

Transport of heavy metals across the supported phospholipid bilayers

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Abstract—As it has been reported in many scientific as well as popular papers, TV, newspapers etc., practically all parts of environment (air, waters, and soils, plants, animals, etc.) have been in increased levels contaminated with various metals, inorganic or organic compounds and species. This effect can be explained as result of human activities in many cases. In combination with pollutions brought about by natural events (volcano eruptions, forest fires, etc.), the human being is fundamentally affected in this respect. To start their negative (or positive) role in plants or in human body, the elements, the compounds and the other species, which are present in polluted environment, must be transported into these organisms, more precisely, into their cells. In other words, each pollution particle, which takes part further in metabolic processes, must be transported across the cell membranes. Similar processes are realized in the opposite way – out of the cells as well as in and out of any sub-cellular structure. Detail elucidation of transport mechanisms is prerequisite for understanding of distribution of pollutants in real cells of more complex organisms (leaves, roots or the whole plants, animals or men) and for their possible control in the future.

Because the real bio-membranes and the transporting processes realized across them are very complicated, the model membranes have been used for elucidation of the basic transporting steps. This article deals with simulation, characterization, description, and elucidation of the above mentioned transport processes on the example of some heavy (hazardous) metals, i.e., of cadmium and of lead. Because cations of these metals do not exist only as separate ions, it is necessary to take into account their existence in complexes. Therefore the attention was paid to their complexes with low molecular weight organic acids (LMWOAs) and their transporting processes. This contribution summarizes the results achieved using supported phospholipid model membranes.

The ion channels, which are responsible for transport of some particles across the cell membranes, were replaced in the reported experiments by ionophores (valinomycin and calcimycin).

The tested membranes and processes were characterized using electrochemical methods (electrochemical impedance spectroscopy, cyclic voltammetry, anodic stripping voltammetry, conductometry, etc.) and optical spectroscopy.

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This manuscript presents new knowledge in this field, first part of which was presented one year ago at this conference.

Keywords—Cell membrane, Charged particles, Electrochemistry, Environment, Ion channels, Ionophores, Phospholipid bilayers, Protoplasts, Trace elements, Voltammetry.

I. INTRODUCTION

THE problems connected with environmental pollution have been studied very intensively by many scientists for more than one century and the investigations have been intensifying in last decades. Simultaneously, the research was split into many branches (chemistry, biology, energetics, economics, etc.). Nevertheless, as it was proved many years ago, the problem is so complex that the top specialists from the various scientific fields have to cooperate very closely to reach the intended goal. Such interdisciplinary research is described in this manuscript too. The present topic requires participation of people with knowledge in electrochemistry, biochemistry, (bio)physical chemistry, agriculture, biology, physiology, medicine, etc. The interdisciplinary approach, in which various scientific branches very close collaborate, has been many times presented in various WSEAS, NAUN and IEEE conferences, journals and books (e.g., [1]-[13]) and in other scientific papers and book (e.g., [14]-[20]).

II. CELL MEMBRANES

Air, waters and soils are contaminated with increasing levels of various metals, inorganic or organic compounds (partly products of human activities), which are transported into plant or animal organisms and more precisely, into their cells. All living cells of plants and of animals (and of some viruses too) are surrounded by lipid bilayer membranes (LBM). These membranes are flat sheets that form a continuous barrier around cells. Similar lipid bilayers surround the sub-cellular compartments. The phospholipids are the basic building elements of most natural membranes. Additionally to phospholipids, the membranes contain a wide variety of biological molecules, primarily proteins and lipids, which take part in many cellular processes, such as ion channeling, conductance, cell signaling, etc. [1], [3]).

A. Phospholipid Bilayers

Lipid bilayers (LBs) (or phospholipid bilayers (PLBs)) are thin, flat membranes consisting of two layers of lipid molecules, with their hydrophobic parts, usually fatty acid

tails, directed toward the centre of the membrane, and with hydrophilic parts located at the inner and outer borders [21]. The LB is the barrier that keeps ions and a great variety of molecules at places, where they are needed, and prevents them from diffusing into areas, where they should not be. LBs are ideally suited to this role because, even though they are only a few nanometers thick (6-10 nm), they are impermeable to most water-soluble (hydrophilic) molecules [22], [23].

The basic brick stones for the formation of the real LB are the phospholipids (as diacylphosphatidylcholine or diacylphosphatidylinositol, etc.), which are composed of two fatty acid tails, phosphate group and choline, inositol, serine, etc. groups. Because the real cell membranes are very complicated, we have started to study the above mentioned processes using model membranes, more precisely, model supported phospholipid (SPL) membranes (SPLM) or bilayers (SPLB), which should represent, in the first approximation, the real protoplast membrane [1], [3], [21].

B. Transport across the Phospholipid Bilayers

The closed bilayer is impermeable for most of water soluble molecules, as they would be insoluble in the hydrophobic bilayer core. There are many different principles, on which the molecules, ions or particles are transported across them. Gases like oxygen, CO₂ and nitrogen – small molecules hardly interacting with solvents – diffuse easily across the hydrophobic part of the membrane. Lipid molecules, e.g., steroidal hormones, permeate the bilayer easily [24]. The rate of diffusion of organic molecules – nonelectrolytes – depends on their lipid-water distribution coefficient. Compounds insoluble in fats are transported across amphipathic proteins and can be dipped into equally oriented lipid bilayer. The proteins form channels for ions and small molecules and serve for transport of bigger molecules, which would not be otherwise able to pass across the bilayer [1]. Transport can be passive or supplied by some external energy. There are some other principles of transport, we can mention endocytosis and exocytosis (e.g., in cases of larger objects and particles, such as bacteria, viruses), electroporation etc. [24].

III. INVESTIGATION OF PHOSPHOLIPID MEMBRANES

A. Method of Investigation of Phospholipid Membranes

Various techniques have been applied to the study of the membrane formation and of the transporting processes, e.g., fluorescence microscopy [25], fluorescence lifetime correlation spectroscopy combined with lifetime tuning [26], combination of fluorescence spectroscopy and *ab initio* calculations [27], solvent relaxation technique [28], or confocal fluorescence correlation spectroscopy [29]. However, the electrochemical methods proved to be very successful in this research. The patch-clamp technique has frequently been used for the study of real membranes structures and of transport of particles across them (e.g., [30], [31]). The investigation of liquid-liquid systems (i.e., systems of two immiscible liquids separated by a phospholipid layer) can be

useful for elucidation of the basic principles dealing with transport of particles across the PLBs too (e.g., refs. [32]-[35]).

B. Applied Electrochemical Methods

This manuscript is focused on the application of electrochemical methods (voltammetry, polarography, electrochemical impedance spectroscopy (EIS)) for the above mentioned purposes.

Electrochemical impedance spectroscopy (EIS) (e.g. [36]) can be very suitable for characterization of the formed PLBs (black lipid membranes (BLM)) [37]. Such relatively simple (however highly sophisticated) non-invasive method provides the detailed structural-functional information on these systems ([38] and the references therein).

Determination of hazardous (heavy) metal ions (Cd²⁺, Pb²⁺, etc.) and their complexes, transport of which across the PLBs is presented in this manuscript, and of many other organic as well as of inorganic compounds using voltammetric methods with various working electrodes, was many times described in literature and our research group has been developing such analytical methods over many decades (e.g., with hanging mercury drop electrode (e.g., [15], [39]-[51]). The formation of model PLBs requires very stable surface, which is flat on atomic scale. Hence, the experiments has been performed on solid electrodes ([4] and the references therein). Moreover, it has been reported that the solid electrodes can be successfully used for voltammetric determinations of mentioned transported environmentally important species. Our research team has been dealing with development and application of solid amalgam electrodes (silver, copper, etc.) (polished, film, mercury meniscus modified etc.) (e.g., [52]-[71]), composite solid electrodes (silver, graphite, gold, etc.) (e.g., [54], [72]-[81]), and solid amalgam composite electrodes for many years (e.g., [54], [77], [82], [83]).

It is well known that in environmental systems metals do not exist as separate ions only, but mostly as complexes with, e.g., low molecular weight organic acids (LMWOAs) ([84]-[86]). It is therefore necessary to utilize such analytical method, which is able to distinguish among cations and their low molecular complexes (e.g., with oxalic (OA) and citric acid (CA)) [1], [85]. Cyclic and stripping voltammetry seem to be very suitable for these purposes [85], [86].

C. Used Apparatus

For quantification of the electrochemical impedances, silver/silver chloride electrodes (silver wire, diameter 1 mm, electroplated with silver chloride) were used. Platinum wire, diameter 1 mm, served as the auxiliary electrode. These measurements were realized using a CHI 650C Electrochemical Analyzer/Workstation, Software: CHI v. 8.1 (IJ Cambria Scientific, Carms, UK) and a potentiostat No. 283 and a FRA No. 1025, No. 5210 (Princeton Applied Research, USA).

The voltammetric determinations of lead ions, cadmium ions and their complexes were carried out using a

PC-controlled voltammetric analyzer ECO-TRIBO polarograph (Polaro-Sensors, Prague, Czech Republic), equipped with MultiElchem v. 2.1 software [83] (J. Heyrovský Institute of Physical Chemistry of AS CR, v.v.i., Prague, Czech Republic) and with POLAR.PRO software v. 5.1 (Polaro-Sensors, Prague, Czech Republic). A pen-type electrode HMDE [87]-[89] was used as the working electrode, an Ag/AgCl/KCl (3 mol·L⁻¹) as the reference electrode to which all the potentials are referred to, and a platinum wire served as the counter electrode (both Elektrochemické Detektory, Turnov, Czech Republic). To determine cadmium ions, the sample was acidified by addition of HNO₃, Suprapur (Merck, Prague, Czech Republic) to pH 1.0 and analyzed using differential pulse anodic stripping voltammetry (DPASV) under the following conditions: Accumulation potential (E_{acc}) = -850 mV, accumulation time (t_{acc}) = 180-360 s, initial potential (E_{in}) = -700 mV, final potential (E_{fin}) = -200 mV, scan rate 10 mV·s⁻¹, pulse amplitude 50 mV, pulse duration 100 ms, the measuring periods over the last 20 ms before the pulse onset and over the last 20 ms of the pulse duration, sample volume 10 mL. A new drop was used for each recording and all the measurements were performed under nitrogen atmosphere. The pH was measured by a digital pH/mV meter MPH 61 with a combined electrode TYPE 01-29 (all from Monokrystaly, Turnov, Czech Republic).

D. Reagents and Materials

The experiments described in this manuscript were realized using porous membranes constructed of two types of phospholipids: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (lecithin, DPPC, GPCCho (16:0/16:0), CAS No. 63-89-8) (Avanti Polar Lipids, Alabaster, USA) (Fig. 1), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE, GPETn(16:0/16:0), CAS No. 923-61-5) (Sigma-Aldrich, Prague, Czech Republic) (Fig. 2). The PLBs were formed by self-assembling in the holes of the Isopore™ Membrane Filters (Millipore, USA) polycarbonate, hydrophilic 8.0 μm, and the supporting membrane thickness amounted to 7-22 μm. The area of one pore amounted to 50 μm², the experimentally found porosity of the membranes was about 25-45 %.

To investigate the transport processes, the ionophores valinomycin and calcimycin were added to the solution of the phospholipids before their application to the support. The ionophores were purchased from Sigma-Aldrich, valinomycin was > 90% (HPLC), and calcimycin (Calcium Ionophore, Antibiotic A23187) was > 98% (TLC). The structures of both ionophores were published, e.g., in [1].

Valinomycin is a dodecadepsipeptide (the cycle consists of three times repeated sequence of L-valine-D-hydroxyisovaleric acid-D-valine and L-lactic acid). It transports ions actively across phospholipid bilayer and is highly selective for K⁺ ion. Calcimycin is a highly selective natural ionophore for divalent cations [21]. Ion transport by this ionophore is mediated by a dimeric form of the molecule that complexes the metal cation. It is suitable for transport of divalent cations (Mn²⁺ > Ca²⁺ ≈ Mg²⁺ > Sr²⁺ > Ba²⁺ as well as of other metallic cations Ni²⁺,

Fe²⁺, Zn²⁺) [90], [91].

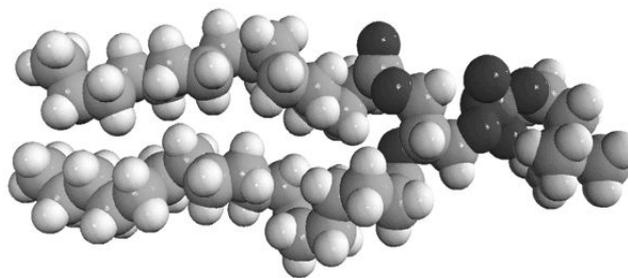


Fig. 1 structure of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (lecithin, DPPC, GPCCho (16:0/16:0), CAS No. 63-89-8) - building stone for construction of PLB in described experiments

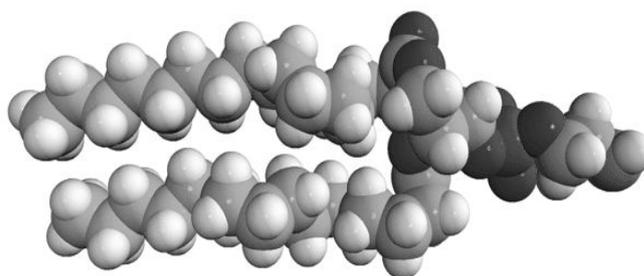


Fig. 2 structure of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE, GPETn(16:0/16:0), CAS No. 923-61-5) - building stone for construction of SPLB in described experiments

E. Cell Designs for EIS Measurements

Two different types of cells were constructed by our research group for investigation of the PLB on porous membranes: A) "U_Cell". The part of polycarbonate membrane (alone or with lecithin solution) is placed between two parts of glass U-shaped cell, which were then filled with a proper electrolyte or sample; B) "Insert" cell. This cell was made from polythene tube cup with a hole of 0.3 cm², on which polycarbonate membrane was sealed with epoxy resin (UHU, Bühl, Germany) onto the plastic cup with a small hole in the centre (0.3 cm²), prior to the application of the phospholipid solution. This cup subsequently formed the bottom of the upper part of the polypropylene electrochemical cell. The schematic and photographic pictures of cells were published in [1], [21], [92].

The electrodes for application of the voltage and evaluation of the impedances were situated in case of the "U-Cell" in the following way: the working electrode (silver wire electroplated with silver chloride) was immersed in the upper part and the reference electrode (silver wire electroplated with silver chloride) and the auxiliary platinum electrode were immersed in the bottom part.

F. Preparation of Supported Phospholipid Bilayers

The way used to preparation of PLBs on porous membranes was described in detail, e.g., in [1], [21], [92]. We mention the

basic points here: A phospholipid solution (20 mg·mL⁻¹ in n-heptane) was applied to both sides of the porous polycarbonate membrane and the solvent was evaporated on the air. The ionophores were added to the phospholipid solutions before their application on the membrane surfaces. To prevent contamination in the experiments, all the parts (polycarbonate membrane, cup, upper and lower part) were exchanged for the new ones prior to each experiment.

In this paper, there is devoted the highest attention to the preparation of SPLBs on porous membranes. Nevertheless, there exist some other possibilities, which can be utilized for these purposes. The relatively simple process of preparation of model PL membrane consists in formation of such a bilayer on some supporting materials (therefore, they are called: supported - phospholipid bilayers), e.g., on a metallic substrate (mercury [37], gold), or on some gel surface, or they can be prepared in the form of a self-supporting membrane, e.g., as a bubble at a Teflon cap [93], by filling a small micro-holes in a plate [94], etc. The selection of a proper solvent allows to obtain the membranes with the thickness and capacity values similar to those of membranes formed of monolayers [32].

The above mentioned preparation of SPLBs on the surfaces of different metals, more precisely on the surface of their alloys – amalgams, has been tested by members of our research team. Solid amalgam electrodes (SAE) covered by a mercury meniscus (m-) or by a mercury film (MF-) (m-AgSAE, MF-AgSAE, m-CuSAE, m-BiAgSAE and m-CdSAE) are convenient for obtaining thiol films, which are bound to different metals. By choosing an appropriate metal for the amalgam preparation, a thiol monolayer can be created. Such monolayer is stable in the given potential range. After a short accumulation time (about 12 min), the area of the reduction peak (charge) practically does not change if the monolayer is created from thiol solution [95]. Statistical results based on many times repeated formation and desorption of thiol monolayers (RSD = 23 %) attest that the stationary SAEs are convenient tools for study of electrochemical properties of thiol films [95]. On such monolayer it is relatively easy to build the reversely oriented PL monolayer, which hydrophilic part is oriented into the solution. Into such PLBs, prepared in this way, it is possible to build-in, the ion channels, ionophores or channel forming peptides, etc., which are able to transport various ions and compounds from the electrolyte to the electrode surface, where they can be registered (electrolyzed, adsorbed). The disadvantage of this way of SPLBs preparation consists in relatively low amounts of transported ions, which can be transported and registered to the electrode surface in time. The so originating currents are on the level of pA and they are distorted by noise.

The preparation of PL on the surface of agar-substrates has been tested in the framework of our research too. Introduction of the polymer gel electrode [96] has stimulated progress in electroanalysis at liquid/liquid interfaces [1]. The gel can be prepared by mixing a suitable polymer (PVC, agar, agarose, etc.) with one of the electrolyte solutions at an elevated

temperature. After cooling the mixture to room temperature in a suitable mould, the gel electrode is formed. It assumes a variety of shapes and it is easy to handle. Polarization measurements at the polymer gel/liquid boundaries can be employed for analysis of the behavior of ISE's, or for direct determination [97] or continuous monitoring [98] of ion concentrations [1].

One of the aims of our investigations is the preparation of SPBLs on an agar gel electrode. Such type of SPBLs can be applied to biosensors, micro- and nano-structures, blood-compatible surfaces, medical implant devices, and to production of catalytic interfaces [1], [99]. Many further applications have been proposed or are currently under study [1]. Systematic studies of the mechanisms of SPB formation, such as the conditions for fusion of adsorbed vesicles, have only been performed over the last few years. In the suggested agar-gel SPBL's, the agar gel is supposed to serve as a new type of "soft polymer cushion" [1], [99].

Other possibility of model PLB formation can be represented by building of vesicles from model PLs. The disadvantage consists in possible adsorption of transported species on the surface of such bilayers.

G. Electric Equivalent Circuits

Two types of electrical equivalent circuits were utilized to characterize the formed SPLBs bilayers and the corresponding transport processes (Fig. 3). The simpler one (composed of one resistor in serial combination with parallel combination of a resistor and a capacitor) was applicable for characterization of the free polycarbonate membranes. The other one was more suitable for characterization of SPLBs formed on the polycarbonate membrane pores. This circuit was similar to the simpler one, but additionally, a parallel combination of one capacitor and one resistor was added to the first capacitor (series-connected).

Each member of the circuits (Fig. 3 (A) and Fig. 3 (B)) could be used for characterization of the system (serial resistor (R_s) correspond to the resistance of the electrolyte; parallel capacitor of the first circuit (denoted as C) and of the second circuit (C_1) correspond to the parasitic capacitance of the membrane; parallel resistors to its resistance; the other parallel combination of a capacitor (denoted C_2) and a resistor in the second type of the circuit describe the electrical properties of the SPLBs [1], [3], [5], [21]). There are a few other possibilities of the used circuits, applications of which are tested in these days (e.g., additional resistor(s) on the right parts of the circuits, or addition of the second parallel combination of capacitor(s) and resistor(s) to the first one in the circuit depicted in Fig. 3 (A)).

IV. RESULTS AND DISCUSSION

This contribution summarizes the results achieved by our research team in last a few years on this topic.

A. Electrochemical Methods of Characterization

After successful experiments, in which a reproducible way

of SPLBs formation was worked-out [32], we realized the tests to characterize these model membranes. The capacitance of a phospholipid bilayer gradually increases after the bilayer exposure to the aqueous phase. This process can be explained by thinning of the SPLBs [100]. This conclusion can be drawn from the changes in recorded Nyquist graphs (the results were independent of the measuring cell type, “U-cell” or “Insert”-cell).

The changes in the calculated capacitances (in both, simpler and more complicated electrical equivalent circuits) increased logarithmically and reached their steady states in 40 to 60 minutes. The most pronounced change (the highest relative increase) was recorded for capacitance C_2 in inserted parallel circuit, which can be therefore used for characterization of the SPLBs. The changes of other capacitances are similar and they are smaller in comparison with the above mentioned one. This is consistent with the presumption that they correspond to the parasitic capacitance of the polycarbonate membrane and confirms the proper selection of the equivalent model [21].

The evaluation of the capacitances for characterization of changes in SPLBs on porous supports seems to be superior to the use of resistances, because any defect in a PLB membrane

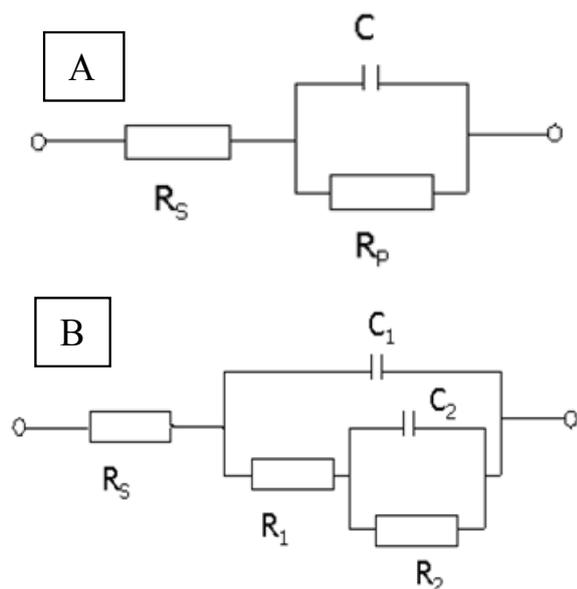


Fig. 3 equivalent circuits used for characterization of the PLBs

can possibly permit charge transfer, which can be realized by cations other than the studied ones (e.g., protons), causing substantial changes in the resistance values.

B. The Effect of Applied Voltage on the SPLBs

In the following experiments, our attention was paid to investigation of the applied DC voltage. The records were realized in steady state of the membrane existence, i.e., after about 1 hour after SPLB formation. With the application of potential in interval from -0.4 V to $+0.4$ V, the increase of capacitance was observed, but changes were reversible. This

confirms that SPLB is not damaged, similarly as has been described for s-BLMS on the tip of Teflon coated silver electrode reported in [101]. An application of a voltage higher than ± 0.4 V can cause a partial damage to the SPLB. The non-idealities are self-repaired after decrease of the applied voltage, i.e., the phospholipids relax, form the PLB membrane again and the membrane parameters (capacitances and resistances) are almost identical with the original ones. The relaxation time constants are relatively short. Their values increase with increasing potential and are generally longer for applied positive potentials (e.g., the membrane is rebuilt (reconstructed) within 30 minutes after 6 min. long application of $+0.4$ V, whereas the relaxation takes only 20 minutes after the same time of application of -0.4 V). The incomplete destruction can be proved by increased capacitance of the SPLB, while its resistance decreased. These effects can be explained by thinning of the membrane and by formation of holes (disturbances) in it, through which the charged particles (e.g., in this manuscript the studied Cd^{2+} cations) are transported.

The details of capacitance-time and resistance-time dependences were published in [21]. Very interesting results were recorded under application of 0 V [1]. There was no substantial difference in behavior of SPLB prepared from DPPC monohydrate or DPPE.

C. Transport of Monovalent and Divalent Cations

In the following step, our attention was turned to investigation of the transport of monovalent (K^+) and divalent (Cd^{2+} and Pb^{2+}) cations across the SPLBs. These model membranes were, similarly as in the above described experiments, formed from DPPC and DPPE, however, two ionophores: valinomycin and calcimycin were added. A similar process of the phosphatidylcholine SPLB formation was recorded as in the case of ionophore absence.

Similarly, an increase in the electric capacitance C_2 with time was observed. Within about one hour, a steady state was attained (the formation of the SPLB is complete; the capacitance values are similar to the capacitance of the SPLB formed in the absence of an ionophore). The capacitances corresponding to the parasitic capacitance of the supporting membrane remain almost constant [21]. In the case of valinomycin presence in SPLB, the parallel capacitance C_2 was decreased owing to the transport of the potassium ion complex with the ionophore across the PL membrane, the effect of which was accompanied by an increase in the electric conductance of the membrane. After exchanging the electrolyte containing K^+ for Ca^{2+} in the presence of valinomycin, capacitance of the SPLB (C_2) begins increasing in time. This effect can be explained by the hypothesis that calcium dications are transported into the membrane. However, valinomycin cannot transport them across the membrane. Therefore, calcium (generally a charge) is accumulated in the membrane, which is manifested by the increase in the capacitance C_2 , i.e., capacitance of the SPLB. Moreover, the accumulated divalent calcium cation stabilizes

the SPLB [102]-[104].

Transport of divalent cations of hazardous (heavy) metals is more complicated, because they are not transported in the form of free cations only, but they form some complexes in the nature (e.g., in the neighborhood of plant roots [1], [6], [84]-[86], [105]). The experiments were realized in this manner: When the steady-state of SPLBs on the polycarbonate support was reached, the Cd^{2+} ions were added to the electrolyte 1. Exactly 1 hour after this application, the amount of cadmium in electrolyte 2 was determined by anodic stripping voltammetry. The results, achieved under different conditions (electrolyte composition, pH, etc.) are summarized in Table I. It is evident that the free, porous membrane does not constitute any barrier for metal transport (almost 30 % of total Cd^{2+} amount was transported from compartment 1 to compartment 2). On the other hand, the SPLBs, formed in the porous membrane, were so perfect that in the absence of calcimycin ionophore, only a negligible amount of cadmium was transported [21]. A similar result was achieved in the case of incorporation of the ionophore calcimycin, which cannot transport the monovalent potassium ions. The results obtained show the positive influence of the applied potential, but, surprisingly, the presence of Ca^{2+} has also a positive effect on the cadmium transport [21]. In our simple system, involving no other metal ions or pump systems, there is a possibility of different effects of 1:1 and 1:2 complexes (CdA^+ and CdA_2), similar to the cadmium transport through a phospholipid bilayer containing a crown ether [106]. These observations have not been explained and confirmed in a satisfactory way, due to the constant concentration of Cd^{2+} ions and a constant ionophore concentration. We can suppose that the other ligand (OH^- or Cl^-) participates in complex equilibria. Such competition, when Ca^{2+} is the central atom, has not been described up to now [107] and it could enhance the cadmium transport in the presence of calcium [21]. Nevertheless, further experiments will be necessary to provide an answer to this question.

D. Transport of Complexes with LMWOA

Very interesting results were reached in case of the transport of heavy metals in the presence of LMWOA, concretely OA. This part of research is very important for elucidation of processes occurring in soils, where the uptake of metals by plant roots is realized. It represents the main entry pathway into animal and human food chain [6]. Generally, the uptake by plants depends on the concentration of metal in the soil, but overall, there is no correlation between soil metal content and plant metal content [6], [108]. Other factors, as pH and the presence of organic and inorganic ligands, influence the size of plant available metal fraction. There is a great difference between cadmium and lead: Whereas cadmium ion is up to pH 7 present as Cd^{2+} or hydrated species, in case of lead, Pb^{2+} is predominant only to pH lower than 6, at higher pH different forms of hydroxylated species prevail, some of them with very low solubility [109]. That is the reason why only Cd is of particular concern in most studies.

As it was reported in [6], OA is the most abundant LMWOA (among other such acids as CA, malic acid, malonic acid, etc.) in soil solution from rhizobag experiment with smith willow and alpine penny-cress. In those real soil solutions (diluted by sodium perchlorate) voltammetric analysis revealed peak of mixed Cd-OA-Pb complex and PbOA complex.

According to the literature sources, higher uptake of lead could be explained by invoking a co-transport of Pb with Cd in the "mixed" complex with oxalic acid. The transport of whole complexes across the plant membrane is preferred over that of single of the cadmium or lead ions as was observed for aluminium [108], [110].

The formed complexes have been studied in model as well as in real solutions very intensively during last a few years by our research team and by collaborating research teams (e.g., [84], [85], [111]-[116]). Various methods have been used for these purposes: DPASV, cyclic voltammetry, pseudopolarography, electrospray ionization - mass spectrometry (ESI-MS). The stoichiometry of the metal complexes can be derived from the spectra by the mass to charge ratios (m/z) in conjunction with the characteristic isotope patterns [111]. It was proved that the combination of DPASV with ESI-MS allows a reliable detection of metal complexes with small organic ligands. The request to exact molecular mass is essential to stoichiometry design simultaneously with low concentration of the analyte in most of biological matrixes. By the analysis of model solutions the structures of many formed complexes (positively ($^{+1}$, $^{+2}$) or negatively charged ($^{-1}$, $^{-2}$)) were suggested. The existence of these complexes was confirmed by analysis of real soil solutions (e.g. [6], [86], [111], [115]). The DPASV results for different accumulation potentials can be also used to construction of pseudopolarograms [117]. Alternatively, the set of curves can serve as a "fingerprint" of the sample [111].

The results, which were achieved by studies of transporting processes of heavy metals across the model SPLBs formed on porous membranes, are presented in Table I. It follows that Cd^{2+} ions are not transported in absence of OA (pH 7.5) and of any ionophore. Nevertheless, the 30 mM concentration of OA in electrolyte can enhance the transport of Cd^{2+} ions as well as of Pb^{2+} ions across the SPLBs. The mechanism of this process has not been elucidated up to now and there exist minimally two or three theories, how to explain the obtained results.

V. CONCLUSION

In correspondence with the earlier published results [1], [21], [32], [92] and the results published in this paper, it can be concluded that the model membranes in the form of SPLBs can be used for simulation of real cell membranes.

TABLE I
TRANSPORT OF Cd²⁺ ACROSS THE DPPC SPLB IN THE PRESENCE
(OR ABSENCE) OF CALCIMYCIN IN DIFFERENT ELECTROLYTES

Conditions	Electrolyte 1 + Cd ²⁺	Electrolyte 2	E [V]	pH of Electrolyte 1	% of transported Cd ²⁺ (=20 µg = 100 %)
Free membrane	0.1M KCl	0.1M KCl	-0.1	5.7	28.73
DPPC	0.1M KCl	0.1M KCl	-0.1	5.7	0.00
DPPC + Calcimycin	0.1M KCl	0.1M KCl	-0.1	5.7	0.01
DPPC + Calcimycin	0.1M CaCl ₂	0.1M CaCl ₂	-0.1	5.7	0.35
DPPC + Calcimycin	0.1M CaCl ₂	0.1M KCl	-0.1	5.7	0.14
DPPC + Calcimycin	0.1M KCl	0.1M KCl	+0.5	5.7	0.24
DPPC	0.1 M KCl+	0.1M KCl	-0.1	7.5	0.22
	30 µM OA				
DPPC	0.1 M KCl+	0.1M KCl	-0.1	7.5	0.17
	30 µM OA				0.76 ^a
	Pb ²⁺ (20 µg)				
DPPC	0.1 M KCl	0.1M KCl	-0.1	7.5	0.00

^aThe value describes the transport of Pb²⁺ ions.

Various reliable and reproducible ways of their preparation were worked out and tested, as well as methods of their characterization using electrical equivalent circuits. Nevertheless, for the more complex systems, containing ionophores and other integral or artificially incorporated parts, more complex circuits will be necessary to apply. The SPLB can be considered as completely formed in about 40-60 minutes after application of phospholipids on the support holder. The values of its capacitance(s) increase after the SPLB exposure to the aqueous phase till steady state is reached. Relatively high stability of SPLB allows the studies of the influence of inserted DC voltage. Applied voltages in interval from -0.4 V to 0.4 damage the consistency of the SPLBs reversibly. Insertion of higher voltage can destroy them irreversibly.

The results achieved using DPPC are almost equivalent to those achieved using DPPE.

The results from both tested cell types ("U-cell" and "Insert") are very similar and from the statistical point of view they do not differ.

We can conclude that the procedure of incorporation of ionophore valinomycin and calcimycin was successfully realized. Valinomycin proved to be suitable for transport of K⁺ (univalent) cation and not for transport of Ca²⁺ (divalent) cation across SPLBs. On the other hand, the addition of calcimycin to the SPLBs enables transport of divalent cations, e.g., Cd²⁺. It was revealed that the presence of Ca²⁺ cations enhances the transport of Cd²⁺ cations across the DPPC SPLB with incorporated calcimycin.

Surprisingly, the presence of a LMWOA (e.g., oxalic acid) enables the transport of cations of heavy metals (Pb²⁺, Cd²⁺) across the DPPC SPLB in absence of any ionophore.

The suitable measuring and analytical techniques for these

purposes have been developed. Because the amounts of ions transported across the membranes are very small, they can be measured using patch clamp technique directly or after accumulation of the transported species using anodic stripping voltammetry. This method enables to differentiate between signals belonging to metallic ions and those produced by their complexes.

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