

# Influence of organic solvent mixtures on biological membranes

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**ABSTRACT** A simple experimental model was used to study the influence of organic solvents and solvent mixtures on the integrity of biological membranes. Radiolabelled membranes were prepared biosynthetically by growing *Escherichia coli* in the presence of  $^{14}\text{C}$ -oleic acid; the bulk of the radioactivity was incorporated into  $^{14}\text{C}$ -phosphatidylethanolamine, the predominant phospholipid species in *E coli* membranes. The radiolabelled bacteria were incubated at  $37^\circ\text{C}$  in the presence of solvent, and the mixture filtrated through a Millipore  $0.45\ \mu\text{m}$  filter. This filtration retained radiolabel associated with the bacteria, and only radiolabel released as a result of solvent action was allowed through the filter. The radioactivity in the filtrate was then counted and expressed as a percentage of the total radioactivity. Results showed that (a) aliphatic alcohols released membrane constituents in relation to their hydrocarbon chain length (1-propanol > 2-propanol > ethanol > methanol); (b) the effects of aliphatic alcohols were potentiated by acetone, ethyl methyl ketone, ethylene glycol, and N,N'-dimethylformamide, and (c) the effects of ethanol were potentiated by 1-butanol, benzyl alcohol, and ethylacetate. These findings point to the possibility that certain mixtures of organic solvents are more damaging to membranes than the components of the mixture would indicate, and suggest that the experimental model used might help in showing mixtures that are particularly harmful.

Although organic solvents are now generally recognised as health hazards, the mechanisms by which they may cause their biological effects are largely unclear. One possibility is that solvents, owing to their lipophilic nature, are able to interact with the lipids of cell membranes and so affect various membrane mediated events.<sup>1-6</sup> Little is known, however, about how different solvents and solvent mixtures may affect the structure and integrity of biological membranes. There are reasons, therefore, to study the influence of different solvents in membranes, and to pay particular attention to the effects of different solvent mixtures.

We have devised a simple and rapid procedure for studying the release of phospholipids from membranes exposed to different solvents and solvent mixtures. The procedure is outlined in fig 1. Radiolabelled *Escherichia coli* membranes are used as the target for solvent action, and a  $0.45\ \mu\text{m}$  Millipore filter is used to separate the phospholipid released from the bacteria. The procedure is rapid

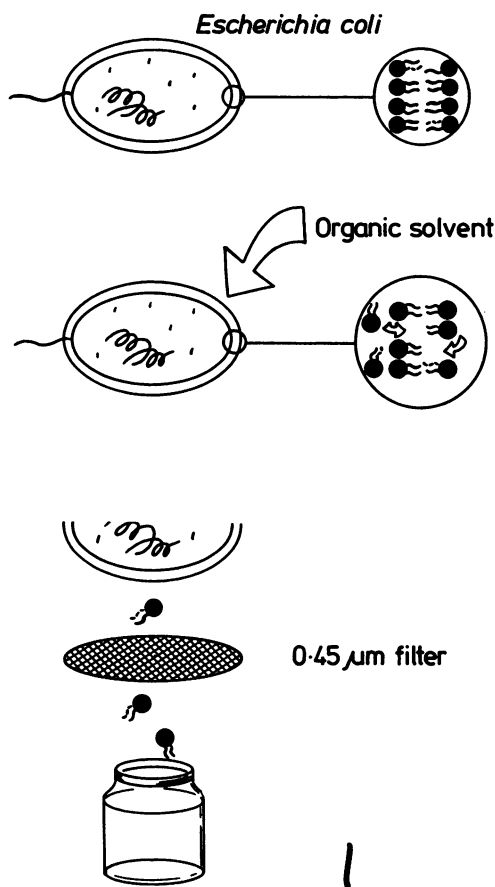
and simple and may readily be used to show that certain mixtures of solvents are more damaging to membranes than their components would indicate.

## Materials and methods

### CHEMICALS

[ $^{14}\text{C}$ ]-oleic acid ( $0.05\ \text{mCi/ml}$ ;  $57.3\ \text{mCi/mmol}$ ) and Riafluor were both purchased from New England Nuclear, Dreieichenheim, West Germany. Toluene, ethyleneglycol, benzylalcohol, chloroform, ethyl methyl ketone, 1-butanol, 1-propanol, 2-propanol, and N,N'-dimethylformamide were all obtained from Merck, Darmstadt, West Germany, whereas ethylacetate was from Kebo-Grave, Stockholm, Sweden, and ethanol, methanol, acetone, and xylene from Apoteksbolaget, Stockholm, Sweden. Bovine serum albumin (BSA) was Sigma type V.

**PREPARATION OF RADIOLABELLED MEMBRANES**  
*E coli K 12* were grown overnight (15-20 h) in nutrient broth (Difco). To prepare diacylphosphoglycerides labelled in the 2-acyl position, 1 ml of the



**Fig 1** Experimental model for studying influence of organic solvents on integrity of biological membranes. Radiolabelled membranes are prepared biosynthetically by growing *E coli* in presence of  $^{14}\text{C}$ -oleic acid (~~~~); bulk of radioactivity is incorporated into  $^{14}\text{C}$ -phosphatidylethanolamine (●~~~~). Radiolabelled bacteria are incubated with solvent(s) under investigation and mixture filtrated through a Millipore 0.45  $\mu\text{m}$  filter. This filtration retains radiolabel associated with the bacteria, and only radiolabel released as a result of solvent action is allowed through filter. Radioactivity in filtrate is then counted and expressed as a percentage of total radioactivity.

overnight culture was mixed with 2  $\mu\text{Ci}$  [ $^{14}\text{C}$ ]-oleic acid. The oleic acid was dispersed in 1 ml of sterile distilled water containing 2 mg BSA (fatty acid free). The mixture was inoculated into 8 ml nutrient broth and the culture incubated at 37°C for four hours. The bacteria were harvested by centrifugation (10 000  $\text{g} \times 10$  min), the supernatant discarded, and the pellet thoroughly resuspended in 5 ml nutrient broth containing 100 mg BSA. The bac-

teria were again centrifuged (10 000  $\text{g} \times 10$  min), the supernatant discarded, and the pellet resuspended in 2 ml saline. Thereafter, the bacteria were autoclaved for 15 minutes, washed (twice in saline with 2% BSA and once in saline without BSA), and stored at -80°C until further use.

The relative amounts of radioactivity found in the major *E coli* diacylphosphoglycerides, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), corresponded closely to the chemical amounts of these compounds in *E coli*.<sup>7</sup> Thus since the predominant species is PE, most of the radioactivity was found as  $^{14}\text{C}$ -PE. Moreover, the incorporated  $^{14}\text{C}$ -oleic acid has been shown to occupy predominantly the 2-position of the diacylphosphoglycerides.<sup>8,9</sup>

#### INFLUENCE OF SOLVENTS ON MEMBRANE INTEGRITY

##### *Aliphatic alcohols*

Radiolabelled *E coli* (about 4000 cpm) were incubated with different concentrations of methanol, ethanol, 1-propanol, or 2-propanol. The reaction mixture also contained  $\text{CaCl}_2$  (11 mM) and Tris-maleate buffer (pH 6.0) in a final volume of 450  $\mu\text{l}$ . The mixture was incubated at 37°C for 90 minutes and 2 ml saline with 1% BSA were added. The mixture was then filtrated through a Millipore 0.45  $\mu\text{m}$  filter. This filtration retained radiolabel associated with the bacteria, and only radiolabel released as a result of solvent action was allowed to pass through. The radioactivity in the filtrate was counted using a liquid scintillation spectrometer and expressed as a percentage of the radioactivity in an unfiltered control sample.

Experiments were also designed to identify the radiolabelled compound(s) in the filtrate. To this end, the filtrate was acidified and extracted with two volumes of ethylacetate. The extract was evaporated under nitrogen, redissolved in chloroform-methanol (2:1), and subjected to thin layer chromatography.<sup>10</sup> After separation, radiolabelled lipids were identified by radioactivity scanning, using a Dünnschicht-scanner II (B 2723; Berthold, Kebo-Grave Labcenter AB, Stockholm, Sweden.) Only  $^{14}\text{C}$ -radioactivity with an  $R_f$  corresponding to  $^{14}\text{C}$ -phosphatidylethanolamine was found.

##### *Mixtures of aliphatic alcohols and polar solvents*

Radiolabelled *E coli* (about 4000 cpm) were incubated with methanol (44%), ethanol (33%), 2-propanol (22%), or 1-propanol (11%), together with acetone (11%), ethyl methyl ketone (11%), ethylene glycol (11%), or  $\text{N,N}'$ -dimethylformamide (11%). The reaction mixtures also contained  $\text{CaCl}_2$

(11 mM) and Tris-maleate buffer (pH 6.0) in a final volume of 425  $\mu$ l. The mixtures were incubated, filtrated and handled as described above.

#### Mixtures of ethanol and non-polar solvents

Tris-maleate buffer (pH 6.0) was saturated with 1-butanol, benzylalcohol, toluene, xylene, chloroform, or ethylacetate. Radiolabelled *E coli* (about 4000 cpm) were then mixed with 250  $\mu$ l saturated buffer and 150  $\mu$ l ethanol. The reaction mixture also contained CaCl<sub>2</sub> (11 mM) in a final volume of 450  $\mu$ l. The final ethanol concentration in the reaction mixture was 33% (vol/vol). The mixtures were incubated and treated as described above.

## Results

### ALIPHATIC ALCOHOLS

The influence of different aliphatic alcohols on the release of <sup>14</sup>C-phospholipid (<sup>14</sup>C-phosphatidylethanolamine) from radiolabelled *E coli* membranes is shown in fig 2; each alcohol, at higher concentrations, released considerable amounts of <sup>14</sup>C-phospholipid. Moreover, for each alcohol the release was drastically augmented at a particular concentration. This concentration was highest for methanol (>44%) and lowest for 1-propanol (<22%), with values for ethanol and 2-propanol in between. The release thus increased with the carbon chain length of the alcohol.

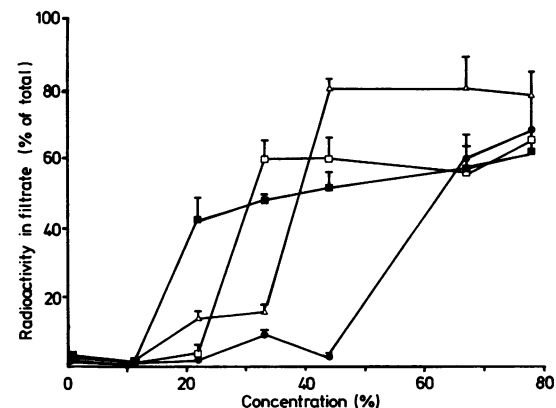


Fig 2 Release of <sup>14</sup>C-radioactivity from radiolabelled *E coli* after incubation with different aliphatic alcohols. (● = methanol, △ = ethanol, □ = 2-propanol, ■ = 1-propanol). Means of three to five experiments, vertical bars indicate standard deviations.

### MIXTURES OF ALIPHATIC ALCOHOLS AND POLAR SOLVENTS

The release of <sup>14</sup>C-phospholipid obtained in mixtures of aliphatic alcohols and various polar solvents is illustrated in table 1. All the polar solvents tested (acetone, ethyl methyl ketone, ethyleneglycol, and N,N'-dimethylformamide) potentiated the effects of all the aliphatic alcohols (methanol, ethanol, 2-propanol, and 1-propanol). In other words, more radioactivity was released in the different mixtures than would be expected from adding together the release obtained by each solvent alone. It also appeared that, on a relative basis, the effect of methanol was potentiated more than the effects of ethanol, 2-propanol, and 1-propanol, in that order. Thus acetone and N,N'-dimethylformamide strongly enhanced the effect of methanol, whereas their effect on 1-propanol was comparatively small (table 2). The effect of ethanol was, however, considerably potentiated by all the polar solvents tested.

### MIXTURES OF ETHANOL AND NON-POLAR SOLVENTS

The radioactivity release in mixtures of ethanol and various non-polar solvents are illustrated in table 3 from which it may be seen that the effects of 1-butanol, benzylalcohol, and ethylacetate were all potentiated by ethanol, whereas no such effect was found in mixtures of ethanol and toluene, xylene, or chloroform.

## Discussion

These findings indicate that the membrane damaging effect of aliphatic alcohols is increased with increasing carbon chain length, and that the straight chain alcohol is more damaging than the corresponding branched alcohol (fig 2). This is in agreement with results from other investigations in which more sophisticated assay systems for detecting membrane damage have been used<sup>11-13</sup> and may be taken to indicate that release in our simple assay does indeed reflect solvent induced membrane damage. As shown by thin layer chromatography, the released radioactivity was due to the presence of <sup>14</sup>C-phosphatidylethanolamine, the predominant phospholipid species in the *E coli* membranes. High solvent concentrations were required, however, to affect overall membrane integrity and release measurable quantities of <sup>14</sup>C-phosphatidylethanolamine. It should be emphasised, therefore, that organic solvents have been shown to affect membranes at concentrations considerably lower than those used in the present study. Still, it is reasonable to hypothesise that the ability of different solvents and solvent mixtures to release

Table 1 Release of  $^{14}\text{C}$ -radioactivity from radiolabelled *E coli* incubated with aliphatic alcohols, alone or together with various polar solvents. The release was determined by Millipore filtration and expressed as a percentage of total radioactivity in the bacteria. (Means  $\pm$  SD of three to six experiments)

Alcohol	Radioactivity release				
	Alcohol alone	Alcohol + acetone (11%)	Alcohol + ethyl methyl ketone (11%)	Alcohol + ethyleneglycol (11%)	Alcohol + N,N'-dimethylformamide (11%)
—		2.3 $\pm$ 2.4	16.3 $\pm$ 4.7	-0.4 $\pm$ 0.4	0.9 $\pm$ 3.5
Methanol (44%)	3.2 $\pm$ 0.9	39.1 $\pm$ 7.6	63.0 $\pm$ 21.1	17.0 $\pm$ 1.8	37.5 $\pm$ 7.0
Ethanol (33%)	7.4 $\pm$ 1.8	66.4 $\pm$ 16.8	72.3 $\pm$ 18.0	22.6 $\pm$ 4.4	42.6 $\pm$ 8.9
2-Propanol (22%)	10.7 $\pm$ 3.8	59.1 $\pm$ 20.0	74.1 $\pm$ 18.0	21.0 $\pm$ 2.9	39.9 $\pm$ 8.9
1-Propanol (11%)	10.0 $\pm$ 4.0	20.2 $\pm$ 12.8	33.0 $\pm$ 18.0	26.2 $\pm$ 5.2	26.5 $\pm$ 6.7

Table 2 Relative radioactivity release from  $^{14}\text{C}$ -labelled *E coli* in various solvent mixtures. The radioactivity release in the different mixtures was determined by Millipore filtration and compared with that released by each solvent alone. Values are quotients between observed values and those obtained by adding the effects of each solvent alone. (Data from table 1)

Alcohol in mixture	Relative radioactivity release			
	Alcohol + acetone	Alcohol + ethyl methyl ketone	Alcohol + ethyleneglycol	Alcohol + N,N'-dimethylformamide
Methanol	7.1	3.2	6.1	9.1
Ethanol	6.8	3.0	3.2	5.1
2-Propanol	4.5	2.7	2.0	3.4
1-Propanol	1.6	1.2	2.7	2.4

phospholipid in our assay may reflect their liability to cause more subtle changes at lower concentrations in, for instance, membrane fluidity and order.<sup>2,4</sup>

The release of radioactivity by mixtures of aliphatic alcohols and various polar solvents was greater than would be expected from simply adding together the effects of each solvent alone (table 2). The damaging effects of the alcohols on the membrane were thus potentiated by other polar solvents. The effect of methanol was potentiated more than that of ethanol, 2-propanol, and 1-propanol, in that order (table 2). This could be because the phospholipid release was exerted through a mechanism in which the alcohol penetrated the membrane. Accordingly, the longer chain alcohols would more easily pene-

trate the membranes by themselves and would be less influenced by the simultaneous presence of another solvent. Nevertheless, the finding that, on a relative basis, the least damaging alcohol was most potentiated should not be entirely overlooked. Thus it appears that a mixture of two solvents—for example, methanol and acetone, each of which causes little damage to the membrane—may cause much more damage than expected.

The effects of acetone, ethyl methyl ketone, ethylene glycol, and N,N'-dimethylformamide were potentiated by ethanol. This finding is worth considering, since the adverse effects of certain chemical substances in industry may be further enhanced by ethanol intake. For instance, ethanol consumption seems to inhibit trichloroethylene metabolism in man<sup>14</sup> and to augment the adverse effects of trichloroethylene on visuomotor performance<sup>15</sup> and mental capacity.<sup>16</sup> Moreover, the potentiation of ethanol toxicity by N,N'-dimethylformamide has recently been reported to occur both in rats<sup>17</sup> and in people working with N,N'-dimethylformamide.<sup>18</sup>

In addition, other investigators have found an interaction between xylene and ethanol metabolism in human subjects<sup>19</sup> and a modification in the behavioural responses of rats exposed to xylene and ethanol as compared with the effects of either agent alone.<sup>20</sup> The combined effects of ethanol and xylene appear complicated, however, since the effect of ethanol and 150 ppm xylene on vestibular function was additive but that of ethanol and 290 ppm xylene antagonistic.<sup>21</sup> We found no synergistic (or

Table 3 Release of  $^{14}\text{C}$ -radioactivity from radiolabelled *E coli* incubated with various non-polar solvents, alone or together with ethanol (33%). (Values are mean  $\pm$  SD of three to six experiments)

Solvent	Released radioactivity	
	Solvent alone	Solvent + ethanol
Ethanol	7.0 $\pm$ 2.2	—
1-Butanol	14.7 $\pm$ 7.4	73.9 $\pm$ 7.9
Benzyl alcohol	5.0 $\pm$ 3.7	50.9 $\pm$ 9.3
Ethyl acetate	ND	45.6 $\pm$ 0.9
Toluene	ND	5.2 $\pm$ 2.1
Xylene	ND	2.0 $\pm$ 1.8
Chloroform	ND	4.7 $\pm$ 0.2

ND = Not detectable.

antagonistic) effect between ethanol and xylene (table 3). This could be because the solubility and, consequently, the concentration of xylene in the present assay system was too low. On the other hand, a synergistic effect was found with mixtures of ethanol and other non-polar solvents, including ethyl acetate and benzylalcohol (table 3). Others have shown that benzylalcohol readily penetrates membranes, orientating with the aromatic part in the hydrophobic interior and the hydroxyl group in the polar region, and that it increases membrane fluidity.<sup>2,4</sup> In addition, benzylalcohol induced membrane changes have been shown to be potentiated by ethanol.<sup>6</sup> These findings point to the possibility that the hydroxyl group of benzylalcohol was essential for its ability to interact with ethanol in our study, and that the lack of polar groups in xylene and toluene made these solvents less likely to penetrate the membrane or to interact with ethanol.

We have thus obtained some evidence to indicate that certain mixtures of solvents are more damaging to membranes than the components of the mixture would indicate. Since the assay system used is rapid and simple, it might be helpful in showing mixtures that are particularly damaging. This is worth considering, since the need for systems by which the effects of mixed exposures may be evaluated, is gradually coming to be recognised.

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