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Interactions of Cyclic Hydrocarbons with Biological Membranes*

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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 Escherichia 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, 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, cytochrome c oxidase was also 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.

Cyclic hydrocarbons, such as aromatics, alicyclics, and terpenes, interact with biological membranes (de Smet et al., 1978; Sikkema et al., 1992; Uribe et al., 1985, 1990). These interactions lead to changes in structure and function of the membranes, which in turn, may impair growth and activity of the cells (Sikkema et al., 1992). The widespread use of cyclic hydrocarbons (e.g. fuels, solvents, starting compounds for organic synthesis) and their release in the environment makes knowledge of their metabolism and toxicity of eminent importance. The toxicity of cyclic hydrocarbons has been well noted (Smith, 1993), 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 (Uribe et al., 1985) and cyclohexane (Uribe et al., 1990) 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 (Sikkema et al., 1992). 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 alicyclic 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 paper). Effects of polar and non-polar compounds on biological membranes have been reported for fatty acids (Rottenberg, 1990), ethanol in yeast (Cartwright et al., 1986; Leao and van Uden, 1984), and anesthetics in erythrocytes (Seeman, 1972). 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 KOW; Leo et al., 1971).

MATERIALS AND METHODS

Preparation of Liposomes—Escherichia coli phospholipids, obtained from Sigma, were washed with acetone/ether (Kagawa and Backer, 1971). The commercially obtained E. coli lipids contained phosphatidylethanolamine (72 mol %), lysophosphatidylethanolamine (5.2 mol %), and cardiolipin (20.5 mol %) (In't Veld et al., 1992). Lipids dissolved in CHCl₃/MeOH (9:1, v/v) were mixed in appropriate quantities and dried under a stream of N₂ gas. Traces of solvent were then removed under vacuum for 1 h. Dried lipid was suspended in 50 mM potassium phosphate (pH 7.0) at a concentration of 20 mg lipid/ml and dispersed by ultrasonic irradiation using a bath sonicator (Sonicec, Sonicec Instruments, New York). Single membrane liposomes (Chapman, 1984; Elferink et al., 1992) were obtained by sonication (probe type sonicator, MSE, West Sussex, United Kingdom) for 300 s at maximal amplitude, using intervals of 15-s sonication and 45-s rest, at 4°C under a constant stream of N₂ gas.

Partitioning of Lipophilic Compounds—Partitioning of lipophilic compounds over membrane and buffer phases was determined in an E. 8022
coli phospholipid liposome/potassium phosphate buffer system (De Young and Dill, 1988; Katz and Diamond, 1974). Increasing amounts of the radiolabeled compounds were added to 50 mM potassium phosphate (pH 7.0) containing liposomes (5.0 mg of phospholipid/ml; final volume 0.5 ml). After equilibration (30 min), the liposomes were spun down in an Airfuge (Beckman Instruments) for 30 min at 135,000 g x g. By this method, liposomes were pelleted at an optical density of 4. To confirm the adequacy of liposome washing, aliquots were washed in 10 ml of 50 mM potassium phosphate (pH 7.0) and centrifuged for 45 min at 280,000 g 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. At pH 7.0, the ΔpH generated by the cytochrome c oxidase-containing proteoliposomes was 0.8-0.9.

**RESULTS**

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

Membrane-buffer partition coefficients in the E. coli phospholipid liposomes/potassium phosphate buffer system were determined for compounds varying in hydrophobicity/lipophilicity, i.e. phenylalanine, benzoic acid, 4-chlorobenzoic acid, 4-toluenesulfonic acid, 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 used (Klaassen, 1988). The octanol-water partition coefficient (log P) is defined as the ratio of the compound concentration in the octanol phase to the concentration in the water phase. For compounds with limited solubility in octanol, a value of 0 was assigned.
coefficients were taken (Leo et al., 1971). The distribution of the lipophilic compounds over the aqueous and the lipid phase was determined at several solvent to lipid ratios. When the measured aqueous concentration was plotted against the lipid to solvent ratio, a saturation curve was obtained (Sikkema et al., 1992). The partition coefficient was calculated, from the linear part of this curve (below maximum aqueous solubility). 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:

$$\log P_{\text{octanol}} = 0.97 \times \log P_{\text{buffer}} - 0.64 \quad (\text{Eq. 2})$$

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 I 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 R18 or 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 (Hoekstra et al., 1984), 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 of toluene, 15 μmol of cyclohexane, and 5 μmol of tetralin/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 R18-labeled liposomes (see Fig. 2) did not result in extraction of more than 10% of the phospholipid content. The data for the different compounds were: decalin, 8.6% of total phospholipid; benzene, 9.4% at 1 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; Ω-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. In a set of control experiments it was shown that the hydrocarbon solvent had no direct effect on fluorescence intensity, which could have occurred as a result of modification of the microenvironment of the probe. In these experiments the same concentrations of solvents (Fig. 2) were mixed with liposomes labeled with non-self-quenching concentrations of the fluorescent probes. Taken together, these results indicate that the observed increase in R18 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 at which the rhodamine fluorescence increased parallels the change in hydrophobicity of the compounds and the partitioning into the membrane. The differences in the extent of the fluorescence increase could be due to differences in maximum solubility of the hydrocarbon in the membrane but may also reflect differences in location in the membrane. Results similar to those presented in Fig. 2 were obtained with N-Rh-PE-labeled liposomes (data not shown).

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 (Lentz, 1989). 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 moieties 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.

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. The permeability for protons and other ions is especially 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 (Δψ) in artificial membranes, beef heart mitochondrial cytochrome c ox-
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<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>M_r</th>
<th>Solubility°</th>
<th>logP</th>
<th>P_CW</th>
<th>P_Meas</th>
<th>P_Metabolic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>C_6H_6</td>
<td>78.11</td>
<td>22.9</td>
<td>2.13</td>
<td>135</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>C_6H_12</td>
<td>84.16</td>
<td>0.683</td>
<td>3.44</td>
<td>2754</td>
<td>498</td>
<td></td>
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<tr>
<td>Toluene</td>
<td>C_7H_8</td>
<td>92.14</td>
<td>6.28</td>
<td>2.69</td>
<td>490</td>
<td>59 ± 8.5</td>
<td>93</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>C_8H_10</td>
<td>106.17</td>
<td>1.27</td>
<td>3.15</td>
<td>1413</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>o-Xylene</td>
<td>C_8H_10</td>
<td>106.17</td>
<td>2.02</td>
<td>3.12</td>
<td>1318</td>
<td>243</td>
<td></td>
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<tr>
<td>Naphthalene</td>
<td>C_10H_8</td>
<td>128.17</td>
<td>0.797</td>
<td>3.37</td>
<td>2344</td>
<td>527 ± 38</td>
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<tr>
<td>Tetralin</td>
<td>C_14H_10</td>
<td>132.21</td>
<td>0.125</td>
<td>3.86</td>
<td>2744</td>
<td>1100 ± 56</td>
<td>1271</td>
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<tr>
<td>o-di-Ethylbenzene</td>
<td>C_16H_14</td>
<td>134.22</td>
<td>0.41</td>
<td>4.10</td>
<td>12590</td>
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<tr>
<td>α-Pinene</td>
<td>C_20H_16</td>
<td>136.24</td>
<td>0.446</td>
<td>28840</td>
<td>4855</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Pinene</td>
<td>C_20H_16</td>
<td>136.24</td>
<td>0.446</td>
<td>28840</td>
<td>4855</td>
<td></td>
<td></td>
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<tr>
<td>α-Terpinene</td>
<td>C_20H_16</td>
<td>136.24</td>
<td>0.446</td>
<td>28840</td>
<td>4855</td>
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<tr>
<td>Limonene</td>
<td>C_20H_16</td>
<td>136.24</td>
<td>0.101</td>
<td>4.46</td>
<td>28840</td>
<td>4855</td>
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<td>Decalin</td>
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<tr>
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<td>C_20H_10</td>
<td>154.21</td>
<td>0.126</td>
<td>4.04</td>
<td>10965</td>
<td>1900</td>
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<td>Anthracene</td>
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<td>178.23</td>
<td>0.040</td>
<td>4.45</td>
<td>28184</td>
<td>4748</td>
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<tr>
<td>Phenanthrene</td>
<td>C_20H_14</td>
<td>178.23</td>
<td>0.025</td>
<td>4.46</td>
<td>28840</td>
<td>4937 ± 86</td>
<td>4855</td>
</tr>
</tbody>
</table>

° Data obtained from Handbook of Chemistry and Physics.
‡ Calculated from P_CW data found in literature and applied to Equation 2.
* Calculated via fragmental constants (Rekker and de Kort, 1979).
& Data not available.
^ Riddick et al., 1986.

**Fig. 2.** Effect of cyclic hydrocarbons on the relief of fluorescence self-quenching of R_flabeled liposomes. The reaction mixture contained liposomes (0.2 mg of E. coli phospholipid/ml) labeled with R_fl (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.

**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 spectrophotometer 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-labeled liposomes (probe to lipid molar ratio in both instances 1–250) in 50 mM potassium phosphate (pH 7.0). A, naphthalene; B, cyclohexane; C, tetralin; D, biphenyl; E, α-pinene; F, decalin.

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The results show that all hydrocarbons tested dissipated the ΔpH and Δψ 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 Δψ 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 Δψ 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 inhibited 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 ΔpH 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, and toluene to benzene. The concentrations of hydrocarbons that affected the proton permeability were in the same range as those that inhibited cytochrome c oxidase.

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 CF was examined. In the presence of various hydrocarbons an increased leakage of CF (Mf = 376) 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).

**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 (Leo et al., 1971; Verschueren, 1983), proved to be the most suitable model system (Lieb and Stein, 1986). 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 (Antunes-Madeira and Madeira, 1984, 1985, 1986, 1987; Katz and Diamond, 1974; Seeman, 1972). 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
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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 \( \alpha \)-pinene and tetralin, although for cyclohexane and naphthalene this effect can also 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. In principle one could envisage an effect of hydrocarbons on the distribution of DPH and TMA-DPH in the membrane. However, if for instance DPH would become intercalated among acyl chains, one would expect a decrease in polarization not only with DPH but also with TMA-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 \( \Delta pH \) and \( \Delta \phi \) generated by cytochrome \( c \) oxidase. Since a 50% reduction of cytochrome \( c \) oxidase activity only causes a small drop in the \( \Delta pH \) and \( \Delta \phi \) (Sikkema et al., 1992), 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 so far. Uribe and co-workers (Uribe et al., 1985, 1990) 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.

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J. Sikkema, W. van den Heuvel, and J. A. M. de Bont, unpublished results.

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). \( \Delta \), benzene; \( \ast \), toluene; \( \triangledown \), ethylbenzene; \( \nabla \), \( \alpha \)-xylene; \( \triangledown \), cyclohexane; \( \circ \), naphthalene; \( \bullet \), tetralin; \( \square \), biphenyl.
Rekker, R. F., and Rekker, R. F., eds), Elsevier, Amsterdam, The Netherlands

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