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Effects of industrial organic solvents on human erythrocyte membrane adenosine triphosphatase activities in vitro

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KORPELA M, TÄHTI, H. Effects of industrial organic solvents on human erythrocyte membrane adenosine triphosphatase activities in vitro. *Scand J Work Environ Health* 13 (1987) 513—517. The effects of some aromatic hydrocarbons, aliphatic chlorinated hydrocarbons, and alcohols on adenosine triphosphatase (ATPase) activity in human erythrocyte ghost membrane were studied in vitro. Both aromatic and chlorinated aliphatic hydrocarbons inhibited this activity dose-dependently, the inhibition of total ATPase activity being clearer than that of magnesium-activated ATPase. Of the alcohols studied, methanol had no effect on the ATPase activity, but ethanol, propranol, and butanol were slightly enzyme-activating at high concentrations. The enzyme-inhibiting potency of organic solvents was generally related to their lipid solubilities, but 1,1,2,2-tetrachloroethane was a potent enzyme inhibitor despite its low lipid solubility. This finding indicates that, eg, the molecular structure of solvents may modulate their enzyme inhibition. In the presence of Triton-X-100, toluene did not cause any changes in the activity of total ATPase, and the combined effect of the two compounds was slight. Triton-X-100 also caused a significant solubilization of membrane proteins although even the highest toluene concentrations did not. These results show that organic solvents may cause their membrane effects by acting directly on membrane-bound integral proteins such as ATPase. This action is not only dependent on the lipid solubility of the compounds, but also on their molecular structure.

Key terms: alcohols, aliphatic chlorinated hydrocarbons, aromatic hydrocarbons, membrane effect.

Organic solvents exert an anesthetic effect on the central nervous system. This effect is based on changes in the cell membrane, and it is nonspecific. Many chemicals are able to induce general anesthesia, and the changes observed in the nerve cell membranes are also seen in peripheral cell membranes, even unexcitable ones.

The anesthetic agents affect both the proteins and the lipids in the cell membrane, but whether their action primarily involves a protein or a lipid is a matter of controversy. The results of some recent investigations on the effects of general anesthetics on some membrane luciferases indicate that the anesthetic site could be a protein (2). The function of proteins can be affected by clinical levels of anesthetic agents, but the changes in the lipid bilayer at such doses are very small. However, changes in proteins have been shown to take place without any marked structural alterations (9).

In our previous studies, we used the erythrocyte membrane-bound enzyme acetylcholine esterase (AChE) as a model in studying the effects of organic solvents on membrane proteins (4). We found that organic solvents inhibit the activity of AChE, which belongs to the integral proteins of the membrane and is situated mainly on the outer half of the lipid bilayer (8).

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In the present study, we investigated the effects of organic solvents on another membrane integral protein, adenosine triphosphatase (ATPase), the location of which differs from that of AChE, ATPase being mainly situated on the cytoplasmic side of the membrane (8). As a membrane model, human erythrocyte ghosts were used under in vitro conditions.

Materials and methods

The organic solvents studied were the aromatic hydrocarbons benzene, toluene, o-xylene and styrene, the aliphatic chlorinated hydrocarbons 1,1,1-trichloroethane, trichloroethylene, 1,1,2,2-tetrachloroethane and tetrachloroethylene, and the alcohols methanol, ethanol, propanol and butanol. Human erythrocyte ghosts were prepared from freshly drawn heparinized venous blood by centrifugation at 1000 *g* for 5 min to separate the plasma and the leukocytes. After the buffy coat was discarded, the cells were washed twice with 0.9 % sodium chloride and hemolyzed with repeated freezing and thawing. Finally, the hemolyzate was diluted with distilled water in the ratio 1 : 1 to give the ghost suspension used.

In the ATPase determinations, a 2-ml reaction mixture was used which had a final concentration of 50 mM tri(hydroxymethyl)aminomethane buffer (pH 7.6), 1.5 mM adenosine triphosphate, 6.0 mM magnesium chloride, 100 mM sodium chloride, 20 mM potassium chloride, and 0.1 ml of ghost suspension. The total ATPase activity was measured with sodium, potassium, and magnesium ions present in the mixture,

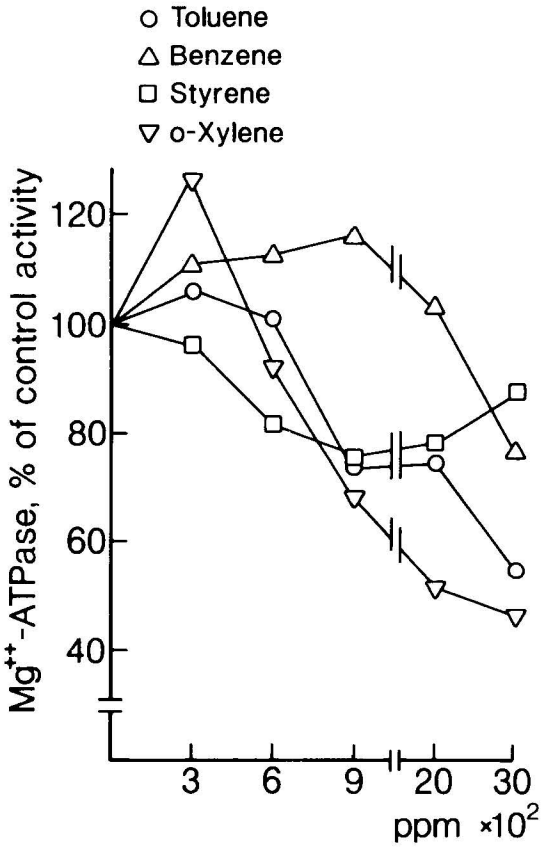

Figure 1. Total adenosine triphosphatase (ATPase) and magnesium-activated ATPase (Mg^{2+}-ATPase) activities of human erythrocyte ghost membranes after incubation in various concentrations of aromatic hydrocarbons. Each point represents the mean of four independent experiments, all made in triplicate.

and the Mg^{2+}-ATPase activity with only magnesium ion (Mg^{2+}) present. The organic solvents were added to the reaction mixture and the test tubes were immediately glass-stoppered. In studies of the effects of Triton-X-100 on the enzyme activities, 0.5 ml of the buffer solution was replaced by 5 % Triton-X-100 solution, and the mixture was incubated for 15 min at room temperature before the addition of toluene.

The treated and control preparations, the reagent, and the enzyme blanks (to correct for inherent phosphate present in the samples) were incubated simultaneously at 37°C for 1 h under continuous stirring. The incubation was stopped by the addition of 2 ml of ice-cold 10 % trichloroacetic acid to the test tubes. Because of the formation of a precipitate, the samples were centrifuged at 3 000 *g* for 5 min, and 1 ml of the supernatant was removed for the assay. The samples were assayed for inorganic phosphate by the Lowry & Lopez method (6) as modified by Phillips et al (11). To 1 ml of supernatant, 3 ml of 0.1 N sodium acetate solution was added as soon as possible. Then 0.4 ml of molybdate/sulfuric acid solution and 2 ml of ascorbic acid were added to each tube. The samples were carefully mixed, and the absorbance was read at 770 nm after 25 min. The enzyme activities were given as millimoles of inorganic phosphate per hour per liter of red blood cells, and the activities of solvent-treated samples were expressed as the percentage of the control values.

The protein determinations were made by the method of Lowry et al (7), and in the presence of Triton-X-100 as modified by Wang & Smith (17).

In the statistical calculations, Student's t-test was used. The significances were evaluated in comparisons of the enzyme activities of treated samples with the enzyme activity of control erythrocyte ghosts.

Results

Of the aromatic hydrocarbons, o-xylene and styrene decreased the total ATPase activity in human erythrocyte ghost membranes at concentrations of 600 ppm and higher ($P < 0.001$) (figure 1). A clear effect of benzene and toluene was seen at concentrations of 2 000 and 3 000 ppm. The Mg^{2+}-ATPase was inhibited by styrene at the concentration of 600 ppm or higher ($P < 0.001$), by toluene and o-xylene at concentrations of 900 ppm and higher, and by benzene at 3 000 ppm (figure 1).

Of the chlorinated aliphatic hydrocarbons, 1,1,2,2-tetrachloroethane and tetrachloroethylene had the most prominent inhibitory effect on both the total ATPase and the Mg^{2+}-ATPase activities. The effects of 1,1,1-trichloroethane and trichloroethylene on the total ATPase activity were smaller, but yet significant, when the solvent concentrations exceeded 300 ppm with the former ($P < 0.01$) and 2 000 ppm with the latter (figure 2). 1,1,1-Trichloroethane had no effect on the Mg^{2+}-ATPase activity, but trichloroethylene inhibited it dose-dependently at the concentration of 600 ppm or higher ($P < 0.01$). Of the alcohols, ethanol, propanol and butanol increased both the total ATPase and Mg^{2+}-ATPase activities at concentrations of 900 ppm and higher (figure 3). Methanol had no effect on the enzyme activities.

The Triton-X-100-treated erythrocytes showed no total ATPase inhibition with different concentrations of toluene (table 1). The effect of toluene on the activity of pure commercial ATPase was as clear as the effect of toluene on the ghost membrane ATPase; only at the concentration of 3 000 ppm was the inhibition of the pure enzyme significantly less than that of the membrane-bound enzyme. The total ATPase activity and the Mg^{2+}-ATPase activity of the control ghost membranes was 16.9 (SE 1.0) and 9.5 (SE 0.50) mmol inorganic phosphate $\cdot h^{-1} \cdot l^{-1}$ red blood cells, respectively.

The protein contents of the samples varied from 17.6 (SE 0.4) mg/ml to 24.9 (SE 1.9) mg/ml. The Triton-X-100 treatment increased the protein content in the supernatant of the samples, but even the highest

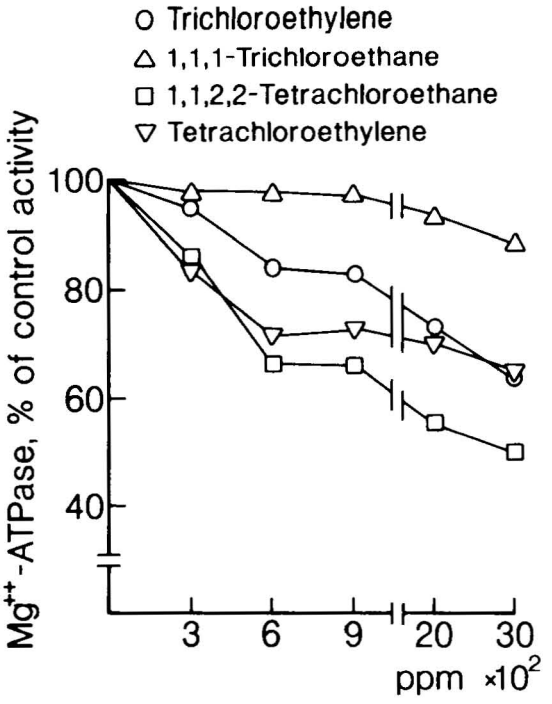

Figure 2. Total adenosine triphosphatase (ATPase) and magnesium-activated ATPase (Mg^{2+}-ATPase) activities of human erythrocyte ghost membranes after incubation in various concentrations of chlorinated aliphatic hydrocarbons. Each point represents the mean of four independent experiments, all made in triplicate.

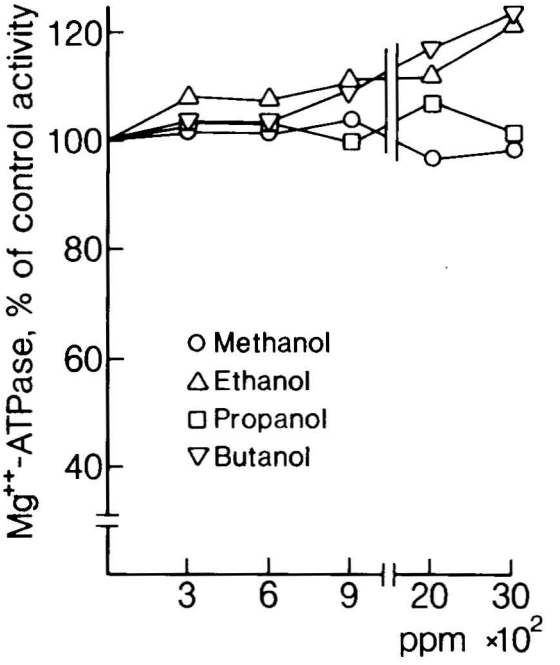

Figure 3. Total adenosine triphosphatase (ATPase) and magnesium-activated ATPase (Mg^{2+}-ATPase) activities of human erythrocyte ghost membranes after incubation in various concentrations of alcohols. Each point represents the mean of four independent experiments, all made in triplicate.

Table 1. Effect of different toluene concentrations on total adenosine triphosphatase (ATPase) activity in human erythrocyte ghosts, on pure commercial ATPase preparate, and on Triton-X-100-treated human erythrocytes.[a]

	Toluene									
	300 ppm		600 ppm		900 ppm		2 000 ppm		3 000 ppm	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Ghosts	93.3	93.0—93.6	89.5	86.3—92.7	82.0	79.9— 84.1	68.8	65.0— 72.6	57.2	53.6— 60.8
Pure ATPase	89.7	88.3—91.1	81.0	78.2—83.8	80.7	74.9— 86.5	73.7	67.9— 79.5	75.3**	66.4— 84.2
Triton-X-100-treated cells	96.2	95.0—97.4	96.9*	94.6—99.2	101.9**	99.0—104.8	104.4***	100.5—108.3	115.1***	110.7—119.5

[a] Means and standard errors (SE) of the means of 12 measurements. The degree of enzyme inhibition is expressed as the percentage of the control value marked as 100 % activity.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ [comparison of the enzyme activity in pure ATPase preparate and Triton-X-100-treated erythrocytes with that in erythrocyte ghosts (Student's t-test)].

Table 2. Effect of Triton-X-100 and different toluene concentrations on the protein content of the samples.[a]

	Control		Triton-X-100		Toluene									
					300 ppm		600 ppm		900 ppm		2 000 ppm		3 000 ppm	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Total protein content (mg/ml)	24.9	23.1—26.7	19.0	18.2—19.8	17.9	16.2—19.6	17.6	17.2—18.0	23.7	21.6—25.8	23.4	21.6—25.2	17.7	3.3— 32.1
Protein in supernatant (%)	19.6	18.8—20.4	37.3***	37.1—37.5	27.5**	27.4—27.6	27.9**	27.7—28.1	19.8	19.3—20.3	20.3	19.9—20.7	13.3**	13.1— 13.5
Protein in pellet (%)	80.4	73.6—87.2	62.7	58.3—67.1	72.5	63.0—82.0	72.1	70.0—74.2	80.2	71.0—89.4	69.7	69.6—69.8	86.7	65.7—107.7

[a] Means and standard errors (SE) of the means of eight measurements.
** $P < 0.01$, *** $P < 0.001$ [comparison of the protein content in the Triton-X-100- and toluene-treated samples with that in the control samples (Student's t-test)].

toluene concentrations (2 000 and 3 000 ppm) did not cause any changes in the protein content of the supernatant (table 2).

Discussion

In the present study, we have used the human erythrocyte membrane as a nerve cell model in studying the effects of some commonly used industrial organic solvents on the cell membrane integral enzyme ATPase, which is one of the minor membrane structural proteins mainly situated on the cytoplasmic side of the membrane lipid bilayer (8). The changes in the nerve cell membrane as a consequence of the anesthetic action of solvents are nonspecific, and we have also seen them in peripheral unexcitable cells (4). Thus, we consider the erythrocyte membrane to be a convenient membrane model, especially for in vitro studies of the mechanisms of anesthesia.

We found that aromatic hydrocarbons and chlorinated aliphatic hydrocarbons inhibit ATPase activities in human erythrocyte ghost membrane dose-dependently. In addition other membrane-active compounds such as free fatty acids (15) and local anesthetics (13) have been shown to cause an inhibiting effect on ATPase in synaptosomal membranes. Of the alcohols tested in the present study, ethanol, propanol, and butanol showed slight dose-dependent ATPase activation. Alcohols have been shown both to inhibit (3, 5, 10, 12, 14) and to activate (1) brain ATPase. Thus the effect of alcohols on this enzyme seems to be influenced by many different factors, eg, by the doses and the exposure times used. In the study of Tanaka (16), benzyl alcohol was found to be ATPase-activating up to a concentration of 70 mM, and inhibiting at concentrations higher than 80 mM. The inhibition was explained as being caused by the denaturation of membrane components at high concentrations of benzyl alcohol. However, in our present study, the ATPase was inhibited even by very low concentrations of aromatic and aliphatic hydrocarbons, which do not induce any protein solubilization or denaturation. Thus the mechanism of enzyme inhibition caused by low solvent concentrations must differ from the protein denaturation induced by high concentrations.

In general, the enzyme inhibiting potencies of the organic solvents tested in the present study seemed to be related to their lipid solubilities. More lipid-soluble agents penetrate the cell membrane and reach the enzyme more easily than, eg, alcohols. However, the molecular structure of a compound may be even more important, because 1,1,2,2-tetrachloroethane, which has a low lipid solubility, was a potent ATPase inhibitor in our study. The organic solvents studied affected both the total and the basal Mg^{2+}-ATPase activities. The effect on Mg^{2+}-ATPase was, however, not as clear as on that total ATPase. Mg^{2+}-ATPase is not easily affected by many agents which are toxic to, eg, Na^+-K^+-ATPase, and the inhibition constants are higher than for other ATPase components (11).

The detergent Triton-X-100 caused at least partial solubilization of membrane proteins. This solubilization was seen as an increase in the protein content of supernatants in our samples. Even the highest toluene concentrations showed no such effect. In addition, in the presence of Triton-X-100, toluene had no total ATPase inhibiting effect. The solubilization of the enzyme by Triton-X-100 probably activates it to such an extent that the inhibition induced by toluene is not seen. Thus the combined effect of these two compounds was a slight ATPase activation.

Toluene also inhibited the pure ATPase preparation significantly. This occurrence may indicate that the solvent molecule can react directly on the enzyme and needs no other membrane components, eg, lipids, for its action.

The present results confirm our previous results on the action of organic solvents on membrane AChE (4), another membrane integral protein, located on the outer half of the membrane lipid bilayer. According to our results the anesthetic effect of organic solvents on the central nervous system may be based on the direct action of solvent molecules on membrane proteins, and the potency of the anesthetic property is dependent not only on lipid solubility, but also on the molecular structure of solvents. Our results support the recent results of Franks & Lieb (2) on the mechanism of anesthesia.

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