APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. **14**, 432–437 (2000)

Direct or indirect influence of triphenyl-lead on activity of Na^+/K^+-ATPase

Stanisław Przestalski,[1]* Halina Kleszczyńska,[1] Zenon Trela,[1] Zofia Spiak,[2] Maria Zamarajeva,[3] Natalia Glazyrina[3] and Andrea Gagelgans[3]
[1]Department of Physics and Biophysics, Agricultural University, Norwida 25, 50-375 Wrocław, Poland
[2]Department of Agricultural Chemistry, Agricultural University, Norwida 25, 50-375 Wrocław, Poland
[3]Department of Biology, Chair of Biophysics, Tashkent State University, 700095, Vuzgorodok, Tashkent, Uzbekistan

We have studied the effect of triphenyl-lead chloride on the lipid phase of erythrocyte membranes, on lipid monomolecular layers and Na^+/K^+-ATPase of the microsomal fraction of rat brain. It was found that the haemolytic effect induced by this compound occurs when its concentration exceeds 30 μM. The minimal lead concentration inducing measurable effects in monomolecular lecithin layers is about 1 μM. Inhibition of Na^+/K^+-ATPase activity begins at a concentration exceeding 0.5 μM. Maximum inhibition is observed at around 40 μM—a concentration at which haemolysis also occurs. It can thus be thought that at very low lead concentrations the main (or exclusive) role in modifying membrane function is played by direct interaction between lead and the sulphydryl groups of ATPase, whereas at higher concentrations two effects seem to overlap: direct interaction between lead and enzymic proteins via their sulphydryl groups and as indirect influence on the proteins via changes in the organization of the lipid phase of the membrane. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: triphenyl-lead chloride; ATPase; SH groups; microsomes; erythrocytes; haemolysis; monolayers

Received 15 May 1999; accepted 15 February 2000

* Correspondence to: Stanisław Przestalski, Department of Physics and Biophysics, Agricultural University, Norwida 25, 50-375 Wrocław, Poland.
Contract/grant sponsor: Polish Research Committee (KBN); Contract/grant number: 6/P04/G0/4313.

INTRODUCTION

It is generally accepted that the level of lead in blood as a measure of absorbed lead is the best biomarker of current exposure.[1] According to WHO (World Health Organization) standards, the maximum admissible dose for humans exposed at the workplace to lead contamination is about 400 μg of lead/dm^3 of blood,[2–4] corresponding to *ca* 2 μM. The level of lead in the blood of humans living in areas free of lead pollution is roughly within the limits 50–200 μg dm^{-3},[1] which is about 0.25–1.00 μM. However, at admissible concentrations (subclinical) some pathological effects are observed, e.g. disturbed mental abilities in humans,[5,6] the initial abnormalities being apparent only in psychological tests.[7] The literature data on contaminations refer mainly to inorganic compounds of lead[8–10] In general the organic compounds of lead are more toxic than the inorganic ones.[11,12] Organic compounds of lead are probably more dangerous to the central nervous system because of their better solubility in lipids, allowing them to penetrate the blood–brain barrier more easily. Of the many organic lead compounds used in practice we have studied phenyl–lead compounds, because they are used in industry and, apparently, in some cases are more toxic than the alkyl–lead compounds;[13] also, our previous investigations[14] showed that these compounds affected the membrane electric potential and electric conductance. Alkyl–lead compounds constitute a greater menace globally to the environment because of their presence in petrol, but this is dealt with elsewhere (e.g. Refs 3, 5 15–21). In the present paper we focus on investigating the effect of triphenyl-lead chloride on membrane lipids and proteins, in order to find out which of the phases plays a decisive role in the interaction. Being amphiphilic, triphenyl-lead should interact with the

lipid phase of membrane, like the other organic lead compounds we have studied (even the inorganic lead compounds, we believe probably interact with membrane lipids[22]). On the other hand, it is known that lead is able to combine with the sulphydryl groups of membrane proteins, thus affecting their activity.[23] Our studies on the effect of triphenyl-lead chloride on the lipid phase of membranes were based on (1) measuring the degree of haemolysis of erythrocyte membranes, according to the accepted premise that haemolysis occurs as a result of disorganization of the lipid phase of the erythrocyte membrane (e.g. Refs 15, 21); and (2) determination of the effect of the compound on monomolecular lipid layers. The influence of triphenyl-lead chloride on enzyme proteins was studied by measuring inhibition of Na^+/K^+-ATPase of membranes from the microsomal fraction of rat brain cells.

MATERIALS AND METHODS

Triphenyl-lead chloride, $(C_6H_5)_3PbCl$, was purchased from ABCR (Karlsruhe, Germany). Chlorotetracycline (CTC) and dithiothreitol (DTT) were from Sigma Chemical Co. (USA). In aqueous solution at pH *ca* 7, triphenyl-lead chloride dissociates with the formation of cationic forms,[15] which we denote as TPhL. Microsomes from rat brain were prepared by a standard protocol.[24] The Na^+/K^+-ATPase activity was determined by accumulation of inorganic phosphate (P_i) in samples in the presence and absence of specific inhibitors. The incubation medium for Na^+/K^+-ATPase assay contained 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 5 mM NaCl, 1 mM ATP and 10 mM Tris–HCl, pH 7.2. Control samples contained additionally 0.1 mM ouabain for Na^+/K^+-ATPase. The protein content in the samples was 40 μg for Na^+/K^+-ATPase. The enzyme activity, expressed in μmol P_1 (mg protein)$^{-1}$ min^{-1}, was determined by the reaction with molyodate reagent as described by Panusz *et al.*[25] The protein content was estimated by the biuret reaction. The content of free SH groups in the membranes was determined by a spectrophotometric method at $\lambda = 412$ nm (OD412) with the use of 5,5-dithiobis(2-nitrobenzoic acid) as sulphydryl-sensitive reagent.[26,27]

In order to decide whether TPhL penetrates the cell membrane and interacts with it, measurements were made of the surface pressure of the monomolecular lecithin layer with the compound present in the subphase. TPhL, dissolved in a small amount of methanol (1 ml), was added to the subphase (at concentrations of 1, 10 and 100 μM, respectively) after a monomolecular phospholipid (DPPC) layer has been formed and pressurized to 30 mN m^{-1}. Changes in the surface pressure induced by the compound added to the subphase indicated that TPhL penetrated the lecithin membrane.

Surface pressure versus mean molecular area isotherms were measured for mixed monolayers composed of lecithin (1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine) and triphenyl-lead chloride. Monolayers were formed in a Teflon rectangular trough with effective dimensions of 280 mm × 82 mm × 10 mm. Twice-distilled water was used as the subphase. Surface pressure as a function of mean molecular area was recorded during compression of a monolayer (compression isotherms); surface pressure was detected by using a platinum plate hung on an electrobalance (Wilhelmy method). Further details of the experiments are described in previous papers.[20,28]

To find out whether the organolead compound dissolves in the subphase when placed together with lecithin on the surface of water, we did the following experiments. The mixture of DPPC–TPhL (TPhL, mole fraction $x = 0.4$) was put on a water surface in a Teflon trough (as in the compression experiments described above). Four experiments were carried out with different amounts of the mixture, so that the mean molecular surface area varied in the range of 1–0.2 nm^2/molecule. Then samples of the subphase were taken and assayed for lead content with a Zeiss AAS-3 atomic absorption spectrophotometer Lead by the non-flame method, with an EA-3 graphite cuvette at 217 nm; the lamp current was 5.0 mA and the slit width 0.06 mm. The results of the measurements did not, in practice, show any lead content in the subphase samples.

The haemolysis experiments were conducted with fresh heparinized pig blood. For washing erythrocytes, an isotonic phosphate buffer of pH 7.4 (1.31 mM NaCl, 1.79 mM KCl, 0.86 mM $MgCl_2$, 11.79 mM $Na_2HPO_4 \cdot 2H_2O$, 1.80 mM $NaH_2PO_4 \cdot H_2O$) was used. Erythrocytes collected from plasma were washed four times in the phosphate buffer and then incubated in the same solution containing appropriate amounts of TPhL at 37 °C for 4 h. The 10-ml samples, containing erythrocytes at 2% concentration, were well stired. During modification, the percentage of haemolysed cells was measured in 1-ml samples taken at 0.5, 1, 1.5, 2.3 and 4 h. These samples were centrifuged and the haemoglobin content was measured in the

supernatant using a spectrometer (Specol 11, Carl Zeiss, Jena) at 540 nm wavelength.[17] The percentage haemoglobin concentration in the supernatant was calculated as the percentage of haemolysed cells relative to a sample containing totally haemolysed erythrocytes. TPhL was dissolved in ethanol in an amount such that after its addition to the erythrocyte suspension the concentration of ethanol in the samples did not exceed 1%. Some experiments were also performed with rabbit erythrocytes.

RESULTS AND DISCUSSION

A decrease in activity was observed when the organolead was tested as an inhibitor of Na^+/K^+-ATPase from rat brain membranes. It was shown (Fig. 1, curve A) that the compound studied inhibits in a dose-dependent manner and the inhibition is correlated with a decrease in the quantity of SH groups in microsomes (Fig. 1, curve B); 50% inhibition was observed at $K_{50} = 4$ μM. Possibly, the decrease of ATPase activity is a result of interaction of the organic lead compound with the SH groups in the membranes.

We also studied the behaviour of DPPC–TPhL monomolecular layers, by taking their compression

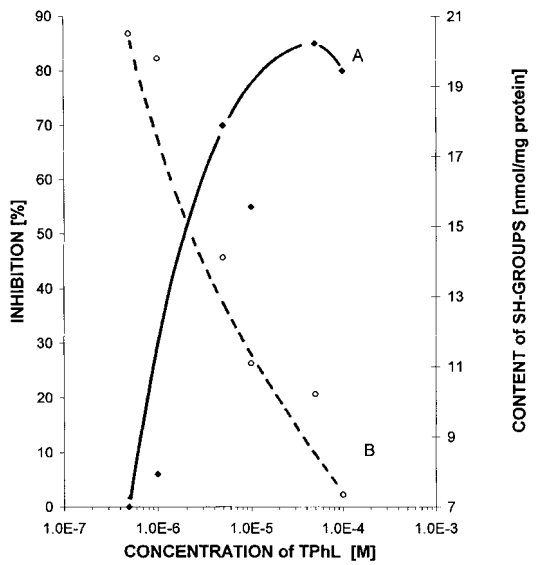

Figure 1 Dependence of Na^+/K^+-ATPase activity (A) and content of SH-groups (B) on the concentration of triphenyl-lead chloride.

isotherms. Compression isotherms for DPPC–TPhL monolayers at molar fractions of TPhL, *x*, of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 are presented in Fig. 2(A). A pure organometallic compound when compressed to a mean molecular area of about 0.15 nm^2/molecule exhibits a relatively small increase in surface pressure (about 5 mN m^{-1} in the isotherm for molar fraction $x = 1.0$, Fig. 2). The dependences of mean molecular area, *A*, on the molar fraction, *x*, of triphenyl-lead at constant pressures of 5, 10, 20 and 30 mN m^{-1} in Fig. 2(B) are almost linear.

TPhL added to a water subphase at 1, 10 and 100 μM caused an increase (fast at 100 μM and slow at 1 and 10 μM; Fig. 3) in surface pressure of the lecithin monomolecular layer by about 1, 4 and 12 mN m^{-1}, respectively.

Haemolytic experiments indicated (Fig. 4) that at 100 μM TPhL 100% haemolysis occured within 4 h. At 40 μM haemolysis is visible but develops more slowly: after 4 h it is not yet 100% (although it continues after that time— not shown in Fig. 4). At concentrations lower than 30 μM we did not observe haemolysis.

The experiments we performed on rabbit erythrocytes indicated that their haemolysis also begins at 40 μM concentration.

Triphenyl-lead chloride has a significant effect on the activity of plasma membrane Na^+/K^+-ATPase in the concentration range 0.5–10 μM. A similar effect was observed with a number of other organolead and organotin compounds.[16] Similar concentrations of these compounds have been shown to block the activity of Na^+/K^+-ATPase in the plasma membrane of HeLa cells.[29]

As we report here, the inhibition of activities of the ion pumps (Na^+/K^+-ATPase) by triphenyl-lead chloride correlates with a decrease in the quantity of SH groups in proteins. These results suggest that the interaction of organolead with thiol residues of enzymes might be responsible for the inhibitory effect.

These data agree with the results of Munter *et al.*,[29] who have reported that 1 mM glutathione can revert the inhibition exerted by triethyl-lead on Na^+/K^+-ATPase plasma membrane. Moreover, it was shown that triethyl-lead can interact with SH groups of acetylcholinesterase in rat brain and inhibit its activity.[30]

An additional explanation of the effects observed can be deduced from monolayer experiments. In these experiments the concentration of TPhL (calculated as the quantity of triphenyl-lead spreading on the water surface per unit volume of the subphase) is almost comparable (within an order of

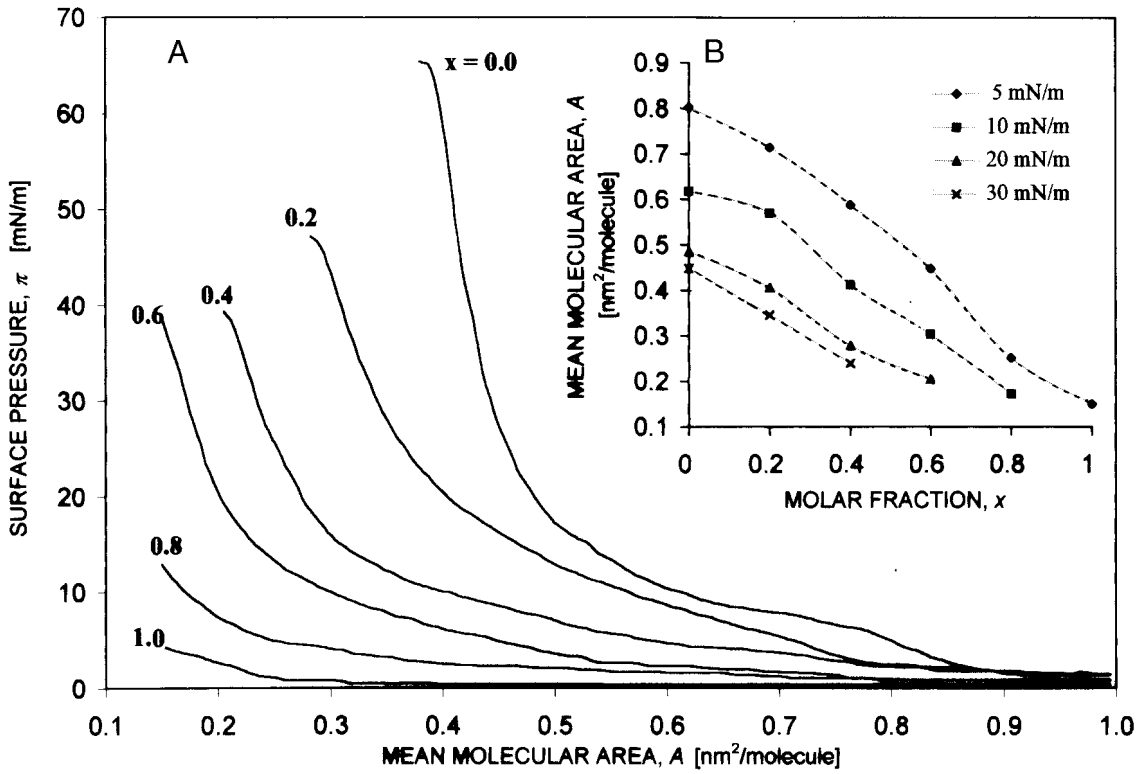

Figure 2 (A) Compression isotherms for two-component mixed monolayers formed from DPPC and TPhL. Each of the curves is an averaged isotherm, obtained from at least five independent experimantal curves; x = molar fraction of TPhL. (B) Dependence of mean molecular area, A, of the DPPC–TPhL monolayer mixture on the molar fraction of TPhL at constant surface pressures of 5, 10, 20 and 30 mN m^{-1} [obtained from (A)].

magnitude) with the concentration at which effects of TPhL on Na^+/K^+-ATPase were observed (0.5 μM). From the thermodynamic point of view, linearity (especially for surface pressures greater than 10 mN m^{-1}) of the dependence of mean molecular area on the molar fraction of TPhL may suggest either ideal mixing of the two monolayer components (the phospholipid and the triphenyl-lead) or phase separation in the monolayer region. Taking into account the fact that pure triphenyl-lead does not yield compression isotherms, the second possibility looks more probable. It may be supposed that modification of the lipid phase of microsome membranes by creation of the triphenyl-lead domains can indirectly influence functions of membrane proteins. There is a close relationship between the chemical composition of domains, their physical state and the function of the membrane.[31–33]

It is generally believed that haemolysis occurs as a result of disorganization of the lipid phase of the membrane. As we can see, the minimum TPhL concentrations for haemolysis coincide with the maximum concentration that inhibits Na^+/K^+-ATPase activity (Figs 1 and 4). That coincidence could be accidental but it is of possible significance. Apparently, disorganization of the lipid phase results, indirectly, in lower ATPase activity (as is commonly known, the lipid–protein interaction may induce modification in enzyme activity[34]). In that situation, two effects would overlap: direct inhibition resulting from the interaction of TPhL with thiol groups of proteins and indirect inhibition via changes in the lipid phase organization. Additionally, at concentrations higher than 40 μM haemolysis increases (Fig. 4) and the number of SH groups decreases (Fig. 1), with Na^+/K^+-ATPase inhibition starting to decrease. This indicates a dominant influence (with respect to direct TPhL action on the proteins) of lipid phase disorganization on the process studied.

Disturbance in membrane function is the main

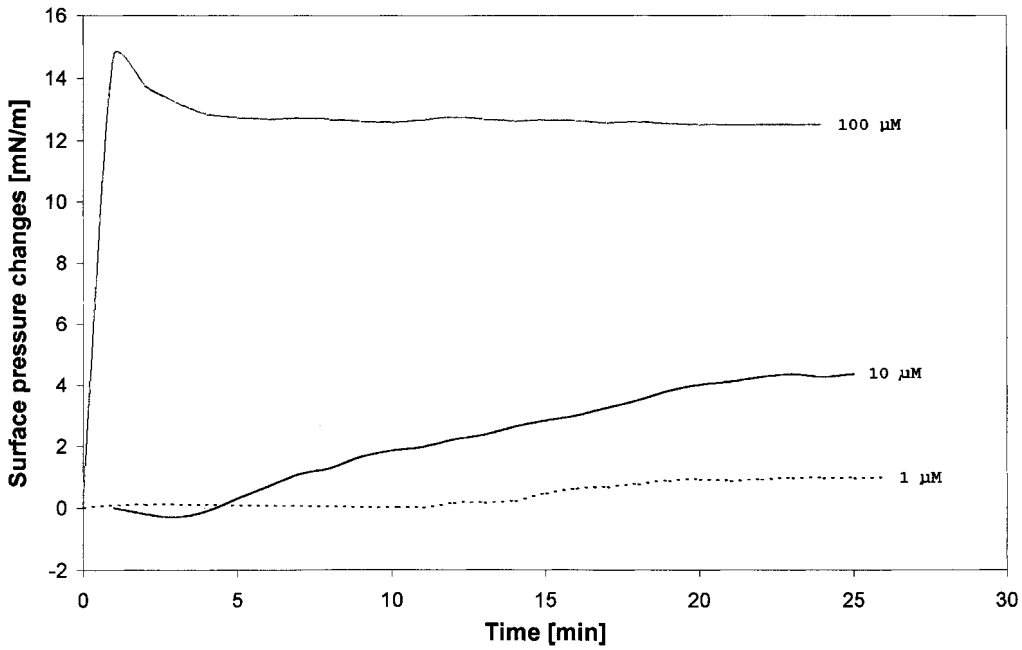

Figure 3 Time course of surface pressure changes of a monomolecular layer (DPPC) after addition of TPhL to the subphase at concentrations of 1, 10 and 100 μM, respectively. The initial surface pressure of monolayer was 30 mN m^{-1}.

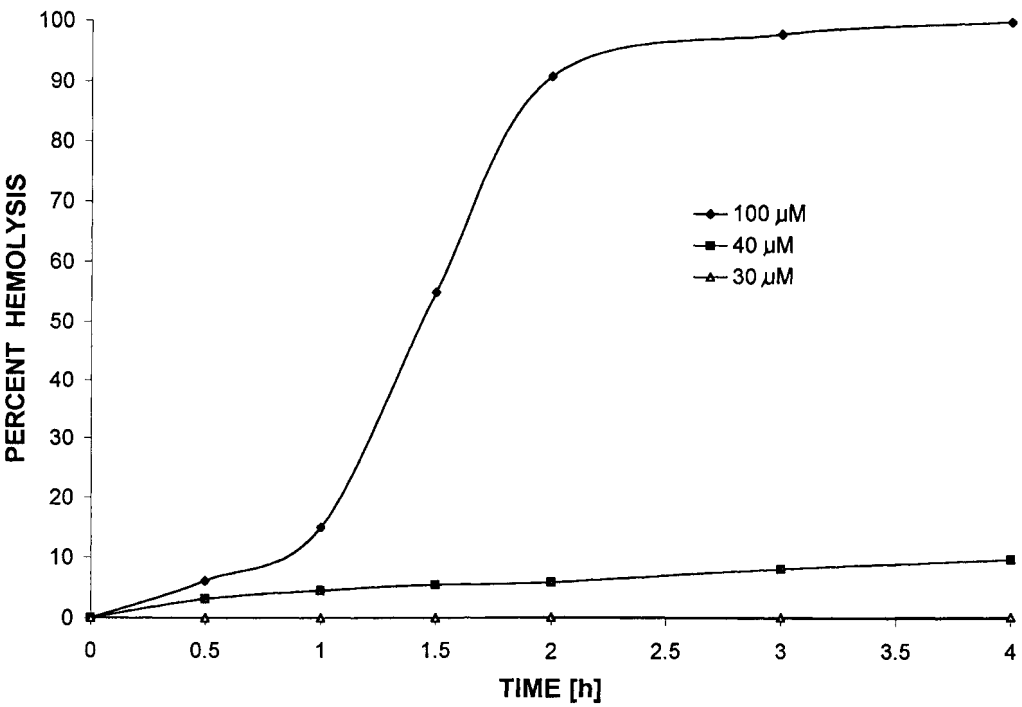

Figure 4 Effect of triphenyl-lead chloride on red cell membranes: dependence of the percentage of hemolysis on the time of incubation.

result of lead compound action on membranes. From the experiments presented here it follows that the compounds studied inhibit the activity of membrane Na^+/K^+-ATPase and disorganize the lipid phase of the membrane. At small enough concentrations, only a slight inhibition of ATPase activity occurs (which may have a connection with early, asymptomatic, neurological effects in humans), and at higher concentrations there is an increasing disorganization of the membrane (both the protein and lipid phase), culminating in its total destruction.

In this situation, one may consider both the direct action of the compounds studied on protein function via modulation of transport enzyme activity, and an indirect one via changes that occur in the lipid phase with probable domain formation.

Acknowledgements This work is sponsored by the Polish Research Committee (KBN), grant no. 6 PO4 GO 4313.

REFERENCES

1. Evers U, Schlipköter HW. Lead. In *Metals and Their Components in the Environment; Occurrence, Analysis and Biological Relevance*, Merian, E (ed.). VCH, Weinheim, 1991; 971–1012.
2. Phoon WH, Lee HS, Ho CK. *Singapore Med. J.* 1990; **31**: 127.
3. Tang HW, Liang YX, Hu XH, Yang HG. *Biomed. Environ. Sci.* 1995; **8**: 23.
4. Heine T, Conrad C. *Psychiatr. Neurol. Med. Psychol. Leipzig.* 1990; **42**: 613.
5. Craig PJ. Environmental Aspects of Organometallic Chemistry. In: *Comprehensive Organometallic Chemistry*, Wilkinson G, Gordon F, Stone A, Abel EW (eds), Vol. 2, *The Synthesis, Reactions and Structure of Organometallic Compounds*. Pergamon: Oxford, 1982; 979–1020.
6. Kleszczyńska H, Hładyszowski J, Pruchnik H, Przestalski S. *Z. Naturforsch. C* 1997; **52**: 65.
7. Antonowicz J, Andrzejak R, Leptow T. *Fresenius' J. Anal. Chem.* 1998; **361**: 365.
8. Alperstein G, Reznik RB, Duggin GG. *Med. J. Aust.* 1991; **155**: 407.
9. Badura L. *Pol. J. Environ. Studies* 1997; **6**(Suppl.): 5.
10. Strużyńska L, Dąbrowska-Bouta B, Rafałowska U. *Acta Neurobiol. Exp.* 1997; **57**: 275.
11. Thayer JS. *J. Organomet. Chem.* 1974; **76**: 265.
12. Andersen O, Grandjean P. *Appl. Organomet. Chem.* 1987; **1**: 15.
13. Radecka H, Sarapuk J, Zielińska D, Przestalski S, Radecki J, Cieciórski B. *J. Med. Prevent.* 1996; **4**: 87.
14. Trela Z, Radecki J, Przestalski S. *Pol. J. Environ. Studies* 1997; **6**: 37.
15. Tobias RS. The Chemistry of Organometallic Cations in Aqueous Media. In *Organometals and Organometalloids—Occurrence and Fate in the Environment*, Brinckman FE, Bellama JM, (eds) *ACS Symp*. American Chemical Society: Washington DC, 1978; 130–148.
16. Endres KP, Faulstich H. *J. Plant Physiol.* 1989; **135**: 531.
17. Nygren O. *Appl. Organomet. Chem.* 1994; **8**: 601.
18. Nerin C, Pons B. *Appl. Organomet. Chem.* 1994; **8**: 607.
19. Gabrielska J, Sarapuk J, Przestalski S. *Z. Naturforsch. C* 1997; **52**: 209.
20. Trela Z Z, Przestalski S, Hładyszowski J. *Pol. J. Environ. Studies* 1997; **6**: 33.
21. Wierzbicka M, Obidzińska J. *Plant Sci.* 1998; **137**: 155.
22. Donaldson WE., Knowles SO. *Comp. Biochem. Physiol. C* 1993; **104**: 377.
23. Silbergeld EK. *FASEB J. C* 1992; **13**: 3201.
24. Skou IC. *Biochim. Biophys. Acta* 1962; **58**: 314.
25. Panusz HT, Graczyk G, Wilmanska D. *Anal. Biochem.* 1970; **35**: 494.
26. Verevkina NV, Tochilin AI, Popova NA. *Modern Methods in Biochemistry*. Medicine: Moscow, 1977.
27. Timonin IM, Petrov VV, Levistsky DO. *Biol. Membr*. 1986; **1**: 60.
28. Przestalski S, Hładyszowski J, Kuczera J, Różycka-Roszak B, Trela Z, Chojnacki H, Witek S, Fisicaro E. *Biophys. J.* 1996; **70**: 2203.
29. Munter K, Athanasiou M, Stournaras C. *Biochem. Pharmacol.* 1989; **38**: 3941.
30. Hoshi F, Kobayashi H, Yuama A, Matsusaka N. *Jpn. J. Pharmacol.* 1991; **55**: 27.
31. Tocanne JF, Cezanne L, Lopez A, Piknova B, Schram V, Tournier JF, Welby M. *Chem. Phys. Lipids* 1994; **73**: 139.
32. Welti R, Glaser M. *Chem. Phys. Lipids* 1994; **73**: 121.
33. Kinnunen PKJ, Jukka AK, Lehtonen YA, Rytömaa M, Mustonen P. *Chem. Phys. Lipids* 1994; **73**: 181.
34. Mouritsen OG. Membranes Display Nano-scale Heterogeneity—Beating the Randomness of the Fluid Lipid Bilayer. In *In Search of a New Biomembrane Model*, Mouritsen OG, Andersen OS (eds). Det Kongelige Danske Videnskabernes Selskab: Copenhagen, 1988; 50.

