

## MEETING REPORT

**THE PHYSICOCHEMICAL BASIS OF ION TRANSPORT THROUGH BIOLOGICAL MEMBRANES**

**Report of a Pre-symposium held in Riga, U.S.S.R., on June 19 and 20, 1970, within the scope of the VII. International Symposium on the Chemistry of Natural Products**

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For the first time in the history of the IUPAC Symposium on the Chemistry of Natural Products, at the initiative of the late M.M.Shemyakin, Honorary President of the VII (Riga) Symposium, was a Pre-symposium devoted to the physicochemical basis of ion transport through biological membranes. Recent achievements in studies of biomembranes are to a great extent associated with studies of the chemical composition and structure of lipids, proteins and other membrane constituents and also of various naturally occurring substances (hormones, antibiotics, toxins, alkaloids, etc.) capable of modifying membrane properties such as permeability, active ion transport, excitability, etc.

Both the introductory lectures and the numerous short reports were grouped around the following major problems of membrane biology: (a) chemical composition and molecular organization of biological membranes, (b) physicochemical properties and ion permeability of membranes, (c) studies of ion permeability by chemical means, and (d) ion transport and the transformation of energy in mitochondria. A considerable portion of the communications was devoted to studies of the properties of model membrane systems: phospholipid bilayers, liposomes and monolayers.

Much attention in the Pre-symposium was devoted to the *lipid composition of biomembranes* and its effect on their properties. L.L.M.van Deenen (Utrecht, Holland) showed that the ability of phospholipid membranes to serve as a diffusion barrier to non-electrolytes is strongly dependent on the structure of the hydrocarbon chains of the lipid: for instance, the permeability

of liposomes to glycerol increases with decreasing chain length and increasing number of double bonds. The permeability of liposomes falls at low temperatures. In *E. coli* cells cultured at reduced temperatures, the decrease in membrane permeability due to the low temperature is compensated by augmentation of the unsaturated lipid content. In van Deenen's laboratory, a series of *E. coli* mutants has been obtained differing in the structure of the fatty acid residues of their membranes. These differences, however, were balanced from the point of view of their effect on the permeability. The membranes of the mutants contained either 80% of residues with one double bond or 40% with two double bonds or a considerably lesser amount with three double bonds. At the same time it was noted by A.D.Bangham (Cambridge, England) that the effect of the lipid composition depends greatly on the nature of the substance undergoing transport. The permeability of liposomes to glycine increases with an increase in unsaturated fatty acid residue content, whereas for phenylalanine it remains practically the same. An original method of modifying the fatty acid composition of lipids was employed by A.P.Johnson (Ryde, Australia). When small doses of fatty acids containing a cyclopropene ring (sterculic and malvalic acids from the seeds of malvales) are introduced into plant and animal tissues, they show a marked decrease in the ratio of oleic to stearic acids owing to irreversible inhibition of the enzymes of fatty acid desaturation. This in turn causes a sharp change in the physicochemical properties of the membranes and tissues.

According to van Deenen, the part played by cholesterol in biological membranes consists in lowering the mobility of the hydrocarbon chains and diminishing the surface area occupied by a phospholipid molecule.

In the communication by L.D.Bergelson (Moscow, USSR), the lipid composition of various organelles (mitochondria, microsomes, nuclei) and of the plasma membrane of normal liver cells was compared in detail with that of hepatoma cells. In contrast to the former where every type of membrane possesses a specific set of lipids, in tumor cells dedifferentiation of the lipid composition of the membranes takes place and the content of the major phospholipids in the various membranes approaches their mean content in the cell. Hence substitution of the polar heads of the phospholipids takes place without associated changes in the functional activity of the membranes. According to the author these results cannot be interpreted in terms of the classical Danielli-Davson membrane mode, or of later models involving specific lipid-protein interactions. Comparative data on the lipid composition of normal liver and hepatoma cells were also presented by C.Theise (Dresden, GDR).

The paper by N.Show (Newcastle-upon-Tyne, England) was devoted to the structure, biosynthesis and functions of glycolipids. The author showed that this class of compounds is widespread in bacterial membranes and usually consists of mono-, di- or oligosaccharides glycosidically bound to 1,2-diglycerides or to fatty acid acylated sugars. The most likely function of glycolipids in the membranes of wall-free bacteria (*L-forms*, *Mycoplasma laidlawii*) is to provide structural rigidity. At the same time glycolipids are possibly also structural components of the hydrophilic pores in membranes and participate as intermediates in the biosynthesis of phospholipids.

Studies of *membrane proteins* were reported to the Pre-symposium by D.W.Urry, L.Masotti and J.Krivacic (Chicago, USA). The authors showed that the difficulties in the interpretation of the optical rotatory and circular dichroism curves of biological membrane suspensions are due to the particulate nature of these systems. They were able to find a way to correct the experimental data; systems of varying dispersity were studied taking account of the densities of the suspensions and model optically active systems with known particle structure were used. It was shown that the corrected CD curves of intact mitochondria, submito-

chondrial particles, plasma and erythrocyte membranes and sarcotubular vesicles differ considerably in circular dichroism, evidence of differences in the conformations of the membrane proteins. In a number of cases, these curves indicate a high content (50%) of  $\alpha$ -helical segments. IR data showing marked helicity of the membrane proteins of *Micrococcus lysodeicticus* and the absence in these proteins of areas with  $\beta$ -structure were obtained by N.C.Gelman, D.N.Ostrovskii and M.A.Lukyanova. According to R.Schön and K.R.H.Repke (Berlin-Buch, GDR), salt treatment of the membranes of heart muscle preparations changes the properties of  $(\text{Mg}^{2+})$ - and  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ .

A lively discussion was started by the communication of D.E.Green and G.Vanderkooi (Madison, USA) in which the authors proposed a new model for the *structure of biological membranes*. According to this model, the membrane consists of two layers formed by protein globules in contact with each other (in each layer and between layers). The space between the globules is filled with lipid bilayers, the lipid molecules situated at the bilayer edges being hydrophobically bound to the protein. The polar heads of the lipid molecules are oriented orthogonal to the membrane plane. The results of studies of membranes by spin and fluorescence probes, and of deuterio exchange in the intact membrane and the isolated proteins reported by D.N.Ostrovski and by L.M.Raikhman (Moscow, USSR) accord with this model. L.M.Raikhman investigated conformational transitions in the membranes of mitochondria and microsomes and showed them to be of a cooperative nature.

A promising novel method for structural studies of membranes has been proposed by V.F.Bystrov, N.I.Dubrovina, L.I.Barsukov and L.D.Bergelson (Moscow, USSR). Investigating the high resolution PMR spectra of aqueous liposome suspensions, these authors showed that the addition of impermeable paramagnetic ions (e.g.  $\text{Mn}^{2+}$ ) leads to abrupt widening of the signals of such groups that are situated on the external surface of the liposomes, but does not affect the shape of the signals of groups situated on the internal surfaces and within the membranes. The proposed method may be particularly useful in studies of asymmetric biological membranes.

A number of participants discussed to what extent artificial phospholipid membranes may be regarded as adequate models of biological membranes. In this con-

nection, Bangham noted that phospholipid micelles (liposomes) are essentially natural systems since their formation is apparently one of the stages in the appearance of life on the earth. He further showed that many parameters of biological lipoprotein membranes can be reproduced with a high degree of accuracy on artificial membranes. S.Kinsky (St. Louis, USA) was even able to reproduce on model membranes such a complex process as the immune lysis of cells. In his experiments, antigen-containing phospholipid liposomes in the presence of antibodies and complement behaved like erythrocytes, pores forming in their membranes leading to increase in permeability.

A large number of the papers read at the Pre-symposium were devoted to the *effect of various chemical agents on ion transport*. R.S.Snart (Sheffield, England) studied the effect of aldosterone and oxytocin on the transport of  $\text{Na}^+$  ions through frog skin and toad bladder. With these tissues, the sodium flux is determined by the relation between the passive permeability ( $E_{\text{act}} = 9 \text{ kcal/geq}$ ) and the  $\text{Na}^+$  pump activity ( $E_{\text{act}} = 13.5 \text{ kcal/geq}$ ). From the plot of the stimulation of  $\text{Na}^+$  transport vs. aldosterone dosage, it appeared that this hormone activates two proteins, of which one is responsible for  $\text{Na}^+$  permeability through the mucosal membranes of the cell and the other affects the permeability of mitochondrial membranes to substrate anions. The action of oxytocin is on the mucosal side and is accompanied by an increase in permeability to  $\text{Na}^+$  ions and water. C.Lippe and D.Cremaschi (Bari and Milan, Italy) showed that effect of amphotericin B on rabbit gall bladder epithelium is manifested only if the antibiotic is introduced on the mucosal side. R. Rybova (Prague, Czechoslovakia) presented data showing that active  $\text{Na}^+$  ion transport in the cells of the fresh water chlorophycean alga *Hydrodictyon reticulatum* is insensitive to ouabain and to dichlorophenyl-dimethylurea (DCMU — an inhibitor of the 2nd photosynthetic system). On the contrary, the transport of chloride anions is directly coupled to the photosynthetic apparatus since it is strongly affected by DCMU.

G.Gardas (Budapest, Hungary) studied the nature of the effect of gramicidin and nigericin on the  $\text{K}^+$  and  $\text{Na}^+$  ion transport through erythrocyte membranes.

Of the various compounds capable of modifying the properties of biological and model membranes, greatest attention was given to the complex-forming cyclodepsipeptides and cyclopeptides, increasing the

membrane permeability to alkali metal ions. Yu.A. Ovchinnikov (Moscow) discussed the present state of conformational studies of these compounds and their complexes in solutions. The use of optical and PMR spectroscopy in combination with theoretical calculations made it possible for him to determine the conformation of antamanide and its  $\text{Na}^+$  complex (see fig. 1). This cyclodecapeptide, which is an antidote for phalloidine and amanitine, the toxic principal of *Amanita phalloides*, was recently isolated from the same fungus by T.Wieland and collaborators and is at present the only known, peptide macrocyclic compound displaying  $\text{Na}^+$ -specificity in complex formation ( $K_{\text{Na}^+/\text{K}^+} \approx 10$ ). It turned out that, like valinomycin, the spatial arrangement of the peptide chain in the antamanide molecule is determined by a system of intramolecular hydrogen bonds, the  $\text{Na}^+$ -specificity of the complexing reaction being due to the spatial hindrance to incorporation of larger cations in the internal cavity of the molecule.

Of general interest were a number of papers devoted to the ion selectivity of complex formation by valinomycin and its cation permeability it induces in model membranes. D.E.Tosteson, B.Gisin, M.T.Tosteson, D. Devis, R.Gunn and P.Coak (Durham, USA) showed that, in the presence of certain lipophilic anions such as trinitrocresolate, valinomycin in non-polar organic solvents binds not only  $\text{K}^+$  but also  $\text{Na}^+$  ions. It was also found that trinitrocresolate reduces the  $\text{K}^+$  selectivity of the valinomycin-induced cation permeability of phospholipid bilayers. A similar phenomenon was noted by L.I.Barsukov in studies of the effect of the anion species on the rate of outflow of cations from nonsonicated liposomes built up from egg lecithin. It turned out that valinomycin and its complexing analogs stimulate the outflow of  $\text{Na}^+$  ions from liposomes containing nitrate, but not chloride anions. This effect was not due to the non-specific detergent-like action of the cyclodepsipeptides.

The paper by A.A.Lev, E.P.Buzhinsky and V.V. Osipov (Leningrad, USSR) was devoted to comparison of the effects of valinomycin on phospholipid bilayers and bulk heptane membranes. Making use of Tosteson's method of separating bulk and surface conductivities of thick membranes by extrapolation to zero thickness, these authors found that the  $\text{K}^+/\text{Na}^+$  selectivity of surface conductivity is much higher than that of bulk conductivity and strongly depends on the concentration of valinomycin.

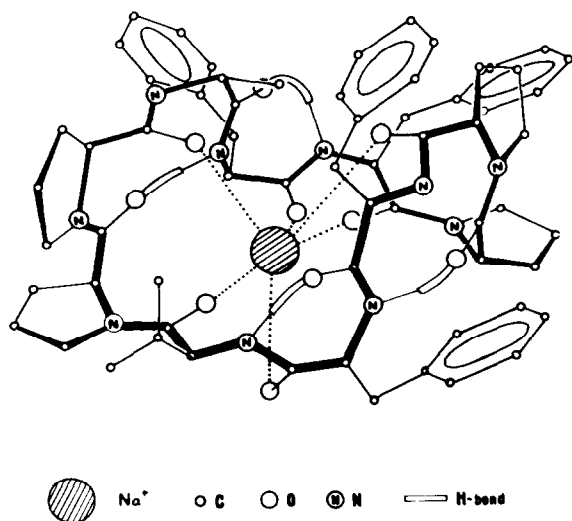


Fig. 1. Conformation of the  $\text{Na}^+$  complex of antamanide

Yu.A.Chizmadzhev and V.S.Markin (Moscow, USSR) discussed the application of impedance measurements in studies of the mechanism of transmembrane ion transport. Comparison of low frequency and high frequency conductivities and capacities of the membranes at varying concentrations in the solution of the transported ion makes it possible to choose between carrier and relay mechanisms of transport. A.M.Shkrob (Moscow, USSR) presented data showing that the nature of the alternating current conductivity and capacity relation of phospholipid bilayers in the range of 50–50000 hz in the presence of valinomycin and some of its analogs correspond to that predicted by Chizmadzhev and Markin for the relay mechanism. The results of these experiments were compared with the results of studies of the formation of  $\text{K}^+$  complexes of these cyclodepsipeptides at the water-air interface. It turned out that increase in the surface activity of the complex cations leads to a fall in the direct-current induced conductivity of the membranes due to increase in the energy barrier to the passage of the complex cations from the surface into the bulk of the membrane. Considerable differences between different cyclodepsipeptides with respect to the value of the complexing constant on the membrane surface were observed.

Yu.A.Chizmadzhev and V.S.Markin, and V.P.Topaly and E.A.Liberman (Pushchino on the Oka, USSR)

discussed the theoretical aspects and experimental data of ion carriers, which in the free state or in complexes with the transported ion carry charges of the same sign. With increasing intensity of the electrical field in the membranes containing such carriers, impoverishment of the zone adjacent to one of the membrane boundaries in both forms occurs, leading to a segment with negative slope on the current-voltage curves. Of interest is that in this region E.A.Liberman, G.M.Frank, G.N.Berestovski and V.Z.Lunevski observed a change in the birefringence of the membrane (for instance with the system dibarenylmercury + iodide) resembling the phenomena in axons caused by the passage of an excitation wave. In this connection it should be noted that studies of excitable membranes were not reported at the Pre-symposium, but Yu.A.Chizmadzhev and V.S.Markin, and S.N.Fishman and M.V.Volkenstein (Moscow, USSR) discussed a number of theoretical models for generating excitation.

E.A.Liberman and A.M.Shkrob noted the part played by the potential jump at the membrane surface in determining the partition coefficient of charged particles. The presence of an effective positive charge within lipid membranes, irrespective of the species of ionogenic groups in the lipid molecules, leads to a  $10^5$ – $10^6$  fold increase in partition coefficient over that for cations of similar structure. The adsorption of complexing and non-complexing cyclodepsipeptides of the valinomycin series on the membrane surface causes an increase in this jump resulting in an increase by these compounds of the membrane permeability to lipophilic anions in NaCl solutions. An alternate explanation of this effect in the case of valinomycin was proposed by Tosteson who suggested that the increased partition coefficient of the lipophilic anion is the consequence of incorporation into the membrane of the  $\text{Na}^+$  complexes of valinomycin.

In a number of papers the effect of the surface charge of a membrane on its ion permeability was discussed. L.L.M.van Deenen and A.D.Bangham discussed the part played by the structure of the lipids in determining the surface charge. In particular van Deenen showed that valinomycin does not accelerate  $\text{K}^+$  ion exchange in liposomes formed by the positively charged lipid lysylphosphatidyl glycerol. F.V.M.Daemen and S.L.Bonting (Nijmegen, Holland) observed an increase in the cation permeability of liposomes from phosphatidylethanolamine on the addi-

tion of retinal and other aldehydes or of dinitrofluorobenzene, due to a decrease in the number of protonated amino groups on the membrane surface. H.Passow (Homburg, GFR) showed that, on treating erythrocytes with dinitrofluorobenzene, 2-methoxy-5-nitropropone or pronase, the cation permeability of membranes increases whereas the anion permeability diminishes, but the permeability to non-electrocytes remains constant. Here, however, the decisive factor is the decrease in the number of protonated amino groups not of the phospholipid but of membrane proteins. It is interesting that D.E.Tosteson and collaborators observed a similar effect on erythrocytes in the presence of a lipophilic anion, trinitroresolate. The important paper by G.Eisenman, S.G.MacLaughlin and G.Szabo (Los Angeles, USA) showed that changes in the lipid composition of the bilayer membrane or in the pH of the solution, leading to increased negative charge of the membrane, is accompanied by its increasing permeability to cations and decreasing permeability to anions, regardless of the ion or carrier species (nonactin, valinomycin, cyclopolyether, iodine). The opposite is observed on increasing the positive charge. A quantitative treatment of these effects was carried out in terms of the Gouy-Chapman double layer theory and it was shown that there is good agreement between theoretical and experimental data on the effect of ionic strength on the permeability of membranes with different signs of the surface charge.

The induced semiconductor properties of phospholipid membranes was discussed by L.I.Boguslavsky, A.V.Lebedev and F.I.Bogolepova (Moscow, USSR). Analysis of the experimental data led these authors to the conclusion that charge transport within phospholipid bilayers of lecithin in the presence of  $I_2$  and  $I$  takes place as the result of movement of positively charged "holes" injected into the membrane owing to the reaction  $I^- + P \rightleftharpoons I^0$  taking place on its surface.

*Energy transformation and ion transport in mitochondria* were problems occupying an important place in the works of the Pre-symposium. According to D.E.Green, J.Blondin and J.Young (Madison, USA), in the presence of oxygen and oxidizable substrates conformational changes occur in the mitochondrial lipoprotein subunits accompanied by electron microscopically observed morphological changes in the mitochondria. The energy accumulated in the subunits due to the con-

formational transitions can be utilized in the synthesis of ATP. The changes in the conformation cause changes in both the number and sign of the charges fixed on the inner membrane surface resulting in changes of pH and in absorption of the anions of weak acids. This last process according to Green and his collaborators is the driving force in the mitochondrial absorption of  $K^+$  ions in the presence of valinomycin. A lively discussion was involved by Green's observation of the ejection of chloride anions from mitochondria accompanying the valinomycin-induced  $K^+$  ion absorption in the presence of acetate. In support of the conformational model of energy transformation in mitochondria, Green stated his opinion on the concurrency of proton transport with the conformational changes in energized mitochondria. The data presented indicate the coupled absorption of weak acid anions and  $K^+$  ions in the presence of valinomycin.

V.P.Skulachev (Moscow, USSR) described the results of mechanistic studies of the generation of a transmembrane electrical potential in mitochondria and sub-mitochondrial particles that were carried out in the author's laboratory together with E.A.Liberman's group. The sign and magnitude of this potential were determined by studying the distribution of the lipophilic cations and anions (tetraalkylammonium and phosphonium cations, tetraphenylborate, phenyldicaborane anions, 1-phenylamino-naphthalenesulfate (ANS), etc.) permeating the membranes.

The redistribution of such permeating ions (as well as of other ions in the presence of corresponding carriers, e.g. valinomycin +  $K^+$ ,  $J_2^{2-} + J^-$  etc.) between the mitochondria and the medium was investigated by a method developed by E.A.Liberman wherein the phospholipid bilayers were used as selective membrane electrodes for the permeating ions. The changes in the inner mitochondrial concentration of the ANS could be followed by measuring the fluorescence intensity which was proportional to the anion concentration within the membrane. A highly important circumstance is that the change in the ANS concentration within the membrane is determined only by the content of the anion in the matrix, since ANS flow into and out of mitochondria has relatively little relation to the ANS concentration in the aqueous phase.

The data obtained indicate that electron transfer along the respiratory chain, or hydrolysis of ATP, is accompanied by the appearance of a negative poten-

tial within the mitochondria and of a positive potential within the ultrasonic fragments. In these circumstances, displacement of penetrating anions and absorption of penetrating cations occurs accompanied by extrusion of protons into the environment. With ultrasonic fragments, having the reverse membrane polarity, the opposite effect is observed, the cations being extruded, the anions being absorbed, and the environment becoming more alkaline. The nature of these effects is independent of the penetrating ion species which is evidence of the electrophoretic character of ion transport through the membrane. It is important that similar ion movements through membranes can also be observed when the electrical potential is due not to the action of enzymatic systems, but to a  $K^+$  ion gradient, in the presence of valinomycin, or to a pH gradient in the presence of proton carriers.

The methods described above made it possible to show that generation of a transmembrane potential is accompanied by electron passage through any of the three points of coupling in the respiratory chain. It has been found, too, that an electrical potential on the membrane also arises as the result of a transhydrogenase transferring hydrogen from NADPH to NAD.

According to the chemiosmotic hypothesis of Mitchell, generation of a transmembrane potential is due to respiration or ATP hydrolysis coupled with proton transport through the mitochondrial membrane. In order to substantiate this hypothesis, it must be shown that an electrical potential can be directly used in the synthesis of ATP and also that the respiratory chain itself can function as a proton pump.

To demonstrate this Skulachev and his collaborators undertook a series of experiments directed at elucidating the location of the separate elements of the respiratory chain in the mitochondrial membrane. The idea behind these experiments is to utilize the appearance of a transmembrane potential as a sign of the passage of electrons through this or that section of the respiratory chain. By this approach it could be shown that the only section of the respiratory chain accessible from the external side of the mitochondrial membrane includes cytochrome *c*. In the "inside-out" ultrasonic fragments flavins and transhydrogenase become accessible to the non-penetrating oxidizing and reducing agents. Hence if it can be shown that transfer of a reducing equivalent from the flavins to cyt. *c* is accompanied by transfer of charge through the mem-

brane (i.e. by electron or hydride ion transfer), it could be regarded as the experimental confirmation of Mitchell's hypothesis.

In connection with the discussion of the chemiosmotic hypothesis, of considerable interest is the study reported by I.I. Severina (Moscow, USSR). It turned out that swelling of mitochondria coupled to ATP hydrolysis, in the presence of the anions of weak acids (acetate, phosphate) and inflowing cations ( $Ca^{2+}$ ,  $K^+$  in the presence of valinomycin, dimethyldibenzylammonium) is completely inhibited in the presence of three respiratory inhibitors: cyanide, rotenone and antimycin, but not by any two of them.

Detection of the transmembrane potential with the aid of penetrating ions was utilized by A.A. Yasaitis, L.L. Grinyuc, M.V. Vladimirova and T.I. Gudzik (Moscow, USSR) for studying the roles of the separate components of the mitochondrial membranes. It was found that acetone extraction of the phospholipid from ultrasonic fragments has no significant effect on the generation of the potential. The so-called ASU particles are devoid of the ability to absorb anions during respiration or ATP hydrolysis but the ability is recovered on addition of oligomycin (only during respiration) or of the  $F_1$  coupling factor.

The data presented in Skulachev's lecture are in good agreement with the results of the study of the effect of anions on the proton absorption by ultrasonic fragments carried out by S.P. Papa, F. Guerrieri, M. Lorusso, S. Simone and E. Quagliariello (Bari, Italy). They showed that anions increase both net proton absorption and the initial rate of this process in the presence of substrates and acids in the order tetraphenylborate,  $SCN^-$ ,  $I^-$ ,  $NO_3^-$ , coinciding with their order of lipophilicity. These anions exert a similar effect on the initial rate of proton outflow in anaerobiosis. In contrast to these anions and those used by Skulachev and Liberman, the substrate anions of the Krebs cycle are transported through the membrane in the form of non-dissociated acids. E. Palmieri, M. Klingenberg and E. Quagliariello (Bari, Italy and Munich, GFR) found that the partition of these anions between the matrix and the medium is determined not by the transmembrane potential but by the pH gradient, their concentration being greater where the pH is higher. In this connection it is interesting that nigericin, removing the pH gradient on the membrane but not affecting the potential, suppresses the effect of pH differences on

the partition of the substrate anions. S.Papa also discussed the transport mechanism for these ions. He showed that the primary process in the active transport of anions is the symport of  $P_i$  and protons (or the antiport with  $OH^-$ ) whereas the transport of substrate anions is due to exchange diffusion of (1)  $P_i$  and the anions of dicarboxylic acids, (2) of malate and the anions of the carboxylic acids, and (3) of  $\alpha$ -ketoglutarate and the anions of dicarboxylic acids.

E.Carafoli (Modena, Italy) discussed the role in the energy-dependent calcium ion transport mechanism of sites with a high affinity for  $Ca^{2+}$  ions ( $K_m = 10^{-6}$  M) present in the inner membrane of mammalian mitochondria. He found that for their functioning these sites require maintenance of the integrity of the phospholipid membrane constituents.