phenol red (20 µg/ml) was added. To initiate the potassium diffusion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

To test whether the decrease in the ΔpH and $\Delta\psi$ by tetralin was due to an increased proton permeability of the membranes, potassium-loaded liposomes were diluted into potassium-free medium in the presence of valinomycin, and the initial rates of H^+ influx in the absence and presence of different amounts of tetralin were determined. As shown in Fig. 4, the proton permeability increased with increasing tetralin concentrations. The proton permeability was highest at concentrations at which the ΔpH and $\Delta\psi$ were maximally affected. These results suggest that the lowering of ΔpH and $\Delta\psi$ by tetralin is primarily caused by an increased H^+ permeability of the membrane and to a lesser extent by the inhibition of cytochrome *c* oxidase.

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

The ΔpH generated by *B. subtilis* and *Arthrobacter* strain T2 was significantly less affected by tetralin than the pH gradient generated by the other organisms (Fig. 5a). The inhibitory effect of tetralin on the $\Delta\psi$ of intact cells was less pronounced (Fig. 5b). As for the (proteo)liposomes, the effect on the ΔpH and $\Delta\psi$ was dependent on the amount of membrane lipid (biomass) present (data not shown). Assuming that a bacterial cell is composed of protein and phospholipids constituting 55 and 9.1%, respectively, of the total cellular mass (data for *E. coli*

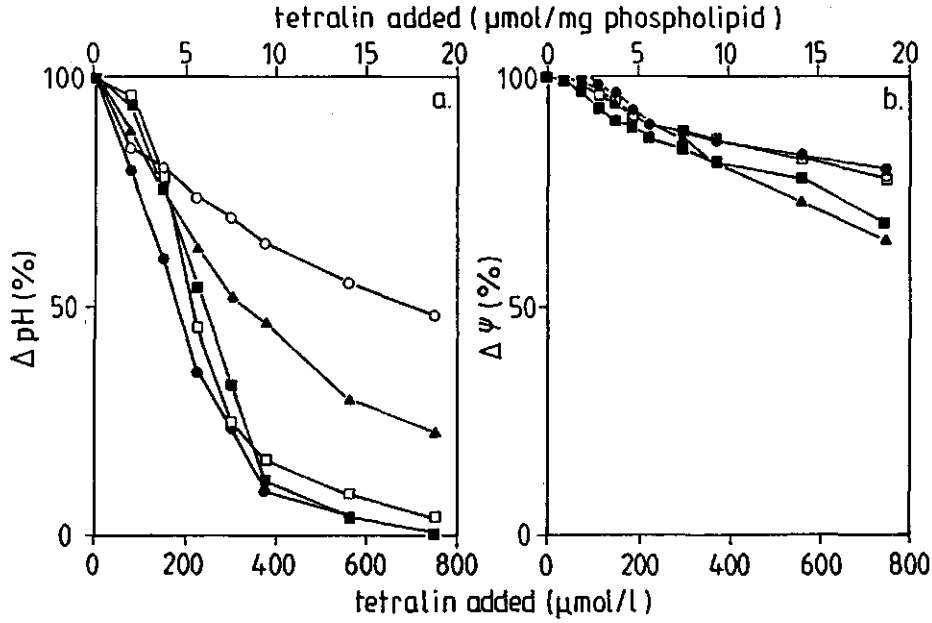

FIG. 5. Effect of tetralin on ΔpH (a) and on $\Delta\psi$ (b) as generated by intact cells. The incubation mixture contained 5 g of sodium succinate per liter in 50 mM potassium phosphate buffer (pH 7.0) and 0.25 mg of cell protein per ml. (●) *E. coli*; (○) *B. subtilis*; (▲) *Arthrobacter* strain T2; (■) *Acinetobacter* strain T5; and (□) *Corynebacterium* strain C125.

[17]), a tetralin concentration of 500 µmol/liter corresponds to approximately 10 µmol/mg phospholipid for the experiment presented in Fig. 5.

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

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

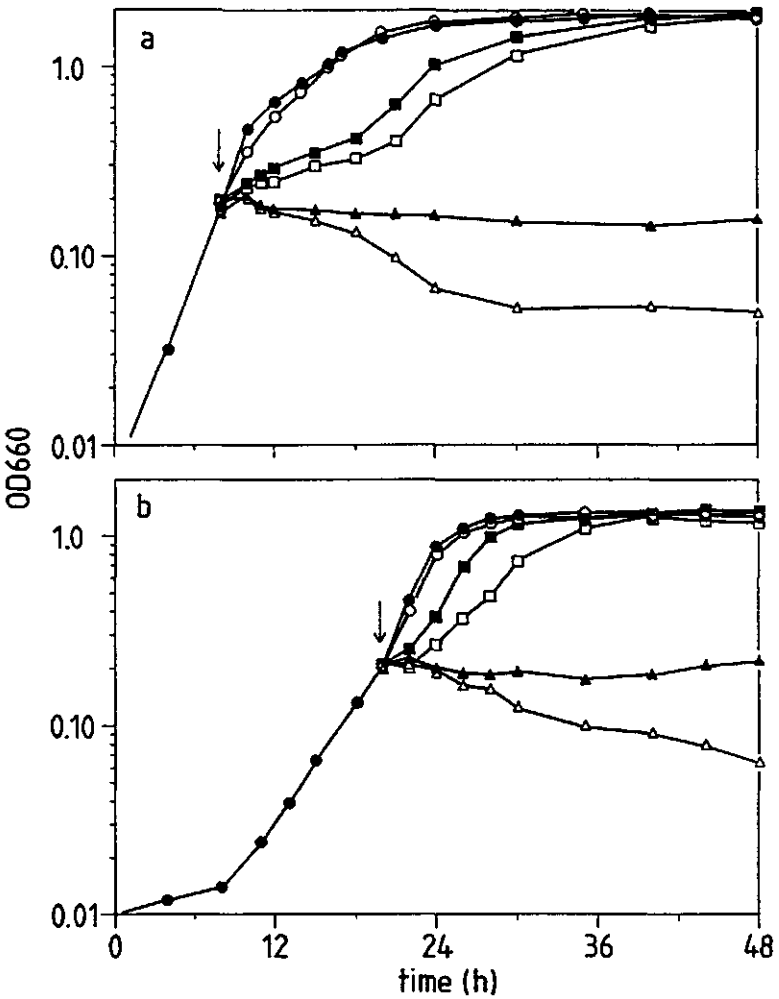

FIG. 6. Effect of tetralin on the growth of *E. coli* (a) and *Arthrobacter* strain T2 (b). Cells were grown in mineral medium (pH 7.0) supplemented with sodium succinate (5 g/liter) and yeast extract (0.5 g/liter) to a cell density of 0.2 at 660 nm. Subsequently, different concentrations of tetralin were added (indicated by arrows), and growth was observed for additional generation times. (●) control, without tetralin added; tetralin added (micromoles per liter): (○) 75; (■) 375; (□) 750; (▲) 1875; (△) 3750. OD, optical density.

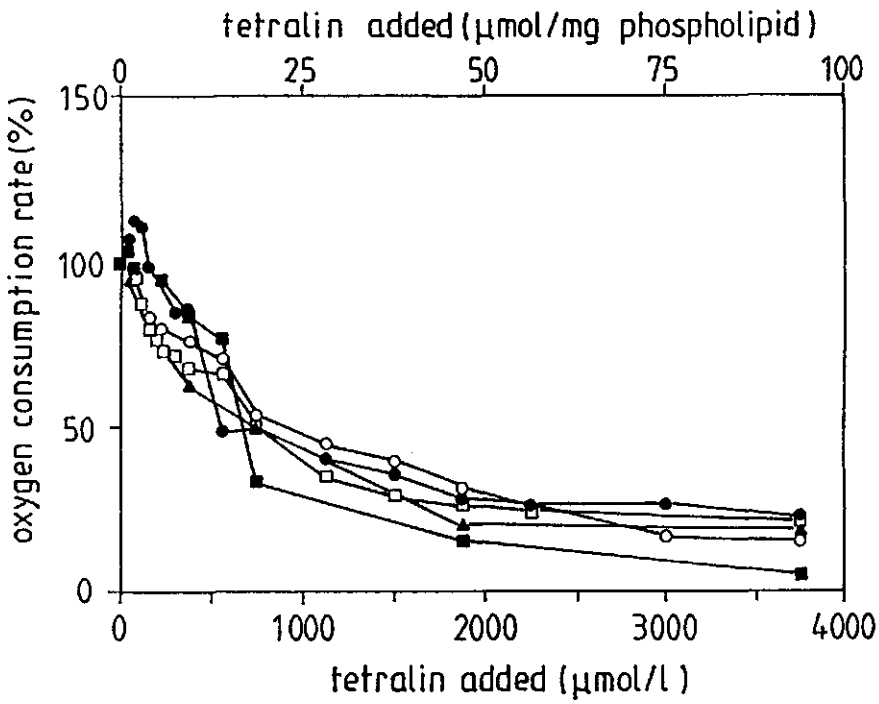

FIG. 7. Effect of tetralin on the rate of oxygen consumption. Cells were grown on succinate-yeast extract medium, and oxygen consumption rates were determined in the presence of succinate (20 mM). The incubation mixture contained 0.25 mg of cell protein per ml. Rates are expressed relative to the rate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

DISCUSSION

Tetralin is a lipophilic compound with a low solubility in water. Therefore, in an aqueous-nonaqueous two-phase system, it will preferentially partition to the nonaqueous phase. From the experimentally obtained partition coefficient of 1,100 for tetralin in a liposome-buffer system ($P_{membrane}$), at a maximum aqueous solubility of 124 µmol/liter (25), a tetralin concentration in the membrane of 0.136 µmol/mg of membrane lipid can be calculated using the following equation: $[solvent_{membrane}] = P_{membrane} \times [solvent_{aqueous}]$. This means that approximately 18 nl of tetralin is dissolved in 1 mg of membrane lipid,

corresponding to 1 molecule of tetralin per 10 molecules of phospholipid. The equation, however, is only valid at low concentrations, i.e., when the behaviour of the solute can be considered ideal, as described by Raoult's law. At higher concentrations, the nonideal behaviour of the solute may lead to a deflection in the partition coefficient (Fig. 1).

One of the principal functions of the cytoplasmic membrane is energy transduction, which involves the generation and maintenance of a Δp. The Δp is composed of a proton gradient (ΔpH) and an electrical gradient ($\Delta\psi$). Dissipation of these gradients leads to a situation in which the cell is unable to screen its cytoplasm from the surrounding medium. The dissipation of the Δp by tetralin can be caused either by leakage of protons and/or other ions or by inhibition of cytochrome *c* oxidase. Although inhibition of cytochrome *c* oxidase occurred, the decrease in cytochrome *c* oxidase activity could not fully account for the decrease in the pH gradient and electrical potential. Since tetralin also affected the proton(ion) permeability of the membrane (Fig. 4), the decrease may in fact be due to an increased proton (ion) permeability and the partial inhibition of cytochrome *c* oxidase. Since more protons have to be translocated across a membrane to generate a pH gradient than to generate an electrical potential of similar magnitude (15), the observation that tetralin affects the pH gradient more severely than the $\Delta\psi$ suggests that the proton influx must partly be balanced by cation efflux or anion influx or both. The effect of tetralin in intact cells also appeared to be more pronounced on ΔpH than on $\Delta\psi$. Analogous to the situation with the cytochrome *c* oxidase-containing proteoliposomes, the effect of tetralin on ΔpH and $\Delta\psi$ in intact cells is likely due to an increase in permeability for protons (ions) and the impairment of the ion-translocating (and/or electron carrier) enzymes. Apart from the effect on the Δp, dissipation of the ΔpH can severely impair normal functioning of the cell because of the lowered internal pH (19). The observation that tetralin reached its maximum effect at concentrations which just saturated the membrane (2.5 μmol/mg of phospholipid) indicates that tetralin is toxic at the molecular level (1). The interaction of tetralin with the membrane (Fig. 2) did not result in full dissipation of the Δp (Fig. 3), not even when the amount of tetralin present exceeded the concentration that saturated the membrane, indicating that tetralin does not fully disrupt the membrane. Therefore, the effects of tetralin must be caused by toxicity of dissolved molecules, and not by the presence of a phase transition due to the water-immiscible second phase (1).

The effect of tetralin on the membrane functioning has consequences for the

overall metabolism and growth characteristics of the cell. Both the lag phase and growth rate of the strains examined were affected by tetralin. Interestingly, cells did eventually overcome the inhibition by low concentrations of tetralin (concentrations of 750 μmol/liter and lower), which most likely is due to an initial slow increase in biomass which subsequently lowers the medium concentration of tetralin as a consequence of the high membrane/buffer partition coefficient. Furthermore, the presence of an additional substrate (succinate) provides the cell with an energy source that may fuel the protection and adaptation of the organism. In the absence of succinate, growth was already inhibited at a tetralin concentration of 100 μmol/liter (29). The inhibition of growth by tetralin could be due to the lowered Δp and the more acidic internal pH. On top of this, tetralin could affect various membrane enzymes directly by disrupting protein-lipid interactions (at maximal inhibitory concentrations, approximately 2 molecules of tetralin are present per molecule of phospholipid). The inhibition of cytochrome *c* oxidase in the proteoliposomes and of respiration in intact cells are indicative for this.

Arthrobacter strain T2 was less susceptible to tetralin, most likely because of the observed production of emulsifying compounds. Another opportunity for a cell to overcome toxic effects of lipophilic compounds is altering the fatty acid composition of the membrane (5, 14). This could lead to different sensitivities, probably as a result of altered partition characteristics of the lipophilic compounds (5). Future studies will be directed toward the effect of lipid composition on the partitioning of lipophilic compounds in biological membranes.

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

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INTERACTIONS OF CYCLIC HYDROCARBONS WITH BIOLOGICAL MEMBRANES

SUMMARY

Many cyclic hydrocarbons, e.g., aromatics, cycloalkanes, and terpenes, are toxic to microorganisms. The primary site of the toxic action is probably the cytoplasmic membrane, but the mechanism of the toxicity is still poorly understood. The effects of cyclic hydrocarbons were studied in liposomes prepared from *E. coli* phospholipids. The membrane/buffer partition coefficients of the cyclic hydrocarbons revealed that these lipophilic compounds preferentially reside in the membrane. The partition coefficients closely correlated with the partition coefficients of these compounds in a standard octanol-water system. The accumulation of hydrocarbon molecules resulted in swelling of the membrane bilayer, as assessed by the release of fluorescence self-quenching of fluorescent fatty acid and phospholipid analogs. Parallel to the expansion of the membrane, an increase in membrane fluidity was observed. These effects on the integrity of the membrane caused an increased passive flux of protons and carboxyfluorescein. In cytochrome *c* oxidase containing proteoliposomes both components of the proton motive force, the pH gradient and the electrical potential, were dissipated with increasing concentrations of cyclic hydrocarbons. The dissipating effect was primarily the result of an increased permeability of the membrane for protons (ions). At higher concentrations, also cytochrome *c* oxidase was inactivated. The effective concentrations of the different cyclic hydrocarbons correlated with their partition coefficients between the membrane and aqueous phase. The impairment of microbial activity by the cyclic hydrocarbons most likely results from hydrophobic interaction with the membrane, which affects the functioning of the membrane and membrane embedded proteins.

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Submitted for publication.

INTRODUCTION

Cyclic hydrocarbons, such as aromatics, alicyclics, and terpenes, interact with biological membranes (34, 35, 38, 39). These interactions lead to changes in structure and function of the membranes, which in turn, may impair growth and activity of the cells (34). The wide-spread use of cyclic hydrocarbons (e.g., fuels, solvents, starting compounds for organic synthesis) and their release in the environment, makes knowledge on their metabolism and toxicity of eminent importance. The toxicity of cyclic hydrocarbons has been well-noted (36), but knowledge about their mode of interaction with cells and the cause of toxicity is scarce. Uribe and coworkers studied the toxicity of β-pinene (38) and cyclohexane (39) on intact yeast cells and isolated mitochondria. Both compounds exerted their action at the level of the membrane and membrane-embedded enzymes. Recently, we have reported the effects of the aromatic hydrocarbon tetralin on the structure and function of both bacterial and liposomal membranes (34). Our data showed that tetralin accumulated in the membrane (partition coefficient approximately 1,100), causing 'expansion' of the membrane surface area, inhibition of primary ion pumps, and increase in proton permeability. As a result the electrical potential and pH gradient were dissipated, which may have been the primary cause of inhibition of cellular growth. Further experiments with other aromatic and alicylic hydrocarbons indicated that the observed effects were not specific for tetralin and that a direct relationship can be found between the partitioning of a particular compound in the membrane and its effect on the structural integrity and functional properties of the membrane (this manuscript). Effects of polar and non-polar compounds on biological membranes have been reported for fatty acids (28), ethanol in yeast (6, 17), and anesthetics in erythrocytes (30). The explanation most given for the observed toxicity of these compounds is disruption of membrane structure by hydrophobic interaction with the lipid bilayer due to their lipophilicity.

In this investigation, the toxic effects of different cyclic hydrocarbons were studied and related to their hydrophobicity and partitioning into the membrane. The results show that effects of cyclic hydrocarbons on structural and functional properties of membranes are closely related to their accumulation in the membrane. The data give a rationale for the frequently observed correlation between the toxicity of lipophilic compounds to microorganisms and the partition coefficients of such compounds in a standard octanol/water system (log P or k_{OW};19).

MATERIALS AND METHODS

Preparation of liposomes. *E. coli* phospholipids, obtained from Sigma Chem. Co. (St.Louis, MO, USA), were washed with acetone/ether (14). Lipids dissolved in $CHCl_3$/MeOH (9:1;v/v) were mixed in appropriate quantities and dried under a stream of N_2 gas. Traces of solvent were then removed under vacuum for 1 h. Dried lipid was suspended in 50 mM potassium phosphate (pH 7.0) at a concentration of 20 mg lipid/ml and dispersed by ultrasonic irradiation using a bath-sonicator (Sonicor, Sonicor Instruments, New York, NY, USA). Liposomes were obtained by sonication (probe type sonicator, MSE, West Sussex, U.K.) for 300 s at maximal amplitude, using intervals of 15 s sonication and 45 s rest, at 4°C under a constant stream of N_2 gas.

Partitioning of lipophilic compounds. Partitioning of lipophilic compounds over membrane and buffer phases was determined in a *E. coli* phospholipid liposome/potassium phosphate buffer system (8, 15). Increasing amounts of the radio-labeled compounds were added to 50 mM potassium phosphate (pH 7.0) containing liposomes (5.0 mg of phospholipid per ml; final volume 0.5 ml). After equilibration (30 min), the liposomes were spun down in an Airfuge (Beckman Instruments, Inc., San Ramon, CA) for 30 min at 135,000 x *g*. By this method all liposomes were pelleted, as was assessed by phosphate analyses (29). The supernatant was removed with a Pasteur pipette, and two portions of 100 μl were pipetted in a scintillation vial. The pellet was resuspended in scintillation fluid. Both the pellet and the supernatant fractions were analyzed radiometrically in a scintillation counter. The results presented are the mean and the standard deviation of 6 independent measurements. Control experiments without liposomes were performed in parallel to account for losses of solvent due to possible evaporation and/or attachment to tubes and pipettes. The internal volume of the liposomes (3 μl per mg phospholipid) was taken into account. Partition coefficients of the non-radioactive lipophilic compounds carvone and tetralin were determined by gas chromatography, as described previously (34).

Membrane expansion and extraction of phospholipids. The expansion of liposomal membranes and extraction of phospholipids from the liposomes due to the addition of lipophilic compounds was monitored in liposomes labeled with the fluorescent fatty acid, octadecyl Rhodamine-β-chloride (R_{18}; Molecular Probes Inc., Junction City, Oreg.) or the fluorescent phospholipid analog, *N*-(lissamine Rhodamine-β-sulfonyl)phosphatidylethanolamine (*N*-Rh-PE; Avanti Polar Lipids Inc., Alabaster, Ala.). The method is based on the relief of fluorescence self-quenching (12) of Rhodamine-β-chloride as a result of expansion of the membrane and/or extraction of the probe from the membrane. The fatty acid probe was incorporated into liposomal membranes at a concentration of 4 mol% phospholipid phosphorous. Maximum Rhodamine-fluorescence was determined upon the addition of 1% (v/v) Triton X-100. Fluorescent changes were measured in a spectrofluorometer (The Perkin-Elmer Corp., Norwalk, Conn.) using the excitation-emission pair 560 and 590 nm. In order to discriminate between fluorescence increases due to expansion of the membrane and extraction of membrane constituents, incubation mixtures with different concentrations of lipophilic compounds were centrifuged at 135,000 x *g* (Beckman Airfuge, 30 min.). Subsequently, the fluorescence of the supernatant was determined relative to the supernatant of an incubation without hydrocarbon added. Additionally, supernatants of incubations containing *E. coli* phospholipid liposomes in MOPS (50 mM; pH 7.0) and varying amounts of cyclic hydrocarbons were assayed for released phospholipids by phosphate analysis (29).

Membrane fluidity measurements/Fluorescence polarization measurements. DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene) steady-state polarization measurements were carried out as described (13). Membrane fluidity is used as a qualitative measure and is defined as the inverse of microviscosity. Microviscosity can be deduced from the steady-state fluorescence polarization of (TMA-)DPH probes (32).

The degree of fluorescence polarization was calculated from equation 1 (18, 32):

$$r_{ss} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad (1).$$

r_{ss}: steady state fluorescence polarization; $I_{\parallel}$: fluorescence intensity at 430 nm, measured parallel to the emitted light; $I_{\perp}$: fluorescence intensity at 430 nm, measured perpendicular to the emitted light.

Reconstitution of cytochrome *c* oxidase into proteoliposomes. Aceton-ether-washed *E. coli* lipid (40 mg) and *n*-octyl-β-D-glucopyranoside (18 mg) in 2 ml of 50 mM potassium phosphate (pH 7.0) was cosonicated until clarity under a constant stream of N_2 gas at 4°C using a probe sonicator. Cytochrome *c* oxidase (9 nmol of heme *a* was added, and the suspension was dialyzed at 4°C for 4 h against a 500-fold volume of 50 mM potassium phosphate (pH 7.0). Dialysis was repeated for another 4 h and continued overnight at 4°C (11).

Internal pH of cytochrome *c* oxidase containing proteoliposomes. Internal pH-changes were measured by following the fluorescence of entrapped pyranine (Eastman Kodak Co., Rochester, NY, USA) (7). To incorporate pyranine into proteoliposomes (20 mg phospholipid/ml), 100 nmol pyranine was added to 0.5 ml proteoliposomes and rapidly mixed. The suspensions were rapidly frozen in liquid nitrogen and subsequently thawed slowly (approximately 30 min) at room temperature. The suspension was sonicated for 8 s using a probe type sonicator at an amplitude of 4. To remove external pyranine, the proteoliposomes were washed in 10 ml 50 mM potassium phosphate (pH 7.0) and centrifuged for 45 min at 280,000 x *g* in a Beckman type Ti 75 rotor at 4°C. Fluorescent changes were measured at excitation and emission wavelengths of 460 and 508 nm respectively. Calibration was performed by titration with acid or base upon addition of nigericin to a final concentration of 20 nM.

Electrical potential across membranes of proteoliposomes. The transmembrane electrical potential ($\Delta\psi$) of cytochrome *c* oxidase containing liposomes was determined by monitoring the distribution of Tetraphenylphosphonium (TPP^+) across the membrane with a TPP^+-sensitive electrode as previously described (22).

Proton fluxes through liposomal membranes. $\Delta\psi$-induced proton fluxes were estimated in liposomes in the presence of varying concentrations of a particular hydrocarbon compound in a well-stirred thermostated 2-ml cuvette, using phenol red (20 μg/ml, final concentration) as external pH indicator. Absorbance changes ($A_{560} - A_{610}$) were converted into H^+ fluxes by calibrating with known amounts of oxalic acid or KOH (41). Valinomycin-induced potassium diffusion potentials were imposed across the liposomal membrane by 100-fold dilution of the liposomes (20 mg of phospholipid per ml) into the same medium, in which sodium ions were substituted for potassium ions, and supplemented with phenol red (20 μg/ml). Generation of the electrical potential was initiated by adding valinomycin (2 μM, final concentration).

Determination of cytochrome *c* oxidase activity. Cytochrome *c* oxidase activity was measured spectrophotometrically by monitoring the decrease in the absorbance of the alpha peak of reduced cytochrome *c*, using an extinction coefficient (reduced minus oxidized) of $\epsilon_{550-540}$ = 19.5 mM^{-1} cm^{-1} (41).

Measurement of carboxyfluorescein efflux. Release of carboxyfluorescein (CF) from liposomes, resulting in relief of its fluorescence self-quenching, was determined in a Perkin Elmer spectrofluorometer equipped with a thermostated cell holder, using the excitation-emission pair 430 and 520 nm respectively. Encapsulation of 5,6-carboxyfluorescein (CF; Eastman Kodak Chemical Co., Rochester, NY) was achieved by preparing liposomes in 50 mM CF following the protocol for the formation of pyranine containing liposomes (see above).

Chemicals. All hydrocarbons used were of the highest available commercial grade.

Radiochemicals were obtained from the Radiochemical Centre, Amersham, England: ^{14}C-phenylalanine, ^{14}C-toluenesulfonic acid, ^{14}C-benzoic acid, ^{14}C-4-chlorobenzoic acid, and Sigma Chemicals (St. Louis, Miss.): ^{14}C-toluene, ^{14}C-naphthalene, and ^{14}C-phenanthrene. ^{3}H-Tetraphenylphosphonium (TPP^+), used for the determination of the partition coefficient, was obtained from Amersham.

Addition of cyclic hydrocarbons. The hydrocarbons were prepared as solutions in *N*-dimethylformamide (DMF). In all cases, the amount of DMF was 2% (vol/vol) of the total volume. In this concentration DMF had no effect on any parameter studied except for the binding of TPP^+ to membranes (binding of TPP^+ was less in the presence of DMF); $\Delta\psi$ values were corrected accordingly (22).

RESULTS

Partitioning of lipophilic compounds. In order to gain insight in the effects of lipophilic compounds, at sub-saturating concentrations in the aqueous phase, on biological membranes it is essential to know the partitioning behaviour of such compounds in a membrane/buffer system. As a model system for a biological membrane, liposomes were used prepared from *E. coli* phospholipids.

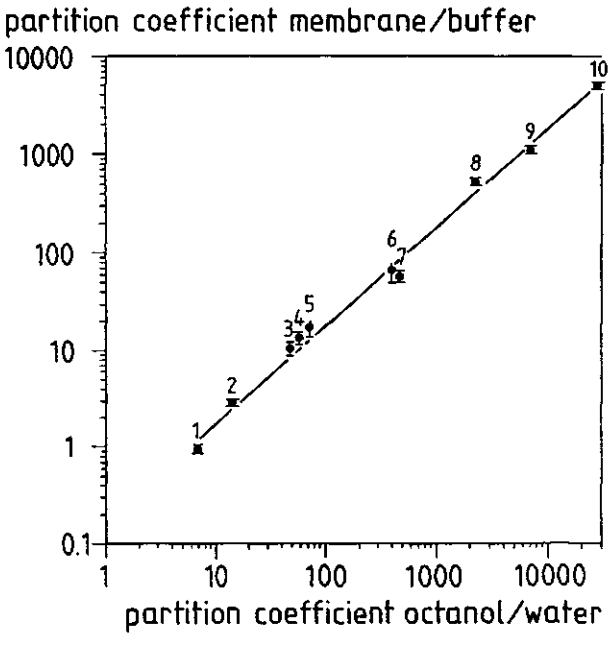

FIG. 1. Relationship between the partition coefficients in an *E. coli* phospholipid membrane/potassium phosphate buffer (pH 7.0; 50 mM) system and the partition coefficients in the standard *n*-octanol/water system of 4-toluenesulfonic acid (1), 4-chlorobenzoic acid (2), benzoic acid (3), phenylalanine (4), tetraphenylphosphonium (TPP^+; 5), carvone (6), toluene (7), naphthalene (8), tetralin (9), and phenanthrene (10). The experimental points represent the mean and standard deviation of 6 independent measurements.

TABLE 1. Physical data of the cyclic hydrocarbons used in this study

Compound	Formula	M_W[a]	Solubility[b] (mmol/l; 25°C)	logP	$P_{O/W}$	$P_{M/B}$exp	$P_{M/B}$calc[h]
Benzene	C_6H_6	78.11	22.9	2.13[b]	135		27
Cyclohexane	C_6H_{12}	84.16	0.683	3.44[b,c]	2754		498
Toluene	C_7H_8	92.14	6.28	2.69[b]	490	59 ± 8.5	93
Ethylbenzene	C_8H_{10}	106.17	1.27	3.15[b]	1413		260
o-Xylene	C_8H_{10}	106.17	2.02	3.12[b]	1318		243
Naphthalene	$C_{10}H_8$	128.17	0.797	3.37[b]	2344	527 ± 38	426
Tetralin	$C_{10}H_{12}$	132.21	0.125[g]	3.86[c]	7244	1100 ± 56	1271
o-Diethylbenzene	$C_{10}H_{14}$	134.22	–	4.10	12590		2173
α-Pinene	$C_{10}H_{16}$	136.24	–	4.46[d]	28840		4855
β-Pinene	$C_{10}H_{16}$	136.24	–	4.46[d]	28840		4855
γ-Terpinene	$C_{10}H_{16}$	136.24	–	4.46[d]	28840		4855
Limonene	$C_{10}H_{16}$	136.24	0.101[e]	4.46[d]	28840		4855
Decalin	$C_{10}H_{18}$	138.25	–	4.83[d]	67608		11094
Biphenyl	$C_{12}H_{10}$	154.21	0.126	4.04[b]	10965		1900
Anthracene	$C_{14}H_{10}$	178.23	0.040	4.45[f]	28184		4748
Phenanthrene	$C_{14}H_{10}$	178.23	0.025	4.46[f]	28840	4937 ± 86	4855

[a] Data obtained from Handbook of Chemistry and Physics (20)
[b] From Eastcott, Shin, and Mackay (9)
[c] From Rekker (25)
[d] Calculated via fragmental constants as described in Rekker and de Kort (26)
[e] From Riddick, Bunger, and Sakano (27)
[f] From Tomlinson and Hafkenscheid (37)
[g] From Sikkema and de Bont (33)
[h] Calculated from $P_{O/W}$ data found in literature and applied to equation 2.

Membrane/buffer partition coefficients in the *E. coli* phospholipid liposomes/potassium phosphate buffer were determined for compounds varying in hydrophobicity/lipophilicity, i.e., phenylalanine, benzoic acid, 4-chlorobenzoic acid, 4-toluenesulfonic acid, tetraphenylphosphonium (TPP^+), carvone, toluene, naphthalene, tetralin, and phenanthrene. The choice of most of these molecules stems from their availability in radioactive form. As a measure for the hydrophobicity of the compounds, the octanol/water partition coefficients were taken (19). The membrane/buffer partition coefficients were plotted as a function of the octanol/water partition coefficients (Fig. 1). Despite differences in structural features of the molecules, a good correlation between the

partitioning in a membrane/buffer system and a standard octanol/water system was observed. The correlation line for lipophilic compounds with logP values between approximately 1 and 4.5, is described by equation 2:

$$\log P_{M/B} = 0.97 \times \log P_{O/W} - 0.64 \qquad (2)$$

The correlation coefficient for the four aromatic hydrocarbons toluene, naphthalene, tetralin, and phenanthrene is 0.9967. With this equation, the membrane-buffer partition coefficients of 20 cyclic hydrocarbons were calculated from their octanol-water partition coefficients. In Table 1 the membrane-buffer partition coefficients of these cyclic hydrocarbons, together with other physical and chemical data of these compounds are given.

Expansion of the membrane. Due to the accumulation of lipophilic compounds in the lipid bilayer, changes in the membrane structure and even swelling of the membrane can be expected. The effect of accumulation in the membrane surface area was monitored by using liposomes prepared from *E. coli* phospholipids that were labeled with octadecyl Rhodamine-β-chloride (R_{18}) or *N*-(lissamine-Rhodamine-β-sulfonyl)-phosphatidylethanolamine (*N*-Rh-PE). The rationale of this method is that expansion of the membrane leads to dilution of the probe in the membrane which can be measured as a relief in fluorescence self-quenching. Since the fluorescence signal is related to the lipid concentration (12), a change in fluorescence will be proportional to a change in surface area. An increase in fluorescence could, however, also be due to extraction of the fluorescent probe from the membrane by the hydrocarbon. Ultracentrifugation of liposomes equilibrated with varying amounts of toluene, cyclohexane, and tetralin showed that at the most 16.3, 11, and 9.4 % of the fluorescence increase with 150 μmol toluene, 15 μmol cyclohexane, and 5 μmol tetralin per mg phospholipid, respectively, could be attributed to probe extraction from the membrane. In addition, supernatants of incubations containing liposomes and varying concentrations of hydrocarbon were checked for the presence of free phospholipids. The highest concentrations of each hydrocarbon applied in the experiments with R_{18}-labeled liposomes (see Fig. 2) did not result in extraction of more than 10 % of the phospholipid content. The data for the compounds were: decalin, 8.6 % of total phospholipid phosphate solubilized at 3 μmol/mg PL; anthracene, 8.4 % at 1 μmol/mg PL; biphenyl, 9.0 % at 2.5 μmol/mg PL; α-pinene, 9.3 % at 2.5 μmol/mg PL; tetralin, 6.2 % at 5 μmol/mg PL; naphthalene,

7.8 % at 6 μmol/mg PL; cyclohexane, 9.9 % at 15 μmol/mg PL; *o*-xylene, 9.6 % at 60 μmol/mg PL; ethylbenzene, 8.7 % at 70 μmol/mg PL; toluene, 9.4 % at 150 μmol/mg PL; benzene, 9.1 % at 250 μmol/mg PL. At higher concentrations solubilization of the liposomes did occur, which was not only detected by a rapid increase of free phospholipids in the supernatant but also by the increase of turbidity of the suspension in the cuvette. These results indicate that the observed increase in R_{18}-fluorescence was primarily due to swelling of the membrane. Different solvents exhibit different concentration dependencies and extents of apparent membrane expansion (Fig. 2). For instance in the presence of decalin the increase in Rhodamine fluorescence not only occurred at a much lower concentration than with benzene, but the extent of fluorescence increase was also higher. The difference in effective concentration parallels the change in hydrophobicity of the compounds and the partitioning into the membrane. Results similar to those presented in Fig. 2 were obtained with *N*-Rh-PE labeled liposomes (data not shown).

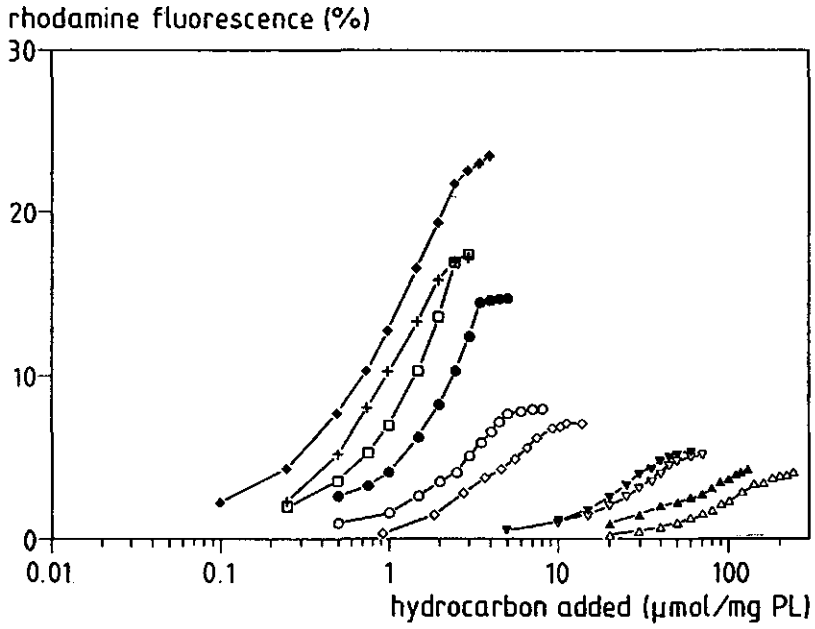

FIG. 2. Effect of cyclic hydrocarbons on the relief of fluorescence self-quenching of R_{18}-labeled liposomes. The reaction mixture contained liposomes (0.2 mg *E. coli* phospholipid per ml) labeled with R_{18} (4 mol%) in potassium phosphate (50 mM, pH 7.0). The changes in fluorescence were monitored using the excitation-emission pair 560 and 590 nm. The temperature of the solution was kept at 30°C. (△) Benzene. (▲) Toluene. (▽) Ethylbenzene. (▼) *o*-Xylene. (◇) Cyclohexane. (○) Naphthalene. (●) Tetralin. (□) Biphenyl. (+) α-Pinene. (♦) Decalin.

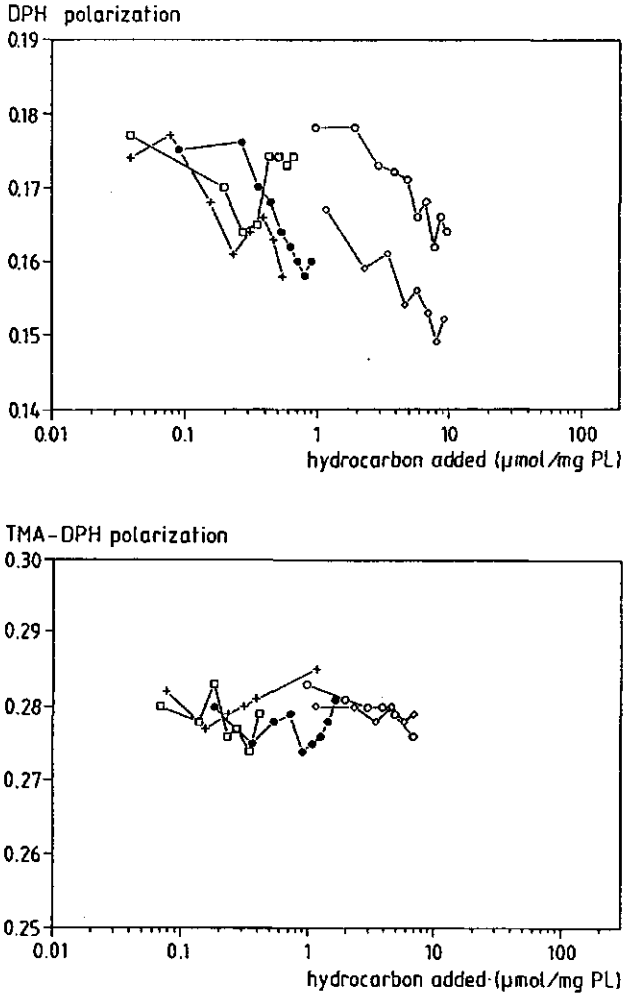

FIG. 3. Polarization of DPH and TMA-DPH as a result of the interaction of different cyclic hydrocarbons with *E. coli* phospholipid liposomes. The measurements were performed in a spectrofluorometer at an excitation wavelength of 360 nm; the emission was recorded at 430 nm. The cuvette, thermostated at 30°C, contained 0.2 mg/ml of either TMA-DPH or DPH labelled liposomes (probe to lipid molar ratio in both instances 1 to 250) in 50 mM potassium phosphate (pH 7.0). (○) Naphthalene. (◇) Cyclohexane. (●) Tetralin. (□) Biphenyl. (+) α-Pinene. (A) Effect of hydrocarbons on the polarization of DPH. (B) Effect of hydrocarbons on the polarization of TMA-DPH.

Changes in membrane fluidity as a result of interaction with hydrocarbons. The fluidity of a membrane bilayer can be assessed by determining the fluorescence polarization of DPH or TMA-DPH. Although the precise location of DPH in the membrane is still not clear, this probe most likely resides near the center of the bilayer (18). Less ambiguities exist about the location of TMA-DPH since its hydrophilic group anchors the molecule at the headgroup region of the bilayer thereby aligning the DPH moiety with the phospholipid acyl chains. All hydrocarbons except biphenyl decreased the polarization of DPH whereas TMA-DPH polarization was not significantly affected (Fig. 3). The different locations in the membrane of DPH and TMA-DPH and the different

effects of tetralin, cyclohexane, naphthalene, and toluene on the fluorescence polarization of DPH and TMA-DPH, suggest that the hydrocarbons perturb the bilayer structure primarily by accumulating into the interior rather than into the peripheral regions of the membrane.

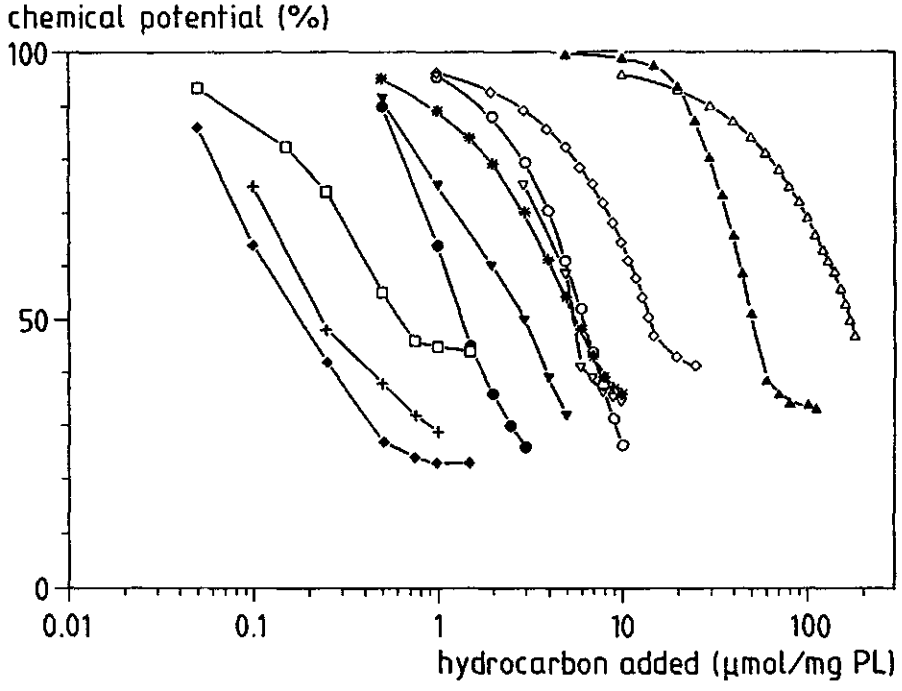

FIG. 4. Effect of cyclic hydrocarbons on the ΔpH generated in cytochrome *c* oxidase containing proteoliposomes. Energization of cytochrome *c* oxidase containing proteoliposomes was achieved in the presence of cytochrome *c* (20 µM), TMPD (200 µM), and ascorbate (10 mM). The assay was performed in 50 mM potassium phosphate (pH 7.0), in a cuvette thermostated at 30°C. The (△) Benzene. (▲) Toluene. (▽) Ethylbenzene. (▼) *o*-Xylene. (○) Naphthalene. (◇) Cyclohexane. (✲) *o*-Diethylbenzene. (●) Tetralin. (□) Biphenyl. (+) α-Pinene. (♦) Decalin.

Effects of hydrocarbons on the proton motive force. The accumulation of hydrocarbons in the lipid bilayer, and the consequent change in membrane-structure due to membrane-expansion, change in membrane fluidity, and/or disruption of lipid-protein interactions could have a strong effect on the

functioning of the membrane as a selective barrier for ions and hydrophilic molecules. Especially the permeability for protons and other ions is of importance, since ion-leaks directly affect the energy transducing properties of the membrane. To analyze the effect of hydrocarbons on the generation of the transmembrane pH gradient (ΔpH) and electrical potential ($\Delta\psi$) in artificial membranes, beef heart mitochondrial cytochrome *c* oxidase was reconstituted into liposomes as proton motive force generating mechanism. At an external pH of 7.0, and in the presence of the electron donor system ascorbate-TMPD-cytochrome *c*, cytochrome *c* oxidase containing proteoliposomes generated a -ZΔpH and $\Delta\psi$ of -54 and -60 mV, respectively. The results show that all hydrocarbons tested dissipated the ΔpH (Fig. 4) and that the inhibitory concentrations directly correlated with the partitioning of the compound into the membrane as well as with the increase in Rhodamine fluorescence and DPH-polarization measurements. The $\Delta\psi$ was found to decrease in a similar way as the ΔpH (data not shown).

Site(s) of action of hydrocarbons. The observed decrease in ΔpH and $\Delta\psi$ could be the result of an increase in passive proton or ion fluxes, and/or inhibition of the energy transducing activity of the cytochrome *c* oxidase. Incubation of cytochrome *c* oxidase containing liposomes with different concentrations of benzene, cyclohexane, tetralin, decalin, and biphenyl showed that indeed inhibition of the enzyme activity occurred. Comparison of the sensitivity of cytochrome *c* oxidase reconstituted in liposomes with the enzyme in Triton X-100 solution indicated that the membrane embedded enzyme was more affected by hydrocarbons (Fig. 5), as could be expected from the accumulation of the molecules in the membrane. Since the enzyme in solution is associated with detergent micelles it is difficult to compare the inhibitory effects on the reconstituted and "free" enzyme quantitatively.

Dissipation of the Δp as a result of an increased proton permeability of the membrane was assessed by determining the passive proton influx across the liposomal membrane. Potassium-loaded liposomes were diluted into potassium-free medium in the presence of valinomycin, and the initial rates of H^+ influx in the absence and presence of different amounts of hydrocarbon were determined (Fig. 6). Increasing amounts of hydrocarbon were needed to increase the proton permeability of the membrane going from anthracene, decalin, tetralin, cyclohexane, toluene to benzene. The concentrations of hydrocarbons that affected the proton permeability were in the same range as those that inhibited cytochrome *c* oxidase.

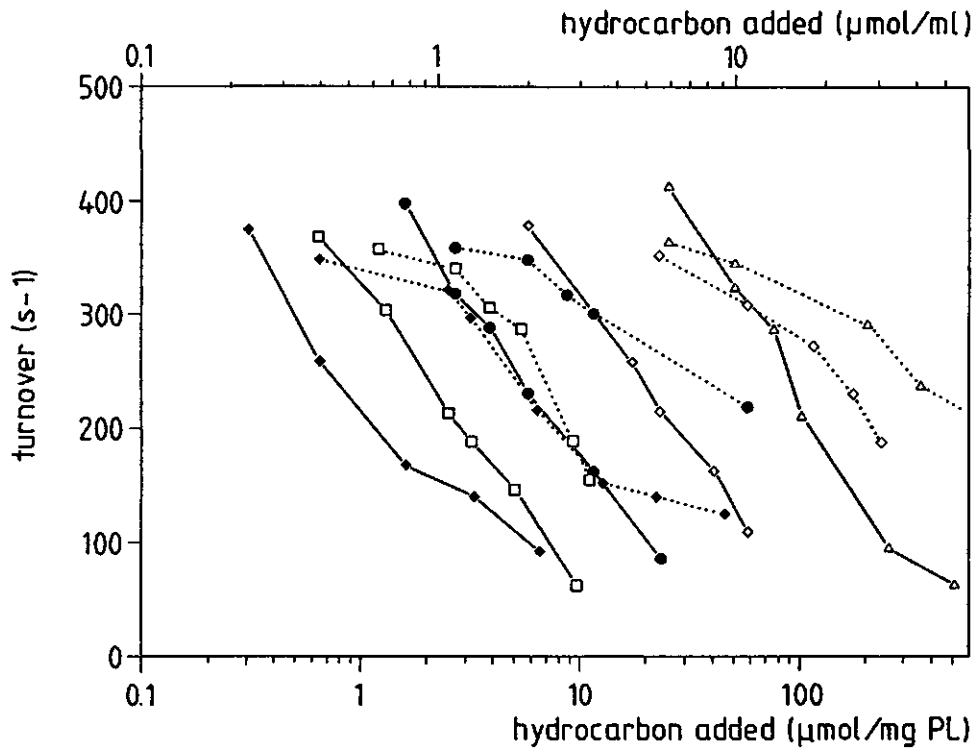

FIG. 5. Activity of cytochrome *c* oxidase as determined by monitoring the oxidation of reduced cytochrome *c* (A_{550}-A_{540}). The reaction mixture contained either proteoliposomes (solid lines) or Triton X-100 (0.5 %, vol/vol) solubilized proteoliposomes (dotted lines), in 50 mM potassium phosphate (pH 7.0); the assay was performed at 30°C. Δp was decoupled by the addition of valinomycin (2 µM, final concentration), and nigericin (0.1 µM, final concentration).
Activity of the cytochrome *c* oxidase is expressed as mole cytochrome *c* per mole enzyme per second. The maximum activity of the cytochrome *c* oxidase in uncoupled liposomes was 425 s^{-1}, and of the enzyme in solubilized liposomes was 370 s^{-1}.
(△) Benzene. (◇) Cyclohexane. (●) Tetralin. (□) Biphenyl. (♦) Decalin.

Permeability of liposomal membranes for CF. To assess the effect of cyclic hydrocarbons on the permeability of the membrane for low molecular weight molecules, the efflux of the fluorescent dye carboxyfluorescein (CF) was examined. In the presence of various hydrocarbons an increased leakage of CF (M_W = 376 Daltons) was observed that paralleled the increase in permeability of the membrane to protons. The concentration at which leakage of

carboxyfluorescein was observed was only slightly higher than the hydrocarbon concentrations needed to increase the proton permeability (data not shown).

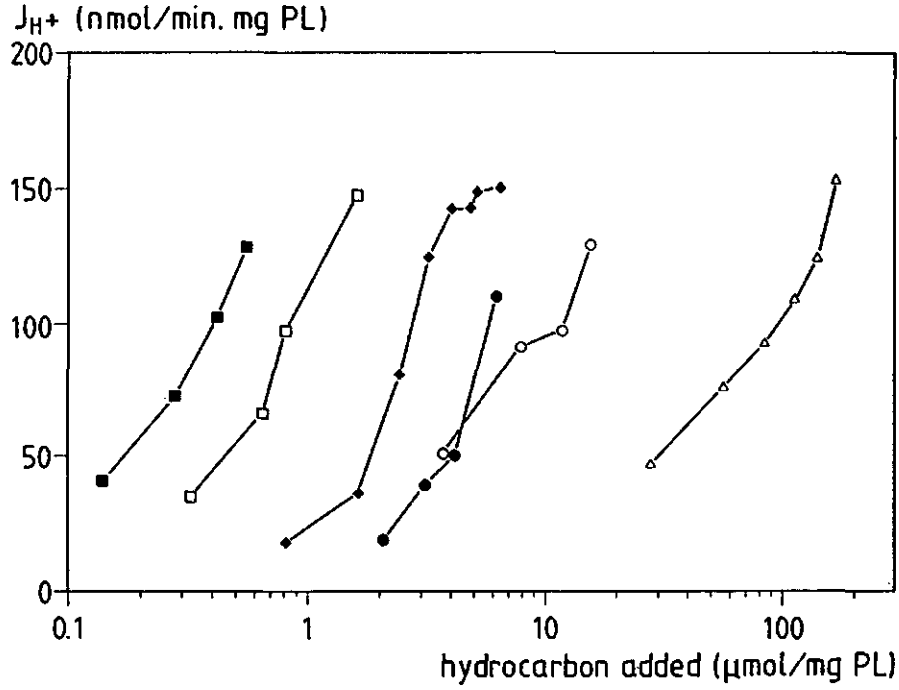

FIG. 6. The effect of cyclic hydrocarbons on the proton permeability of *E. coli* phospholipid membranes. Liposomes (0.2 mg of phospholipid per ml) were washed and resuspended in a medium in which sodium ions were substituted for potassium ions and to which phenol red (20 µg/ml) was added. To initiate the potassium diffusion potential, valinomycin (2 µM, final concentration) was added. Subsequently, absorbance changes were measured at $A_{560} - A_{610}$ to determine the external pH changes caused by proton influx as a compensatory effect on the imposed diffusion potential. (△) Benzene. (○) Naphthalene. (●) Tetralin. (□) Biphenyl. (♦) Decalin. (■) Anthracene.

DISCUSSION

Due to the hydrophobic character of hydrocarbons the primary site of their toxicity is the membrane. Hydrocarbons accumulate in the lipid bilayer according to a partition coefficient that is specific for the compound applied. Since

partitioning of a compound between a membrane and an aqueous phase is difficult to determine, and may vary with the composition of the membrane, attempts have been made to find a parameter for partitioning. The octanol/water system, which has been applied for many years in anesthesiology and environmental biology (19, 40), proved to be the most suitable modelsystem (21). For the *E. coli* phospholipid liposomal membrane/potassium phosphate buffer system, the octanol/water partition coefficient of a wide variety of compounds showed good correlation with the membrane/buffer partition coefficient (Fig. 1). The ratio between these partition coefficients, however, may differ significantly depending on the type of membrane (1-4, 15, 30). Therefore, each membrane system should be tested before quantitative estimations of the partition coefficients can be made.

The cyclic hydrocarbons were dissolved in DMF in order to increase the dissolution rate of the hydrocarbons. The use of a cosolvent is especially relevant for solid hydrocarbons, such as naphthalene, biphenyl, phenanthrene, and anthracene. By using a cosolvent the distribution of the hydrocarbons in the aqueous phase and the membrane phase will come to equilibrium rapidly.

Accumulation of compounds in the membrane may lead to alteration of the membrane structure and function. An important change is the apparent increase in surface area of the membrane, due to swelling of the membrane upon accumulation of lipophilic compounds (23, 30). The expansion observed with hydrocarbons was more than 2 times higher than the expansion by alcohols (31). This variation is probably due to differences in the type of hydrophobic interaction and part of the membrane where lipophilic compounds reside (see also below). Differences in the methods applied to determine the increase in surface area were of less importance, since experiments with *n*-alcohols (butanol to decanol) in the *E. coli* phospholipid/R_{18} system gave results that did not significantly differ from the data reported by Seeman and coworkers (Sikkema et al., unpublished results). The hydrocarbon concentrations that are present in the membrane can be calculated from the estimated membrane-buffer partition coefficients (Table 1). When the R_{18} fluorescence data from Fig. 2 are plotted against the membrane concentrations of the hydrocarbons a concentration range at which 'swelling' occurs can be seen (Fig 7). Up to a concentration, in the membrane, of approximately 0.5 μmol per mg phospholipid ($\pm$ 1 hydrocarbon molecule per 2 phospholipids) an increase in membrane surface area is observed, after which an apparent maximum is reached. The extent of R_{18}

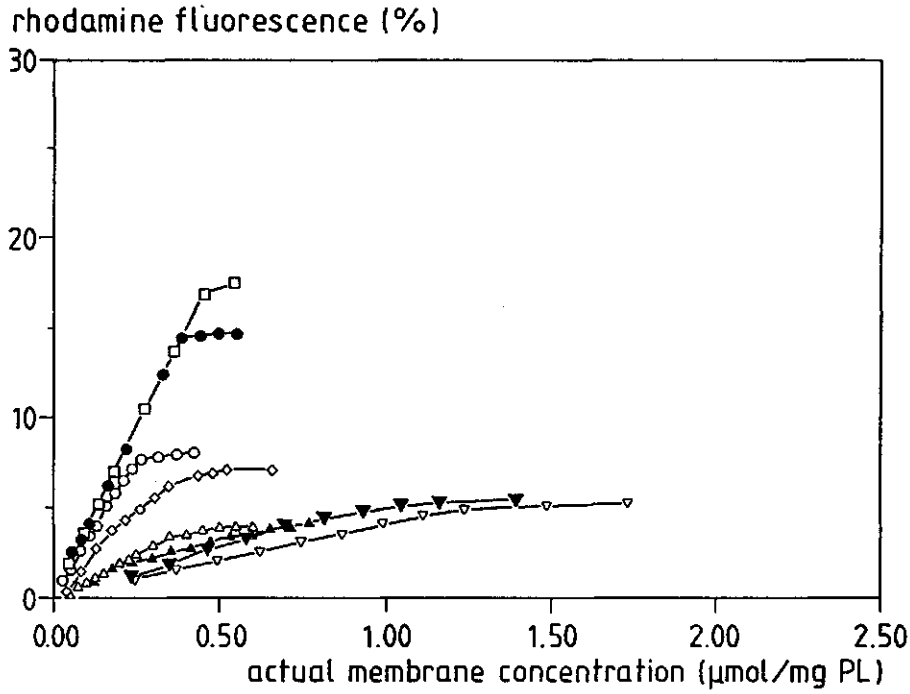

FIG. 7. Increase in R_{18} fluorescence as a function of the concentration of cyclic hydrocarbons in the membrane as calculated from the membrane-buffer partition coefficient (Table I). (△) Benzene. (▲) Toluene. (▽) Ethylbenzene. (▼) *o*-Xylene. (◇) Cyclohexane. (○) Naphthalene. (●) Tetralin. (□) Biphenyl.

fluorescence increase at an actual membrane concentration higher than 0.5 µmol/mg phospholipid (Fig. 7) was highest for the compounds with the highest $P_{M/B}$, i.e., biphenyl and tetralin; naphthalene and cyclohexane were intermediate, whereas *o*-xylene, ethylbenzene, toluene, and benzene were lowest. The cause of this phenomenon is not readily understood although the extent of the R_{18} fluorescence increase parallels the molar volumes of the molecules (Table 1).

The increase in membrane fluidity as estimated from DPH polarization measurements (Fig. 3) is already apparent at slightly lower cyclic hydrocarbon concentrations than the increase in membrane surface area (Fig. 2). This is most clear for α-pinene and tetralin, although also for cyclohexane and naphthalene this effect can be seen. No significant effect of the hydrocarbons on the

polarization of TMA-DPH was observed. These results suggest that the hydrocarbons partition to the central part of the membrane, which directly affects the polarization of the DPH.

As a result of accumulation of hydrocarbons in the membrane the activity of cytochrome *c* oxidase is lowered and the proton (ion) permeability increases. Both effects act synergically on the magnitude of the ΔpH and $\Delta\psi$ generated by cytochrome *c* oxidase. Since a 50 % reduction of cytochrome *c* oxidase activity only causes a small drop in the ΔpH and $\Delta\psi$ (34), the drop in the components of the proton motive force will primarily be caused by the increased proton permeability. To our knowledge the effects of hydrocarbons on the generation and maintenance of the proton motive force have neither quantitatively nor qualitatively been analyzed sofar. Uribe and coworkers reported results which are in accordance with ours and support the view that an important part of the toxicity of hydrocarbons is exerted by effects on the proton motive force (38, 39).

The action of general anesthetics on cell functioning, which is similar to the effects observed for cyclic hydrocarbons, is often ascribed to interaction of the anesthetic compounds with the membrane (24, 30). This hypothesis, which ascribes the inhibitory action of anesthetics fully to changes in membrane integrity, is named the lipid-theory of anesthesia. The competing theory is the protein-interaction theory, which states that anesthesia is a result of interaction of anesthetic molecules with various enzymes involved in cellular metabolism. Our studies clearly indicate that the effects of hydrocarbons on the functioning of biological membranes involves both effects on the permeability [protons (ions) but also larger molecules, e.g., CF] and the activity of membrane enzymes (cytochrome *c* oxidase). The effects on enzyme activity can be due to altered protein-lipid interactions (hydrogen bonding and others), membrane thickness, fluidity, and/or phospholipid headgroup hydration (42). Therefore, it is remarkable that the obvious combination of the lipid-theory of anesthesia (24) and the protein-interaction theory advocated by Franks and Lieb (10, 16) has not gained more attention so far.

A remarkable outcome of our studies is the observation that the effect of cyclic hydrocarbons on the structural and functional properties of biological membranes [(proteo)liposomes] is directly related to accumulation in these membranes; the effect is independent of the structural features of the molecules. The accumulation of cyclic hydrocarbons in the membranes is proportional to the concentration in the aqueous phase and the membrane/aqueous phase partition coefficient. This latter parameter relates directly to the partitioning of

these cyclic hydrocarbons in a standard octanol/water system, which allows predictions to be made about the toxicity of other lipophilic compounds on basis of their logP values. Since bacteria highly differ in their sensitivity towards cyclic hydrocarbons it will be important to establish how the membrane bilayers of these organisms differ and how the $P_{M/B}$ is affected by the phospholipid composition of the membrane. Future studies are aimed at addressing these questions.

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CONCLUDING REMARKS

Regioselective oxidation of cyclic hydrocarbons by biocatalysts is an area with many opportunities for the production of fine chemicals. However, as outlined in the Introduction, biocatalytic systems still suffer from some major drawbacks preventing their commercial application. In this thesis different microbiological aspects that are of importance in establishing such a biotechnological process have been studied. The specific hydroxylation of tetralin to 5,6,7,8-tetrahydro-1-naphthol has been investigated as a model reaction. Two basic questions have been addressed i) selection of suitable biocatalysts, and ii) mechanism of the inhibitory action of the hydrocarbon substrate. The biocatalytic potential of some tetralin-utilizing bacteria has been evaluated, and the biotransformation of tetralin in one strain, *Corynebacterium* sp. strain C125, has been studied in more detail. In this chapter, both aspects are discussed in view of the results presented in this thesis.

Selection of suitable biocatalysts

The tetralin-utilizing strains that have been isolated exhibited two different modes of initial attack, i.e., monooxygenation of the benzylic carbon atom, and dioxygenation of the aromatic nucleus. To obtain the desired phenol, 5,6,7,8-tetrahydro-1-naphthol, only *Corynebacterium* sp. strain C125 was shown to be of interest. The first intermediate in the degradative pathway of tetralin employed by this strain, is a *cis*-dihydro diol (1,2,5,6,7,8-hexahydro-*cis*-1,2-naphthalene diol) with

its hydroxyl groups proximal to the substituent. Since *cis*-dihydro diols are known to rearrange in an acid environment to phenols, 1,2,5,6,7,8-hexahydro-*cis*-1,2-naphthalene diol may yield 5,6,7,8-tetrahydro-1-naphthol and/or 5,6,7,8-tetrahydro-2-naphthol. However, the chemically preferred rearrangement is the formation of 5,6,7,8-tetrahydro-2-naphthol, thus preventing application of *Corynebacterium* sp. strain C125 and also other strains which dioxygenate the aromatic nucleus for the specific production of the leather fragrance. Direct, specific hydroxylation of the aromatic nucleus at the 1-position does not seem feasible in view of the presence of the activated benzylic carbon atom. Experimental data on the monooxygenation of tetralin by either bacteria or fungi suggest that monooxygenases prefer the attack at the benzylic carbon atom (17, 26). Oxidation of indan, a 'cyclopentane-substituted' aromatic that resembles tetralin, by toluene dioxygenase from *Pseudomonas putida* F1, yielded indanol which is the product of the monooxygenation of the benzylic carbon atom (7, 54). Also the naphthalene dioxygenase of *Pseudomonas* sp. NCIB 9816 preferred hydroxylation of the benzylic carbon atom (54). These observations suggest that only highly specific dioxygenases will catalyze the oxidation of the aromatic nucleus of tetralin and indan.

Strains that start with an initial specific oxidation of the benzylic carbon atom may yield other interesting oxidized products from tetralin. The observed stereospecificity of the hydroxylation of the benzylic carbon atom by *Acinetobacter* sp. strain T5 and the fungi studied by Holland et al. (26) show that biocatalytic hydroxylation of tetralin is highly specific in contrast to present chemical processes which produce mixtures of α-tetralol and α-tetralone (49). In some instances also the highly explosive tetralin hydroperoxide is accumulated as an intermediate in the formation of the alcohol and ketone (36). The large scale application of a biocatalytic process for the oxy-functionalization of tetralin, however, is severely hampered by the deleterious effects of tetralin on cellular viability in general and the capability to regenerate cofactors in particular.

Mechanism of the inhibitory action of cyclic hydrocarbons

Our attempts to obtain microorganisms that are able to grow on tetralin, were seriously hampered by the toxicity of the substrate (Chapter 3). The toxicity of tetralin also impaired other attempts to obtain microorganisms capable to grow on this compound (47). From various reports on the microbial transformation of cyclic

hydrocarbons, an inhibitory effect of these substrates on the microorganisms can be concluded (Chapter 2). However, the indications about the nature of the toxicity are vague. Questions about the mechanism of the (aspecific) toxcity of tetralin urged us to evaluate some events that are involved in the inactivation of microorganisms by cyclic hydrocarbons.

From our results it can be concluded that the toxic action is the result of the accumulation of hydrocarbon molecules in the membrane bilayer which depends on the aqueous concentration and membrane/buffer partition coefficient of the molecules (Chapter 7). The extent of the toxic effect is related to the actual concentration in the membrane, the location in the membrane, and the interaction with the membrane constituents. The actual concentration in the membrane can strongly differ as a result of mass transfer characteristics, and possible metabolism of the compounds. In the liposome model system, the actual membrane concentration equals the equilibrium concentration since mass transfer limitation is relieved by the addition of cosolvents and stirring in the cuvette, and no hydrocarbon-metabolizing enzymes are present.

Limited transfer of the lipophilic molecules as a result of low dissolution rates and low solubilities may cause delay of the toxic effects, but does not prevent the toxic action *per se*. However, in a batch system to which a (solid) hydrocarbon substrate with a low dissolution rate is supplied after inoculation, cells can remain viable as a result of the low aqueous concentration. When the cells start to consume the dissolved hydrocarbon molecules, as a result of either adaptation or the presence of constitutive enzymes, the transfer of molecules from the hydrocarbon bulk phase to the aqueous phase may become limiting (53). When the hydrocarbon substrate is allowed to equilibrate in the aqueous medium prior to inoculation, toxic effects do occur (9). Relief of mass transfer limitation may also explain the synergistic toxicity of biphenyl and ethylbenzene to a pseudomonad capable of utilizing either substrate present in higher concentrations than present during incubation with the dual substrates (50).

Addition of an easy degradable carbon source may facilitate an even more rapid depletion by combining consumption of the potentially toxic substrate with increase in biomass. The increase in biomass can cause depletion of the hydrocarbon molecules from the aqueous phase as a result of high partition coefficients between membrane and aqueous phase (Chapter 2, 6, and 7).

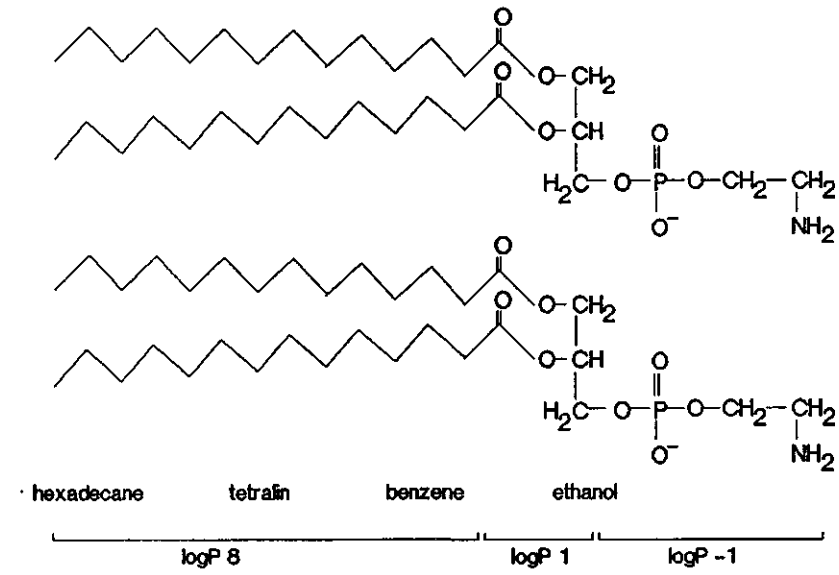

FIG. 1. Proposal for the location of hydrophobic molecules in phospholipid bilayer membranes. The phospholipid bilayer can be seen as a ordered second phase in an aqueous environment. As a result of the amphiphilic character of the constituting phospholipid molecules no homogeneous distribution of the accumulating lipophilic molecules will occur. Depending on the hydrophobicity, solutes will penetrate more or less deep into the bilayer. In this figure the location of some lipophilic compounds in the lipid layer is given depending on the relative hydrophobicity of the phospholipid and the solute. The logP-values for the different parts of the phospholipid molecule are estimated on the basis of the logP-values of related structures.

Accumulation of lipophilic compounds occurs at varying depths in the bilayer depending on the hydrophobicity and presence of functional groups such as hydroxyl-, carboxyl-, or phenyl groups (Fig. 1). Depending on the location in the membrane either the head group or the acyl chain interactions may be disturbed. The hydrophobic cyclic hydrocarbons most probably accumulate in the acyl region of the membrane (Chapter 7). Interaction of lipophilic compounds with the hydrophobic end of the acyl chains has the most pronounced effect on the surface area of the membrane (12), whereas more hydrophilic compounds affect the hydration of the head groups (46). Relatively small hydrophobic compounds such as tetralin, decalin, biphenyl, and anthracene intercalate with the acyl chains,

disturbing the acyl chain interactions which results in 'swelling' of the membrane (see for instance Fig. 7 in Chapter 7), and an increase in bilayer fluidity (Chapter 7). Large, non-toxic, hydrophobic molecules such as longer alkanes also intercalate with the acyl chains, but probably compensate the disturbing effects by interacting with both the inner and outer leaflet of the lipid bilayer (38). The resulting increase in lipid ordering reduces the membrane permeability and fluidity, but does cause an increase in bilayer width (38).

Studies on highly apolar solvents with logP values higher than 5 have shown that these molecules have no toxic effects on cells (8, 32, 51, 57). The observation that solvents with a high partition coefficient to the membrane (e.g., hexadecane) are not toxic to cells seems to be contradictory to observations that toxicity of lipophilic compounds increases with increasing partition coefficients. For *n*-alkanols, the anesthetic efficacy increases upto *n*-dodecanol, whereas *n*-alkanols with more than 12 carbons lack anesthetic potency (39). For microorganisms the 'cut-off' in toxic effect is around logP 4 to 5 (32, 52) but significant differences are observed for different organisms and different solvents. The explanations given for the 'cut-off' in toxicity or anesthesia either involve low solubility of the solvents in the membrane bilayer, or absence of a membrane disturbing effect.

For extremely hydrophobic compounds, e.g., fluorocompound 40 (logP $\approx$ 11.4), the low solubility in the membrane bilayer and the high transfer resistance of the hydrophilic phospholipid head groups may prevent accumulation in the membrane. However, experimental evidence on the fate of these compounds in biological membranes is not available.

Experiments by Miller and coworkers (39) showed that nonanesthetic alkanols accumulate in the membrane, suggesting that lack of anesthetic potency is not a result of low solubility in the lipid bilayer. Partition coefficients of long chain alkanols appear to correspond with the higher logP values of these compounds. Similar observations were reported by Requena and Haydon (45) for *n*-alkyltrimethylammonium ions. Whereas for the nonanesthetic alkanols no deviation of the partitioning could be observed, a significant change in the effect of the alkanols on the ordering of the membrane lipids occurred (39). These data are in accordance with the hypothesis of Lee (33), who postulated that anesthesia is not merely a result of accumulation of lipophilic compounds in the membrane but is also caused by an increase in the proportion of lipids in a fluid state. It has been shown in several instances that interaction of lipophilic compounds with anesthetic or toxic potency causes an increase in lipid fluidity (Chapter 7). On the other hand,

long chain alkanols that have no anesthetic potency may produce a cataleptic state (33).

Implications of substrate toxicity to biotransformation processes

Studies on the membrane interaction of tetralin with intact cells indicated that also in the whole cell system dissipation of both components of the proton motive force occurred. The collapse of the proton motive force will affect various vectorial energy transducing processes such as solute transport, ATP synthesis/hydrolysis, and others (23, 40). In addition to the effect on the energy status of the cell, the maintenance of cellular homeostasis is affected by the increased membrane leakage (6). The inability to control the intracellular pH may severely impair proper functioning of the cell (44), and may be even more important than the maintenance of a high proton motive force as 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 mM range (21). Recently, Cardoso and Leão (10) reported that the inactivation of cells of *Saccharomyces cerevisiae* by monocarboxylic acids 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. In Fig. 2 the effect of the external pH on the growth rate of cells of *Escherichia coli* in the presence of tetralin is presented. It appeared that cells incubated in media with a low pH (< 7) are more sensitive to the inhibitory action of tetralin than cells that are incubated in media with higher pH (7.5-8). These results demonstrate that the toxic effects of tetralin can be reduced when the external pH approximates the internal pH of cells under physiological conditions, and when the amount of Na^+ in the medium was limited (data not shown). Although the results with tetralin-treated cells suggest that the regulation of the intracellular pH is impaired, the limited reduction of the toxic effects demonstrates that other sites of toxic action (dissipation of Δp, inactivation of enzymes) are more important.

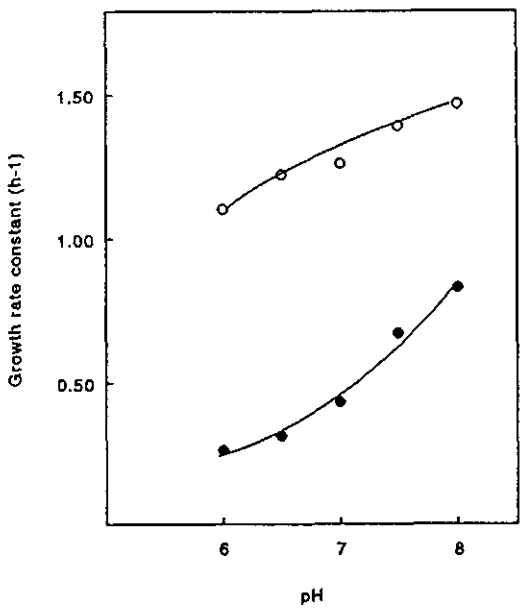

FIG. 2. Effect of tetralin on the growth rate of *Escherichia coli* K-12. Cells were grown in yeast extract/glucose medium (0.1 % (wt/vol) yeast extract, 1 % (wt/vol) glucose), buffered with 200 mM potassium phosphate buffer. Symbols: (●) growth rate in the presence of 1 mM tetralin; (O) growth rate in the absence of tetralin.

As a result of the changes in membrane structure the functioning of proteins that are embedded in the membrane lipid bilayer is affected. Transport and other Δp requiring processes may be severely hampered by the effects of these compounds on the energy status of the cell, but also the changes in the lipid environment of the (transport) proteins may lead to decreased turnover rates (28, 58).

Efficient oxy-functionalization of cyclic hydrocarbons can be severely hampered by the impairment of the barrier function of the membrane. Depletion of NAD(P)H by respiratory control mechanisms impairs activities of oxygenases, and also changes in intracellular pH decrease activities of these enzymes. Since NADH is the main substrate for the respiratory chain, whereas NADPH is the most important electron donor in reductive biosynthesis, especially NADH requiring oxygenases (e.g., hydroxylating dioxygenases) will be affected. Furthermore, the functioning of membrane embedded enzyme complexes involved in hydroxylation of (cyclic) hydrocarbons may be hampered as a result of changes in lipid-protein interactions (27).

In the environment cyclic hydrocarbons may severely impair microbial transformation processes which play an essential role in the various elemental cycles (15, 24). Especially pulses of high concentrations of these compounds disturb

microbial populations, which otherwise could have adapted to the new nutrients. Due to the sudden shock of high concentrations of cyclic hydrocarbons, microorganisms will be inactivated and even fail to generate energy from their normal substrates. Similar problems occur in waste treatment facilities when hydrocarbon-containing wastes are discharged. Also the isolation of microorganisms from the environment by selective enrichment on cyclic hydrocarbons can be impaired by the toxicity of these compounds to the cells (3, 20). Substrate concentrations of around 1% of e.g., benzene, toluene, and tetralin effectively (chemo)sterilize the incubation media (3; Chapter 3), whereas at low concentrations (lower than approximately 100 μg/l) the amount of organic carbon is too low to sustain distinguishable growth.

TABLE 1. Activities of oxygen radical scavenging enzymes in bacteria that have been grown on aromatic hydrocarbons as sole source of carbon and energy. Cells grown on succinate were taken as control.

Organism Growth substrate	Catalase activity[a] ($u \cdot mg\ protein^{-1} \cdot min^{-1}$)	SOD activity[b] ($u \cdot mg\ protein^{-1} \cdot min^{-1}$)
Corynebacterium sp. strain C125		
Succinate	102 ± 3	11 ± 2
Glucose	114 ± 6	13 ± 2
o-Xylene	255 ± 4	51 ± 3
Ethylbenzene	535 ± 12	70 ± 6
Tetralin	657 ± 9	68 ± 7
Arthrobacter sp.strain T2		
Succinate	101 ± 1.5	10 ± 2
Toluene	620 ± 50	66 ± 8
Tetralin	703 ± 16	61 ± 11
Pseudomonas sp. strain NCIMB 10643		
Succinate	144 ± 18	26 ± 1
Biphenyl	820 ± 35	74 ± 5

[a] Catalase activity was determined by monitoring the decrease in A_{240} as a result of the removal of H_2O_2, as described by Aebi (1).

[b] Activity of the superoxide dismutase was determined by monitoring the decrease in the rate of reduction of cytochrome *c* by superoxide radicals as described by McCord and Fridovich (37).

In addition to effects (dissipation of proton motive force, inhibition of membrane embedded enzymes) of cyclic hydrocarbons on microorganisms, listed in Chapters 6 and 7, other targets in the cell can be affected. In several instances induction of

the synthesis of stress proteins upon incubation with hydrophobic compounds has been observed (41, 42). Blom et al. (5) reported that treatment of cells of *Escherichia coli* with pollutant compounds induces a large number of proteins. Although some of these proteins overlapped with heat shock and carbon starvation proteins, most were unique for the chemical compound supplied. In cell free extracts of bacteria grown in the presence of several aromatic hydrocarbons we have observed increased levels of catalase and superoxide dismutase (Table 1; Sikkema and Smith, unpublished results). Similar observations have been made by Zhang and Yonei (59), who showed that treatment of cells of *Escherichia coli* with membrane binding drugs (chlorpromazine, procaine) resulted in an increase of the activity of superoxide dismutase. The mechanism of the induction of catalase and SOD, and the source of oxygen radicals remains to be established. A possible explanation is the induction of a general alarm response upon disturbance of cellular homeostasis as observed with cells that have been exposed to ethanol (43) or uncouplers (2).

The 'heat shock response' is possibly triggered by the decrease of the internal pH (11). Acidification of the cytoplasm is known to affect various metabolic processes (44), and to significantly decrease cellular viability (10). Many of the factors that induce the heat shock response, including ethanol, lowered external pH, and increased temperature, are known to affect the internal pH (11). Proteins that are induced include radical scavenging enzymes that prevent adverse side effects, but also enzymes that are involved in the regulation of membrane fluidity can be expected (16; see also Chapter 2 for references).

Methods to prevent toxic effects

In order to be able to profit from the transformation capacity of microoganisms, methods to prevent the toxicity have to be found. Since the toxicity results from the accumulation of the lipophilic hydrocarbon compounds in the membrane bilayer, the transfer of such compounds to the lipid bilayer should be minimized. At the same time, enough hydrocarbon molecules should be available to be transformed by intracellular enzymes. Reduction of the hydrocarbon accumulation can be achieved by i) limiting the transfer across the cell envelope, ii) lowering of the partition coefficient between the membrane bilayer and the aqueous environment, or iii) decreasing the aqueous concentration of the cyclic hydrocarbon. Basically,

two approaches can be followed, starting either from the microorganism or from the process.

The selection of microorganisms that are able to thrive in high concentrations of toxic organic solvents by forming a capsule around the cell may not always be useful for application in biotransformation processes. The isolation of a *Pseudomonas* strain that is extremely resistant to toluene (29) may help to illustrate this. Although this strain is able to grow on yeast-glucose medium in upto 50 % (vol/vol) toluene, it can not transform toluene.

Recently, other strains of *Pseudomonas* were obtained that were able to grow on cyclic hydrocarbons, e.g., *p*-xylene (14), styrene (55), and α-pinene (Sikkema et al., unpublished results), that were present in super-saturating amounts. The mechanism of the adaptation has not been elucidated sofar, however, data obtained by Weber indicate that the characteristics of the cell surface as well as the composition of the lipid bilayer are modified. Modification of the lipid composition of the bilayer is a common mechanism in cells to compensate for changes in membrane fluidity induced by environmental stimuli (48). For a *Pseudomonas* strain able to grow on high concentrations of phenol, a marked change of the fatty acids from *cis* to *trans* conformation has been observed (16, 22). It is known that an increase in the content of *trans* fatty acids results in a higher ordering of the lipid layer (see for references Chapter 2). The higher degree of ordering of the lipid bilayer provides the cell with a mechanism to compensate for loss of membrane integrity as a result of accumulation of lipophilic compounds. Furthermore, an increased lipid ordering also opposes partitioning of lipophilic compounds in membranes (35). The application of such strains as plugbugs may facilitate efficient transformation of cyclic hydrocarbons in simple fermentors. Furthermore, the application of these adapted strains in waste treatment will significantly reduce the sensitivity of such biological processes. The influent concentration of the cyclic hydrocarbon pollutants needs to remain at a high level in order to outcompete other, non-adapted, bacteria.

The technological approach which concentrates on reducing the amount of toxic cyclic hydrocarbon in the direct environment of the cells has received a great deal of attention in the last decade. Much effort has been directed towards the selection of biocompatible organic solvents, that can serve as a non-toxic second-phase that contains the toxic cyclic hydrocarbon substrate. An additional advantage is that the second phase can be used for the continuous extraction of product(s) from the aqueous phase. However, the application of two-phase systems also has some major limitations, e.g., restricted application in the production of pharmaceuticals and food

additives, problematic bioreactor design, usage of large amounts of solvents (e.g., phthalates, fluorocarbons, etc.). Moreover, non-toxic solvents are usually highly apolar and consequently partitioning coefficients for oxy-functionalized compounds are relatively low (52). Studies on the prevention of toxic effects of steroids by the addition of liposomes showed that significant increases in biocatalytic activity could be obtained when the total lipid content of the incubation is raised (17, 18). These results are not surprising since they elegantly reflect the effect of accumulation of lipophilic compounds in the membrane which reduces the aqueous concentration of these compounds. However, the use of liposomes is technically and economically not feasible. Application of cell recycle systems (4) or addition of easy degradable cosubstrates in order to increase the biomass is more realistic. Addition of a cosubstrate together with careful buffering of the medium at approximately the same pH as the optimal cytoplasmic pH of the cells, may provide enough phosphate-bond energy and a suitable environment, in order to perform biocatalytic reactions.

Limitation of toxic effects by immobilization of the biocatalysts in hydrophilic support materials has been observed for different organic solvents (25, 56). Data obtained by Hocknull and Lilly (25) indicate that immobilization protects the cells against toxic effects of distinct second phases. Immobilization looks promising for application in waste treatment, in order to prevent toxic effects of pulses of lipophilic compounds. However, biocatalytic processes may be impaired by accumulation of products. Furthermore no protection against molecular toxicity can be expected.

The above mentioned approaches focus on limiting the aqueous concentration or the transfer of hydrocarbon molecules to the cells. These approaches both involve the addition of a third phase to the medium (non-miscible organic solvent, solid support), leading to a more complex process. Addition of substrates via the vapor phase is an easy method to prevent substrate toxicity (13), but large scale applications are limited to gaseous compounds. Process engineering methods to increase the cellular tolerance to lipohilic compounds have not received a great deal of attention. A very interesting characteristic of narcotics is the reversal of anesthesia upon application of hydrostatic pressure (30, 34). This feature can be very well explained by the increase in lipid ordering in response to the applied pressure (35). Application of hydrostatic pressure in biotransformation processes might provide an efficient method to increase lipid ordering and thus prevent toxic effects of compounds that disturb the lipid order.

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SUMMARY

Biocatalytic oxidation of cyclic hydrocarbons has many potential applications in the production of fine chemicals. Especially regioselective hydroxylation of aromatics and the stereospecific formation of secondary alcohols is of interest for the pharmaceutical and flavoring industries. Hydroxylating enzymes are active under mild reaction conditions allowing the controlled transformation of less stable substrates and formation of easily oxidizable products (e.g., catechols). Furthermore, application of microorganisms for the removal of cyclic hydrocarbons from waste streams provides a highly versatile method for the removal of toxic cyclic hydrocarbons.

Major drawbacks in the application of biocatalysts for these processes are the need for cofactor regeneration and the low stability as a result of inhibitory effects of the hydrocarbon substrates. The investigations described in this thesis have dealt with microbiological aspects of the design of a biocatalytic hydroxylation process. As a model for microbial oxy-functionalization, the dioxygenation of tetralin to 1,2,5,6,7,8-hexahydro-*cis*-naphthalene diol and the subsequent chemical rearrangement to 5,6,7,8-tetrahydro-1-naphthol has been studied. Tetralin provides a perfect model compound for specific hydroxylation since different sites of initial oxidative attack can be envisaged (Chapter 4). Moreover, different oxygenated derivatives of tetralin are of interest to pharmaceutical industries as precursors for the production of hormone-analogs, sedatives, and tranquilizers. Also for the production of fragrance compounds, oxygenated tetralins may be useful (Chapter 1 and 4). Special attention has been given to the mechanism of the toxicity of cyclic

hydrocarbon substrates. In Chapter 2 literature data concerning inhibitory effects of cyclic hydrocarbons and other lipophilic compounds on microorganisms has been reviewed.

Selection of suitable biocatalysts. Chapter 3 describes the procedure that has been followed to obtain microorganisms that are able to use tetralin as sole source of carbon and energy. Enrichment cultures on tetralin were set up with soil samples from polluted areas, and also cyclic hydrocarbon-utilizing strains from culture collections were tested. Initial attempts were unsuccessful, which was attributed to substrate inhibition. By lowering the concentration of tetralin in the incubation media, growth occurred in several enrichment cultures, but no pure strains were isolated. Eventually, a pure culture was isolated by supplying tetralin in sub-saturating concentrations (lower than 125 μmol/liter). Initial studies on the inhibitory action of tetralin on this strain, *Arthrobacter* sp. strain T2, indicated that an aqueous concentration of approximately 100 μmol/liter already impaired growth, whereas quantities above the saturation concentration (approximately 125 μmol/liter) fully inhibited growth of the starved cells. These findings were taken into consideration in new attempts to isolate tetralin-utilizing strains. Addition of tetralin via the vapor phase, thus limiting the aqueous concentration, resulted in the selection of another bacterium from an enrichment culture set up with soil from a land farming facility. Furthermore, four strains that were previously isolated on *o*-xylene, styrene, or mesitylene respectively were also shown to degrade tetralin. Alternatively, enrichment cultures were set up with tetralin added in a non-miscible, non-biodegradable, and non-toxic organic solvent (Fluorocompound 40) which limits the aqueous concentration by serving as a reservoir for the toxic substrate. From these enrichment cultures, two different strains were obtained that were able to use tetralin as sole source of carbon and energy.

In Chapter 4 a survey of initial oxidation steps that may be involved in the biotransformation of tetralin is presented together with experimental data on the accumulation of intermediates oxygenated intermediates from tetralin. The knowledge on the initial oxidative steps has been used to evaluate the potentialities of the selected strains. It appeared that five strains started with an initial oxidation of the benzylic carbon atom, resulting in the formation of α-tetralol and α-tetralone. Two strains exhibited aspecific oxidations yielding products characteristic of both oxidation of the aromatic and the alicyclic moiety. Only one strain, *Corynebacterium* sp. strain C125, degraded tetralin by initially oxidizing the benzene nucleus. The metabolic pathway of tetralin in *Corynebacterium* sp. strain C125 has been studied

in detail. The results, which are presented in Chapter 5, show that the aromatic moiety is attacked by a dioxygenase at the carbon atoms proximal to the cycloalkane substituent. The *cis*-dihydro diol that is formed in this reaction is further metabolized via a dehydrogenase to 5,6,7,8-tetrahydro-1,2-dihydroxynaphthalene, which is a substrate for an extra-diol cleaving catechol dioxygenase. Acid-rearrangement of 1,2,5,6,7,8-hexahydro-*cis*-1,2-naphthalene diol yielded the corresponding phenols, 5,6,7,8-tetrahydro-1-naphthol and 5,6,7,8-tetrahydro-2-naphthol, in a ratio of 1:6. The apparent preference for the 2-naphthol limits the feasability of this system for the formation of the desired fragrance compound, 5,6,7,8-tetrahydro-1-naphthol. However, the specificities of the different tetralin-transforming strains enable the formation of some high-value precursors for the synthesis of pharamaceuticals (Chapter 1 and 4).

Mechanism of the inhibitory action of tetralin and other cyclic hydrocarbons. From the results presented in Chapter 3 it is clear that tetralin has a deleterious effect not only on tetralin-utilizing strains but also on other organisms. From incubations with possible intermediates of tetralin metabolism it appeared that tetralin, and not an intermediairy reaction product, was responsible for the observed toxicity. Similar observations for other cyclic hydrocarbons suggested that the inhibitory action of these compounds resulted from interaction with the membrane(s) of microbial cells (Chapter 2).

In Chapter 6 the mechanism of the toxic action of tetralin on microorganisms has been investigated in intact cells of tetralin-utilizing and non-utilizing bacteria, as well as in liposomes. The results of these investigations indicated that tetralin was accumulated in the membrane, which lead to a significant increase in membrane surface area. Similar studies with other cyclic hydrocarbons, as described in Chapter 7, showed that in addition to the increase in surface area also an increased membrane fluidity was observed. The effective concentrations of the cyclic hydrocarbons necessary for disturbing membrane integrity decreased with increasing hydrophobicity (measured as partition coefficient in an octanol/water system). The hydrophobicity of the hydrocarbon compounds provides a good measure for the partition coefficients of these compounds between the aqueous environment and the membrane (Chapter 7). From the estimated membrane/buffer partition coefficients the actual concentrations of the different in the membrane could be calculated and related to their effects on the membrane properties.

As a result of the membrane expansion and increase in bilayer fluidity, the integrity of the membrane was impaired. Consequently, the passive permeability of

the membrane to protons (ions) was increased and the activity of the membrane embedded proton-pump cytochrome *c* oxidase was reduced. As a result of an increased permeability for protons and impairment of proton-pumping activity, the proton motive force was dissipated and internal pH homeostasis was disturbed. The dissipation of the proton motive force may result in the depletion of metabolic energy, but lowering of the internal pH may lead to complete inactivation of enzymes.

In Chapter 8 some implications of the postulated mechanism of the toxic action of cyclic hydrocarbons have been discussed in relation to the application of microorganisms for the biotransformation of such compounds. Also some aspects of the adapation of the cells have been treated in connection with a general response of cells to stress. Finally, some methods to prevent deleterious effects of cyclic hydrocarbons have been discussed in view of the proposed toxicity mechanism.

SAMENVATTING

Het in dit proefschrift beschreven onderzoek was gericht op microbiologische aspecten die van belang zijn voor het opzetten van een biokatalytisch hydroxyleringsproces. Het proces van biokatalytische oxydatie biedt veel mogelijkheden in de produktie van fijnchemicaliën. Voor de geneesmiddelen- en de geur- en smaakstoffenindustrie biedt vooral de regiospecifieke hydroxylering van aromaten en de stereoselektieve vorming van secundaire alkoholen interessante mogelijkheden. Hydroxylerende enzymen zijn werkzaam onder milde reaktieomstandigheden, waardoor ook meer labiele substraten kunnen worden gebruikt (b.v. aminozuren) en bovendien, gemakkelijk (auto)oxydeerbare produkten (catecholen) kunnen worden gevormd. Daarnaast zijn micro-organismen vaak uiterst efficiënte en veelzijdige opruimers van cyclische koolwaterstoffen. Deze veelal giftige verbindingen komen veelvuldig voor in afvalstromen. Echter, het gebruik van biokatalysatoren voor zowel synthetische- als zuiveringsprocessen wordt bemoeilijkt door de remmende werking van deze koolwaterstoffen op de microbiële cel (Hoofdstuk 2).

Als model voor microbiële oxygenering is in dit onderzoek de oxydatie van tetralien (1,2,3,4-tetrahydronaftaleen) tot 1,2,5,6,7,8-hexahydro-*cis*-1,2-naftaleendiol en de daaropvolgende chemische omzetting in 5,6,7,8-tetrahydro-1-naftol bestudeerd. Tetralien werd enerzijds als modelverbinding voor specifieke hydroxylering gekozen, omdat verschillende aangrijpingspunten voor de initiële oxygenering mogelijk zijn (Hoofdstuk 4). Anderzijds, omdat een verscheidenheid van geoxygeneerde tetralien-derivaten in de geneesmiddelen- en de

geurstoffenindustrie toepassing vinden (Hoofdstuk 1 en 4).

Speciale aandacht is besteed aan het mechanisme van de toxische werking van cyclische koolwaterstoffen. In Hoofdstuk 2 wordt een overzicht gegeven van de literatuur over de toxiciteit van cyclische koolwaterstoffen, waarbij met name aandacht is besteed aan de invloed van deze verbindingen op de membraanfunctie.

Selektie van geschikte biokatalysatoren. Het experimentele onderzoek was aanvankelijk gericht op het selecteren van micro-organismen die tetralien kunnen gebruiken als enige koolstof- en energiebron. In Hoofdstuk 3 is de procedure beschreven die voor de selectie van biokatalysatoren gevolgd is. Vervuilde grond werd gebruikt als entmateriaal om ophopingen op tetralien in te zetten. Ook werden uit cultuurcollecties stammen geselecteerd, die op gelijksoortige cyclische koolwaterstoffen kunnen groeien. De eerste pogingen om tetralien-afbrekende bacteriën te verkrijgen hadden geen succes, naar later bleek als gevolg van remming door het substraat. Na een geringe verlaging van de tetralienconcentratie in de cultuurvloeistof vond in enkele ophopingen groei plaats. Maar er konden geen reincultures worden verkregen. Pas door toediening van tetralien in concentraties beneden de verzadigingsconcentratie (≤125 μmol/liter) kon *Arthrobacter* stam T2 worden geïsoleerd. In onderzoek naar de oorzaak van de giftigheid van tetralien voor *Arthrobacter* stam T2 werd gevonden, dat bij concentraties van ca. 100 μmol/liter al groeiremming optrad, en dat hoeveelheden boven de verzadigingsconcentratie (± 125 μmol/liter) resulteerde in een volledige remming van de gehongerde cellen. Deze resultaten werden gebruikt in het vervolgonderzoek naar methodieken om cultures te verkrijgen van micro-organismen, die op tetralien kunnen groeien. De hoeveelheid in de waterfase werd verminderd door tetralien in de dampfase toe te voegen. Uit een ophopingsculture met een grondmonster van een 'land farming' werd op deze wijze een bacterie geïsoleerd. Onder deze omstandigheden bleken ook vier stammen, die tevoren op respectievelijk *o*-xyleen, styreen en mesityleen waren geïsoleerd, op tetralien te kunnen groeien. Als alternatief werd voorts een aantal ophopingscultures op tetralien ingezet in de aanwezigheid van een met water onmengbaar oplosmiddel (Fluorocompound 40). Dit produkt is niet giftig, maar biologisch ook niet afbreekbaar. Het diende als een reservoir voor het giftige substraat, zodat hun hoeveelheid in de waterige fase werd beperkt. Uit deze ophopingen werden twee bacteriën geïsoleerd, die tetralien kunnen gebruiken als enige koolstof- en energiebron. Uit de verdelingscoëfficiënt van tetralien tussen water en het oplosmiddel kon worden berekend, dat de concentratie tetralien in de waterfase van deze ophopingen iets lager was dan de

verzadigingsconcentratie.

Hoofdstuk 4 geeft een overzicht van de oxydatiestappen die door micro-organismen kunnen worden gebruikt voor de omzetting van tetralien. Hier zijn voorts de resultaten van proeven beschreven met de geselecteerde bacteriën over de ophoping van initiële oxydatie-produkten. Identificatie van de opgehoopte intermediairen was nodig, om de toepassing van de geselecteerde organismen voor de vorming van specifieke gehydroxyleerde tetralien-derivaten te beoordelen. Vijf stammen startten de afbraak met de oxydatie van het benzylisch koolstofatoom, waarbij α-tetralol en α-tetralon werden gevormd. Twee bacteriën gaven oxydatieprodukten, die duidden op de oxydatie van zowel de aromatische als de verzadigde ring van het tetralienmolecuul. Alleen *Corynebacterium* stam C125 begon de afbraak van tetralien met een oxydatie van de aromatische ring.

De afbraakroute van tetralien in *Corynebacterium* stam C125 is verder onderzocht. De resultaten hiervan zijn in Hoofdstuk 5 beschreven. De oxydatie door het dioxygenase grijpt aan op de twee koolstofatomen die het dichtst bij de substituent liggen. Het gevormde 1,2,5,6,7,8-hexahydro-*cis*-1,2-naftaleendiol wordt omgezet door een *cis*-dihydrodiol dehydrogenase tot het bijbehorende catechol (5,6,7,8-tetrahydro-1,2-naftaleendiol). Vervolgens wordt de aromatische ring geopend door een catecholdioxygenase, dat aangrijpt naast de beide hydroxylgroepen (exo-splitsing). Omzetting van 1,2,5,6,7,8-hexahydro-*cis*-1,2-naftaleendiol in een zure omgeving resulteerde in de vorming van 5,6,7,8-tetrahydro-1-naftol en 5,6,7,8-tetrahydro-2-naftol in een verhouding van 1 op 6. Het organisme toonde een duidelijke, chemische voorkeur voor het 2-naftol. Dit beperkt de toepasbaarheid van een dergelijk systeem voor de (gewenste) vorming van de geurstof 5,6,7,8-tetrahydro-1-naftol. Niettemin kunnen de waargenomen specificiteiten van de verschillende tetralien-omzettende bacteriën behulpzaam zijn bij de vorming van enkele waardevolle grondstoffen voor de produktie van geneesmiddelen (Hoofdstuk 1 en 4).

Toxische werking van tetralien en andere cyclische koolwaterstoffen. De resultaten van het in Hoofdstuk 3 beschreven onderzoek gaven aan, dat tetralien giftig is voor een groot aantal stammen en zelfs voor micro-organismen die op tetralien kunnen groeien. Aan de hand van incubaties met mogelijke intermediairen van tetralienafbraak kon worden geconcludeerd, dat het tetralien zélf de toxiciteit veroorzaakte. Vergelijkbare waarnemingen voor andere cyclische koolwaterstoffen wijzen erop, dat de giftigheid een gevolg is van de beïnvloedingvan de structuur en het functioneren van het celmembraan door deze verbindingen (Hoofdstuk 2).

In Hoofdstuk 6 zijn de achtergronden van de toxische invloed verder uitgewerkt aan de hand van onderzoek aan cellen van zowel tetralien-afbrekende bacteriën, als aan die van stammen die niet op tetralien kunnen groeien. Bovendien zijn liposomen van *E. coli* fosfolipiden voor dit doel bestudeerd. De uitkomsten wezen er op, dat tetralien werd opgehoopt in het membraan. Dat leidde tot een toename van het membraanoppervlak. Uit vergelijkbare experimenten met een aantal andere cyclische koolwaterstoffen bleek, dat naast het membraanoppervlak ook de vloeibaarheid van het membraan was toegenomen. De hoeveelheid koolwaterstof nodig om de membraanfunctie te verstoren nam af naarmate de hydrofobiciteit (gegeven als de verdeling in een octanol-water mengsel; logP) van de verbinding toenam. De hydrofobiciteit van de koolwaterstoffen bleek tevens een betrouwbare maat te zijn voor de verdeling van deze verbindingen tussen de waterige fase en het membraan (Hoofdstuk 7). De werkelijke concentraties van de koolwaterstoffen in het membraan, bepaald uit de membraan-buffer verdelingscoëfficiënten, konden worden uitgezet tegen de effecten van deze verbindingen op het membraan. De toename in het membraanoppervlak en in de vloeibaarheid was gerelateerd aan een afname van de integriteit van het membraan. Dit ging gepaard met een toename van de doorlaatbaarheid van het membraan voor protonen en andere ionen. Tevens werd een afname in de aktiviteit van het in het membraan gesitueerde, protonen-pompende, cytochroom *c* oxidase gemeten. Door de toegenomen doorlaatbaarheid voor protonen en ionen en de remming van protonen-pompende enzymen, liep de protonen drijvende kracht (Δp) terug. Het verval van de protonen drijvende kracht leidt tot een afname van de energetische potentiaal van de cel. Bovendien werd hierdoor de controle van de intracellulaire pH verstoord, wat kan leiden tot inactivatie van enzymen.

Het in dit proefschrift beschreven mechanisme verantwoordelijk voor de toxische eigenschappen van cyclische koolwaterstoffen heeft grote consequenties voor de toepassingsmogelijkheden van microorganismen in biotransformatieprocessen. Enkele van deze implicaties zijn in Hoofdstuk 8 beschreven. Daarnaast worden hier enkele adaptatie-mechanismen van de cellen besproken in relatie tot algemene aanpassing aan stress. Aan het eind worden, in het licht van het mechanisme dat er aan ten grondslag ligt, enkele methoden om de toxiciteit van cyclische koolwaterstoffen te voorkomen besproken.

LIST OF ABBREVIATIONS

$\Delta\tilde{\mu}_{H}+$	:	electrochemical gradient of protons across the membrane
Δp	:	protonmotive force ($\Delta p = \Delta\psi - Z\Delta pH$)
$\Delta p_{Na}+$	:	sodiummotive force
$\Delta\psi$	:	transmembrane electrical potential
ΔpH	:	transmembrane proton gradient (pH_{in}-pH_{out})
ΔG_P	:	phosphate potential
J_{H+}	:	initial rate of H^+ influx
$P_{O/W}$	:	partition coefficient in a standard octanol / water system
$P_{M/B}$	:	partition coefficient in a *E. coli* phospholipid membrane / potassiumphosphate buffer system

ATP	:	adenosine 5'-triphosphate
BCECF	:	2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein
CF	:	carboxyfluorescein
DBP	:	dibutylphthalate
DEP	:	diethylphthalate
DMF	:	*N*-dimethylformamide
DMSO	:	dimethyl sulfoxide
DOP	:	dioctylphthalate
DPH	:	1,6-diphenyl-1,3,5-hexatriene

EDTA	:	ethylenediaminetetraacetate
FC 40	:	fluorocompound 40
LPS	:	lipopolysaccharide
MDR	:	multidrug resistance
MOPS	:	4-morpholinepropanesulfonic acid
NADH	:	reduced nicotinamide adenine dinucleotide
NADPH	:	reduced nicotinamide adenine dinucleotide phosphate
PAH	:	polycyclic aromatic hydrocarbon
PL	:	phospholipid
R_{18}	:	octadecyl rhodamine β-chloride
N-Rh-PE	:	*N*-(lissamine rhodamine β-sulfonyl)-phosphatidyl-ethanolamine
SOD	:	superoxide dismutase
TMA-DPH	:	1-[4-(trimethylamino)-phenyl]-6-phenylhexa-1,3,5-triene
TMPD	:	N,N,N',N'-tetramethyl-*p*-phenylenediamine
TPP^+	:	tetraphenylphosphonium ion

CURRICULUM VITAE

Jan Sikkema werd geboren op 27 juli 1964 te Surhuisterveen. Na het behalen van het Gymnasium β diploma aan de Rijksscholengemeenschap "Het Drachtster Lyceum" te Drachten begon hij in 1982 met de studie Biologie aan de Rijksuniversiteit te Groningen. Met als hoofdvakken Microbiologie en Biochemie, werd deze studie in 1987 afgerond.

Van oktober 1987 tot oktober 1992 was hij werkzaam bij de sectie Industriële Microbiologie van de Landbouwuniversiteit te Wageningen waar het onderzoek zoals beschreven in dit proefschrift werd verricht. Dit onderzoek werd uitgevoerd in samenwerking met de werkgroep Moleculaire Microbiologie van de Rijksuniversiteit Groningen.

Sinds oktober 1992 is hij werkzaam bij Snow Brand European Research Laboratories in Groningen.