IUPAC SERIES ON ANALYTICAL AND PHYSICAL CHEMISTRY OF ENVIRONMENTAL SYSTEMS

Series Editors
Jacques Buffle, *University of Geneva, Geneva, Switzerland*
Herman P. van Leeuwen, *Agricultural University, Wageningen, The Netherlands*

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IUPAC Series on Analytical and Physical Chemistry of Environmental Systems. Volume 3

Metal Speciation and Bioavailability in Aquatic Systems

Edited by

ANDRÉ TESSIER,
Université du Québec, Sainte-Foy, Québec, Canada

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Contents

André Tessier is professor of aquatic chemistry at INRS-Eau, a multidisciplinary research center of the Institut National de la Recherche Scientifique (INRS), Université du Québec, Québec, Canada since 1970; he has also been an adjunct professor at the Department of Geology of McMaster University, Hamilton, Ontario, Canada since 1983. Dr Tessier received his DSc degree in physical chemistry from Laval University in 1970. He is an associated member of the Commission on Environmental Analytical Chemistry of IUPAC since 1989. He has also served on grant committees of the Natural Sciences and Engineering Research Council of Canada and of the Québec Fond pour la Formation de Chercheurs et l'Aide à la Recherche.

Dr Tessier has published over 75 papers and chapters in books and has presented short courses at institutions in Mexico, Switzerland, Turkey and Hungary. His main research contributions have been in the field of trace element biogeochemistry with a strong emphasis on *in situ* validation. He has done extensive research on trace element reactions in recent lake sediments, with particular reference to their sorption on diagenetic material, and on the prediction of trace metal accumulation in benthic organisms. He has a strong interest in contributing to the development of quality criteria for the management of aquatic systems based on an understanding of the biogeochemical processes occurring in field situations.

David R. Turner is Professor of Marine Chemistry at the University of Göteborg, Sweden. He attended Queen's College, Oxford, where he obtained a Bachelor of Arts degree in Chemistry in 1973. This was followed by research into the chemistry of the superoxide ion and its interaction with iron porphyrins, leading in 1977 to the award of a DPhil degree from Oxford University. In 1976 he moved to the Marine Biological Association in Plymouth to work with Dr Michael Whitfield on the electroanalytical chemistry of trace metals in seawater, subsequently transferring to Plymouth Marine Laboratory on its formation in 1988. He was granted a sabbatical year in 1982, and with the support of a Royal Society European Fellowship spent a year in Sweden at the Department of Analytical and Marine Chemistry in Göteborg. In 1993 he returned to Göteborg to take up the newly created Chair in Marine Chemistry.

Professor Turner's initial research topic in Plymouth was concerned with the effects of the chemical speciation of trace metals on the response obtained using electroanalytical methods. This led to a wider interest in trace metal speciation and in particular the development of chemical speciation models, first for inorganic complexation reactions and subsequently for complexation and humic substances. This was paralleled by a strong interest in the analogy between electrochemistry and biological uptake and in the relationship between chemical speciation and bioavailability. His current research in chemical speciation is concerned with the development of models for the adsorption of trace metals on natural particles in estuarine and sea waters. During the late 1980s he developed a second major research area in carbon dioxide chemistry in seawater, with particular emphasis on primary production and air/sea gas fluxes. In this connection he was an active participant in the UK contribution to the Joint Global Ocean Flux Study (JGOFS), and was chief scientist on cruises in the North Atlantic and the Antarctic Oceans. He has over 60 scientific publications in the field of marine chemistry.

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Series Preface

Most environmental systems are *living* systems in the basic sense: they are not immobile in thermodynamic equilibrium, but continuously *changing* thanks to the input of external energy (ultimately solar or geothermal). Such changes occur at all spatial (and time) scales, from the molecular level to the global scale, through micro and macroscales, for instance in weathering processes which clearly encompass processes at all scales. Organisms, particularly microorganisms, often play a dominant role in causing these changes, by generating different chemical reactions and conditions in different environmental niches (or compartments). This spatial diversity of chemical conditions may extend from a molecular to a macroscopic level and is generally organized into environmental *structures*. An obvious example is the assembly of soil horizons with various redox and acid–base properties; environmental systems include many other micro and macro structures. Finally, these different chemical conditions maintained in neighboring compartments by the external input of energy, dictate the *fluxes of chemical compounds* between the compartments, either small or large.

A key feature of environmental chemistry is to understand and predict fluxes of vital and detrimental compounds. Hence the description of the various physical structures and of their chemical constituents as well as their reactivity and environmental roles are key questions for investigation. This topic is dealt with in the first and the second volumes of this Series, namely *Environmental Particles*, Volumes 1 and 2, which describe colloids and biopolymers, in the nm to 100 μm range, their aggregation rates and structures, and their properties and environmental roles, in air, soils, waters and sediments. Two other volumes specifically on air and soil particles respectively are in preparation. Solid–water interfaces, colloids and colloid aggregates however are only a few examples of environmental structures. Other very important examples are sediment–water interfaces in lakes, rivers or ocean, air–water interfaces in soils, lakes and ocean, oxic–anoxic boundary layers in lakes or ground water, liquid–air interface in fog or rain droplets, soil porosity structure, etc. Not only is more information needed on these structures, but new analytical tools must be developed to study them in detail, without causing perturbation. A large number of recent techniques based on surface spectroscopies, electron microscopies, atomic force microscopies, light scattering, and many others should be adapted to the specific needs of environmental sciences, for the description of the most important structures relevant to the functioning of ecosystems.

The second major issue of environmental chemistry is the determination of fluxes of (bio)-active compounds. Long-term biological productivity and diversity in environmental systems depend on compound fluxes at least as much as on their concentration. Estimation of pollution impacts therefore should not be based solely, as it is done presently, on the analytical measurements made in the laboratory, of compound concentrations in homogenized samples. Determination and study of fluxes is quite complicated. They result from the coupling of chemical kinetics and physical diffusion processes, often linked to microbially catalyzed transformation processes. The understanding of their origin therefore requires quantitative integration of chemical, physical and biological concepts. In addition, it is very difficult to reproduce, under controlled laboratory conditions, fluxes representative of natural systems, and their experimental determination is complicated, both in the laboratory and *in situ*, because very steep gradients occurring over submillimeter ranges may affect ecosystems at the macroscale level. Development of specific analytical techniques for *in situ*, high resolution (submillimeter) concentration gradient measurements are hence closely linked to the improvement of our knowledge of environmental processes, and is a major challenge for analytical chemistry in general.

Another specificity of environmental chemistry is the important role of chemical heterogeneity. Sediments, soils and aquatic colloidal material include an extremely large number of components, all of which may contribute to a different extent to a particular chemical property (acid–base, redox, complexation) of the target environmental system. The net result is an efficient buffering of the corresponding intensity parameter value (pH, redox potential, free metal ion), which plays a key role in maintaining the stability of biological ecosystems. The chemical description of these intensity parameters on a rigorous basis, however, is quite complicated, since their value results from a weighted average of the contributions of all the constituents. In such cases, statistical approaches of chemical reactivity should be developed. Again, this physical chemistry problem is closely linked to an analytical challenge: indeed the analytical tool necessary to determine environmentally meaningful intensity parameters based on well-defined weighting averages has yet to be developed.

A further important aspect is mathematical modeling. Understanding specific environmentally relevant physico-chemical reactions at the molecular or microscopic level, would not be useful, if it could not be coupled to other physical or biological processes. This is crucial in evaluating the overall behavior of the ecosystem of interest, in making impact predictions, and in particular in evaluating the relative importance of the chemical processes. This is only feasible by means of numerical models. It is therefore of utmost importance that submodels, including detailed descriptions of the most relevant physico-chemical processes, be integrated into general macroscopic models of ecosystem behavior. This is highly lacking at present.

The above discussion points out that the development of general predictive models enabling a correct evaluation of man's activities, requires detailed biophysicochemical studies, in particular in relation to structures and fluxes. Unfortunately, the traditional method for training chemistry students is still largely based on studies of homogeneous systems. Hence, students are not familiar with the techniques and concepts required for understanding physically heterogeneous systems including processes encountered in other disciplines. This may be one of the reasons why environmental quality control is still largely based on determination of total concentrations of compounds in homogenized samples, while speciation and fluxes would be key parameters for pollution evaluation.

The main purpose of this series is to make chemists and other scientists aware of the most important bio-physico-chemical processes which influence the behavior of environmental systems, in terms of sound quantitative theoretical concepts. The various volumes of this series emphasize processes which are specifically related to environmental systems, and which are therefore often unfamiliar to chemists with a main background in homogeneous reactions in solutions. The second major issue of this series is to discuss in parallel (either in the same book or in separate books), (i) the important bio-physico-chemical processes and structures discussed above, and (ii) the analytical tools which exist or should be developed to study them. As pointed out earlier, there is indeed a great need for methods developed specifically for this field in close connection with the corresponding process studies. We feel therefore that inclusion in the same series of both analytical aspects and process studies is essential. The development of specific analytical tools for molecular biology, biophysics, biochemistry, and physiology has been done in parallel with the understanding of cell and organism functioning, and this has enabled great progress in this field. Similar tools are badly needed in environmental chemistry. It is hoped that this series will help promoting this development, for deepening our knowledge in environmental biophysical chemistry.

We would like to acknowledge here, the strong and constant support of Prof. G. den Boef, Secretary General of IUPAC. His encouragement has been very much appreciated for the creation of the Commission of Environmental Analytical Chemistry and of the present IUPAC Series, as well as on several other occasions. We also would like to thank Prof. A Hulanicki, President of the Analytical Chemistry Division, and Dr M. Williams, executive secretary of IUPAC, for their warm support and help with administrative matters. Thanks are due to the International Council of Scientific Unions (ICSU) for financial support of the work of the Commission, within the frame of the program 'Influence of multifold chemical interactions in ecosystems'.

J. Buffle and H. P. van Leeuwen
Series Editors

Preface

Metal contamination of aquatic ecosystems is a matter of concern to governmental authorities because it is widespread and because many metals are persistent and potentially deleterious to aquatic life. It is probable that our industrialized civilization will continue to produce metal wastes, perhaps even at increasing rates. Remediation of contaminated sites or reduction in waste disposal are possible but they are costly; the justification of such measures, on the basis of present environmental quality criteria, is usually difficult. It is clear that the development of rational, effective, and economical strategies to solve the problems of contaminated environments will depend greatly on our ability to predict how remedial actions will improve environmental quality and how these changed conditions will affect aquatic organisms. The formulation of such predictive models presupposes an understanding of the geochemical and biological processes involved in trace metal bioaccumulation and in the manifestation of their toxic effects.

Substantial progress has been achieved over the past 20 years in our understanding of metal interactions with aquatic organisms. In early studies designed to measure trace metal toxicity, solution chemistry was generally overlooked; oversaturated solutions, uncontrolled complexation of trace metals, pH variations during the experiments, adsorption and contamination by the labware, etc., were more the rule than the exception. Recent work has stressed the importance of controlling chemical speciation in the exposure medium external to the organism, i.e. the actual partitioning of the trace metal among various ligands present. Bioassays performed in carefully controlled systems have revealed the central role played by the free-metal ion activity, and this has led to the formulation of what is known now as the free-ion activity model. This awakening to the crucial role of solution chemistry gave rise to a strong interest in the development and use both of thermodynamic models to calculate the speciation of trace metals and of analytical methods for speciation measurements. In parallel to these noteworthy chemical developments, important progress has also been achieved in our knowledge of metal detoxification mechanisms, the importance of food as a route of trace metal uptake, the transfer of metals along food chains, and the role of various biological factors in metal accumulation in organisms.

It is perhaps trite to point out that the study of the interactions of trace metals with aquatic organisms requires interdisciplinary approaches. An

appreciation of the importance of the biological, biochemical, geochemical and chemical aspects involved is a prerequisite to studying trace metal–organism interactions; none of these aspects can be ignored. The main purpose of this book was therefore to bring together specialists from various disciplines to cover, in a comprehensive and critical manner, various aspects of trace metal–organism interactions. It was our intent to identify some of the assumptions and shortcoming of past research, and thus to provide some suggestions for future research.

The chapters of the book are organized in a rational fashion, so that the reader progresses from fundamental concepts and models to speciation measurements and field applications. Organisms are in contact with trace metals through their cell wall and plasma membranes. Accumulation of metals or the expression of their toxicity starts with their reaction at sites on the membrane and with their transport across the plasma membrane; the mechanisms of these processes are discussed in Chapter 1. The free-ion activity model is often used to describe the uptake of trace metals by aquatic organisms and the manifestation of their toxic effects. Chapter 2 describes this model and summarizes the available literature supporting it for a variety of aquatic organisms; most of the apparent exceptions to the free-ion activity model are also rationalized, based on the nature of the dissolved ligands and on the metal species formed at physiologically active sites at the plasma membrane. Chapter 3 focuses on organometals which, as a class, do not conform to the free-ion activity model.

From Chapters 2 and 3 it is obvious that the effects of trace metals on aquatic organisms (accumulation, nutrition, toxicity) depend on metal speciation in the external environment. Chapter 4 considers the chemical complexity of natural waters and reviews the current status of the thermodynamic approaches to trace metal speciation, i.e. the calculation of the partitioning of trace metals on the basis of their total concentrations and the chemical composition of the medium. Three chapters then describe electrochemical (Chapter 5) and non-electrochemical (Chapter 6) methods for measuring labile chemical species and chromatographic methods (Chapter 7) for measuring non-labile species.

Animals can potentially obtain trace metals from ingestion of food as well as from water; Chapter 8 examines the transfer of trace metals in food webs and its implications for trace metal cycling. In relating metal concentrations in organisms to environmental levels, it is necessary to take into account biological factors that contribute to the variability of trace metal concentrations in organisms at a given site; these aspects are critically reviewed in Chapter 9. Once trace metals enter the intracellular environment, they encounter a variety of ligands and binding of the metal to some of these ligands may perturb the normal cell metabolism. Chapter 10 discusses the mechanisms of metal toxicity and the detoxification strategies that organisms

have developed to prevent metal toxicity; it also stresses the analytical problems associated with the intracellular metal speciation studies.

Many types of bioassays, ranging in complexity from individual organisms exposed to a single trace metal in a beaker to manipulations of a whole lake ecosystem, have been used to study trace metal–organism interactions. Chapter 11 shows that bioassays have been very useful tools for understanding fundamental aspects of trace metal toxicity, but that extrapolation from laboratory experiments to nature is difficult. It also addresses the limitations of bioassay techniques that are often used as a basis for management decisions, for assessing damage to the natural environment and for predicting how changed environmental conditions will affect the ecosystem.

We gratefully acknowledge the cooperation of the various authors which has greatly facilitated the preparation of this book. We are indebted to the various copyright holders who kindly granted leave to use Tables and Figures. A financial contribution from the International Council of Scientific Unions (ICSU) is gratefully acknowledged. It is our sincere hope that the critical synthesis of available information presented in this book will facilitate the study and eventual mitigation of the effects of trace metals in aquatic ecosystems.

A. Tessier
D. R. Turner

1 Transport of Metals Across Membranes

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Metal Speciation and Bioavailability in Aquatic Systems. Edited by A. Tessier and D. R. Turner

1 INTRODUCTION

All living cells are delimited from their environment by a selectively permeable membrane. The existence and basic properties of this barrier were determined over 100 years ago by tearing it with needles, exposing it to dyes, and subjecting it to osmotic stresses so that its presence was generally accepted, even though it could not be seen until the electron microscope was invented. Two crucial sets of experiments established the physical basis of this membrane. It was shown, initially by Overton,[1] and subsequently by other groups,[2,3] that the permeability of this membrane was a function of the oil/water partition coefficient of the permeant material (Figure 1). Gortner and Grendel[4] later demonstrated that the lipid content of the erythrocyte membrane was sufficient to form a monolayer with ro[illegible] twice the surface area of the cell itself. This suggested that the cell membrane was a bimolecular layer composed of lipids which functioned as amphipathic molecules, with their hydrophilic ends pointing out and their hydrophobic chains forming the center of the sheet. The membrane is typically 4 to 10 nm thick. This conceptual model has formed the basis for most of the subsequent ideas of membrane structure, from the rigid, sandwich form of Danielli and Davson[5] to the fluid mosaic model of Singer and Nicolson.[6] If, however, a lipid bilayer of the type shown in Figure 2 is constructed in the laboratory it is virtually impermeable to small ions such as Na^+, K^+, Cl^-, etc. (Table 1), in contrast to their permeability in cell membranes.[7] Clearly, the properties of ion permeability are not a simple reflection of the general structure of this plasma membrane.

Permeation is generally attributed to the presence of a variety of proteins that occur as important components of these cell membranes. The proportions of protein to lipid vary on a weight basis from roughly 1 : 4 (bovine myelin) to 4 : 1 (myxoviruses) in different membranes. On a molecular level this may represent 50 lipid molecules to 1 protein molecule in a plasma membrane whose mass is 50% protein. Some of these proteins are relatively easy to remove, i.e., they can be extracted with a salt solution and are probably surface bound (extrinsic or peripheral proteins), whereas others are extremely difficult to extract and are released only by disruption of the bilayer via detergents or organic solvents (intrinsic or integral proteins). Freeze fracture experiments show the extrinsic proteins to be relatively superficial while the integral

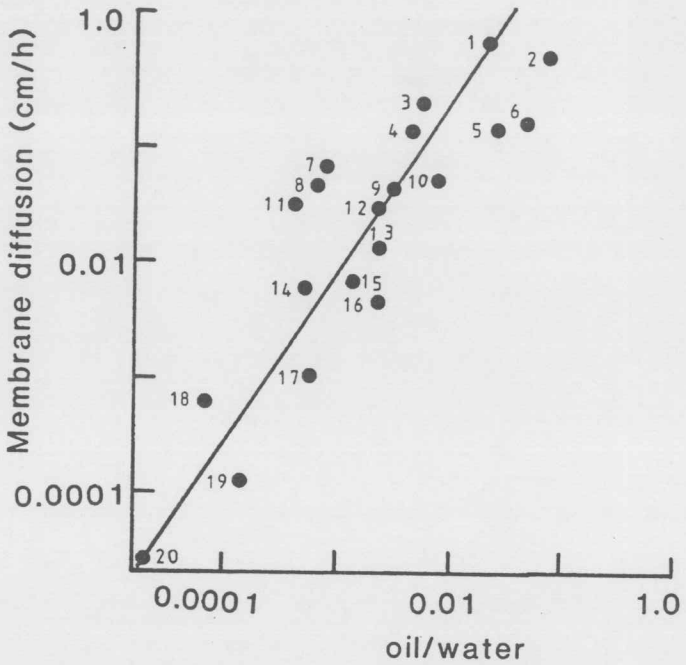


Figure 1. The permeability of a range of organic molecules showing how the oil/water partition coefficient relates to their rate of diffusion through the cell membrane: 1, ethyl alcohol; 2, urethane; 3, cyanamide; 4, propionamide; 5, antipyrin; 6, valeramide; 7, formamide; 8, acetamide; 9, glycerol methyl ether; 10, diethylurea; 11, glycol; 12, dimethylurea; 13, ethylurea; 14, methyl urea; 15, thiourea; 16, dimethyl malonamide; 17, dicyanodiamide; 18, glycerol; 19, malonamide; 20, erythritol. (Modified from Collander, R., *Trans. Faraday Soc.*, **33**, 985, 1937 and from Collander, R., and Bärlund, H., *Acta. Bot. Fenn.*, **11**, 1, 1993)

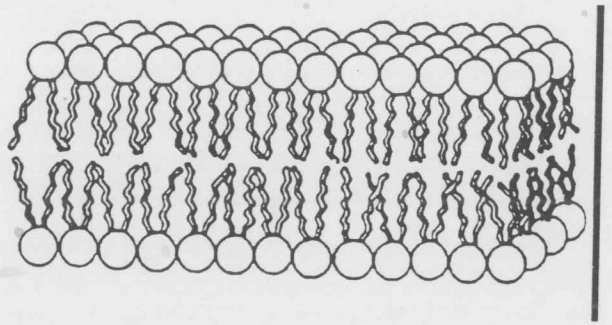


Figure 2. Packing of the hydrocarbon chains in a phospholipid bilayer as in an artificial membrane

Table 1. Membrane permeability to Na^+, K^+, and Cl^-: permeability coefficient ($cm\ s^{-1}$)

Membrane	K^+	Na^+	Cl^-
Phosphatidylcholine	3.4×10^{-12}	—	$10^{-9}-10^{-12}$
Phosphatidylinositol	2.7×10^{-11}	—	$10^{-9}-10^{-12}$
Human erythrocyte	2.4×10^{-10}	—	2.0×10^{-4}
Cat erythrocyte	3.4×10^{-9}	—	—
Squid axon	5.6×10^{-7}	1.5×10^{-8}	1.0×10^{-8}
Frog sartorius	1.6×10^{-7}	1.4×10^{-7}	9.5×10^{-8}

Modified from Reeves, J. P. in *Biological Membranes* (Boston: Little, Brown, 1969).

proteins are firmly embedded in the membrane and pass right through it. Integral proteins often contain a high proportion of hydrophobic residues which provide an interface with the lipid phase. This is in keeping with their penetration through the bilayer. The transport of materials across cell membranes is basically a phenomenon associated with their competitive binding to the various ligands in the membrane. Thus, the structure of the cell membrane is largely determined by the lipids while its functions are determined mainly by the protein content. The lipid and protein components of the cell membrane are described here in some detail both in chemical and structural terms.

2 LIPID COMPONENTS

2.1 CHEMICAL

There are a variety of ways of classifying membrane lipids, e.g. as glycolipids and phospholipids, charged or neutral lipids, and esters or nonesters, but we follow the classification of Kotyk *et al.*[8] This is a structure-based system which divides membrane lipids into derivatives of glycerol (glycerolipids), aminoethanol (sphingolipids), and sterols.

Glycerolipids are esters based on the general formula

$$\begin{array}{l} CH_2OR^1 \\ | \\ CHOR^2 \\ | \\ CH_2OR^3 \end{array}$$

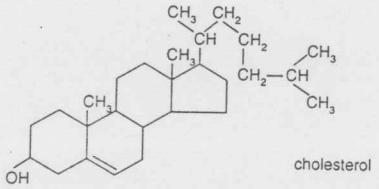
where R is derived from one, two, or three fatty acids, R^1 and R^2 may be acyl groups, and R^3 phosphate which may be substituted further to form a variety of phospholipids.

Sphingolipids are derived from 1-amino-2-hydroxyethane

$$\begin{array}{l} CH_2OR^3 \\ | \\ CHR^1 \\ | \\ NHR^2 \end{array}$$

where R^1 can be one of a number of sphingosines and R^2 is an acyl or α-hydroxyacyl group of 16 to 26 atoms. A common sphingolipid is sphingomyelin, the phosphorylcholine derivative. The substitution of R^3 as a sugar produces cerebrosides (glucosyl or galactosyl) or gangliosides (oligosaccharides). The sugars contained in gangliosides include glucosamines, galactosamines, and sialic acid. These two types of glycolipids are on the outside of the membrane only and are probably important recognition sites.

Sterols differ from other lipid components in both size and shape. The best known examples are cholesterol (animal membranes), ergosterol (lower eukaryotes), and β-sitosterol (plants).



cholesterol

Cholesterol can stabilize the membrane structure and modify its permeability.[9] There are large variations in the composition of membranes between organelles, tissues, and species of organisms (Table 2).

2.2 STRUCTURAL

Membranes are asymmetric structures such that the composition of the two faces differs according to the functions at each surface. A large number of techniques including X-ray diffraction, fluorescent probe, and rotational diffusion measurements have confirmed the fluidity of the plasma membrane. Movements of lipids within the two-dimensional plane of the membrane are relatively rapid, with a typical diffusion coefficient of $10^{-8}\,cm^2\,s^{-1}$.[10] Movements of lipids across the thickness of the membrane from one monolayer to the other are relatively rare. A number of factors influence these rates, of which temperature is the best understood. Frequently, the homeoviscous nature of the cell membrane is modified so as to reduce the effects of such changes. Thus, falling temperatures can induce modifications of acyl chain composition, alterations in phospholipid components, and a modulation of the phospholipid cholesterol ratio,[11] all of which maintain

Table 2. Lipid composition of selected membranes (in mol %)

Type of membrane	PC	PE	PS	PI	PG	CL	Glycoglycerolipids	Sphingolipids	Sterols	Others
Sarcina lutea	—	—	—	8	90	1	—	—	—	1
Micrococcus roseus	—	—	—	—	66	32	—	—	—	2
Salmonella typhimurium										
Cytoplasmic	—	60	—	—	32	7	—	—	—	1
Outer	—	81	—	—	15	2	—	—	—	2
Bacillus subtilis	—	—	—	—	78	3	—	—	—	19
Anacystis nidulans	—	—	—	—	14	—	70	—	—	16
Saccharomyces cerevisiae										
Plasma membrane	27	10	14		—	—	—	—	40	9
Tonoplast	33	15	43							
Rat hepatocyte										
Plasma membrane	28	17	6	6	—	1	—	12	27	3
Smooth endoplasmic reticulum	41	16	5	10	—	1	—	7	17	3
Erythrocyte										
Rat	30	13	7	2	—	—	2	16	25	5
Pig	15	18	11	1	—	—	10	18	27	—
Lobster nerve plasma membrane	29	18	8	—	—	—	—	9	36	—
Rabbit muscle plasma membrane	23	9	1	—	—	—	—	4	57	6
Human myelin sheath	11	14	5	1	—	—	—	32	25	12
Inner mitochondrial membrane										
Rat liver	40	35	1	5	2	17	—	1	1	1
Cauliflower	37	34	—	4	3	13	—	—	5	1
Baker's yeast	36	29	2	8	1	19	—	—	1	4
Outer mitochondrial membrane										
Rat liver	46	21	1	10	2	5	—	2	10	3
Cauliflower	36	21	—	17	8	2	—	—	12	4
Baker's yeast	43	22	3	20	1	6	—	—	1	4
Spinach chloroplast										
Outer membrane	22	—	—	1	9	—	68	—	—	—
Inner membrane	6	—	—	1	8	—	79	—	—	6
Thylakoid	5	—	—	1	9	—	78	—	—	7

Note: PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PG = phosphatidylglycerol; CL = cardiolipin.
From Kotyk, A. *et al.*, *Biophysical Chemistry of Membrane Functions* (Chichester: Wiley, 1988) With permission.

Figure 3. The various ways that phospholipids may be asymmetrically oriented in the membrane: 1, spontaneous flip-flop; 2, phospholipid flippase; 3, active transport by a translocase; 4, asymmetrical synthesis; and 5, asymmetry due to charges on an adjacent protein. (Modified from Zachowski, A., and Devaux, P. F., *Experientia*, **46**, 644, 1990.)

membrane fluidity. It might be expected, therefore, that because the cell membrane is basically a fluid system it would be uniform in its composition. This, however, is conspicuously not the case. The accumulation of proteins at certain sites, such as intercellular junctions and attachments to the cytoskeletal proteins, clearly impede lipid diffusion. There are, however, even more fundamental factors that influence the movement of lipids in cell membranes. Thus, lipids appear to be synthesized and inserted into the membrane from the cytoplasmic face, and it is only through the action of special enzymes (flip flop) that they appear on the noncytoplasmic face (Figure 3).[12] In addition, certain regions of the cell membrane, the so-called domains, may be richer in some lipid species than others because of mutual solubility. Thus, for example, cholesterol molecules appear to associate preferentially with particular lipid species,[13] while certain integral proteins are surrounded by a layer of relatively immobilized lipids to form the so-called annulus layer.[14] Removal of this boundary layer of lipids severely modifies the activity of many membrane proteins.[15]

2.3 ELECTRICAL

Phospholipids such as phosphatidylserine contain ionizable groups which can create regions on the membrane surface with a net negative charge. This is especially important on the cytoplasmic surface of the cell membrane. Although the surface charge density is defined as the number of electric charges per unit area, it is usually expressed as a unit charge over a stated area. Therefore, if 20% of the lipids carry a negative charge over a mean surface area of 0.6 nm^2 the surface charge density σ is one electron charge per 3 nm^2. According to the Gouy-Chapman theory this charge density gives rise to a membrane surface potential and a transmembrane potential which depend on the ionic strength of the electrolytes in the external or internal aqueous medium and the charge on the cations, but is independent of the nature of the cations.[16,17] The membrane potential ψ_s can be calculated from

$$\sigma = \{2\varepsilon_o \varepsilon RT \Sigma_i c_i [\exp(-z_i \Psi_s F/RT) - 1]\}^{0.5} \quad (1)$$

where σ denotes the surface charge density, ε is the relative permittivity of the medium, ε_o is the vacuum permittivity, and c_i and z_i are the concentration and charge on species i, respectively (see Chapter 4). Therefore, for a lipid membrane with a surface charge density of one electron charge per 2 nm^2, the surface potential at an electrolyte concentration of 100 mmol dm^{-3} is -60 mV. The cations in the electrolyte will be attracted to the negatively charged surface and the anions repelled. An equilibrium will be set up between the coulombic forces of the electric field and thermal diffusion away from the region. The region is referred to as the diffuse electrical double layer and the electric field decays exponentially to a distance, the Debye length, which is approximately 1 nm from the membrane at physiologically relevant electrolyte concentrations. The concentration of cations in the diffuse double layer leads to the screening of the surface negative charge and a lowering of the surface potential. Polyvalent cations are more efficient in this screening process than are monovalent cations. The size of the cation will determine the closest approach to the membrane. In biological membranes there appear also to be specific binding sites on the membrane, and cations are adsorbed, which once more leads to a decrease in the surface potential. Stern included these additional effects into the Gouy-Chapman-Stern theory (Figure 4). The implications of these theories are important in the approach of cations at the membrane surface, the selectivity of channels in the membrane, and the transport of cations through the membrane and are discussed in Section 3.2.[18]

3 PROTEIN COMPONENTS

Many membrane proteins are held in the lipid bilayer by hydrophobic interactions. Because of this the polypeptide chains are thought to be arranged mainly as α helices or β sheets, with polar groups tending to bond internally. The degree and type of protein interaction with a given lipid membrane is determined, therefore, partly by its amino acid composition and partly by its primary and secondary structure. The degree of hydrophobicity of individual amino acids and their arrangement in the secondary structure enable good predictions to be made about the position of a protein in the membrane. Membrane proteins, particularly peripheral proteins, can move within the fluid mosaic membrane, with diffusion coefficients of around 10^{-10} cm^2 s^{-1}, i.e., roughly 1% of the typical values of lipids,[13] depending of course upon protein size. In addition, these proteins usually show vibrational movements which may be important in finding and binding to a variety of ligands.

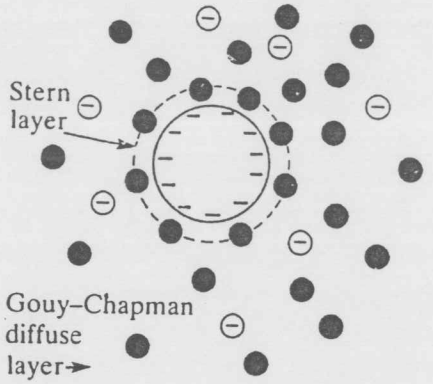


Figure 4. The distribution of ions around a charged particle showing the tightly bound Stern layer and the more loosely attached Gouy-Chapman region. Cations shown as solid spheres

3.1 PERIPHERAL PROTEINS

For the purpose of this review the best example of a peripheral protein is the transferrin receptor, although it actually straddles the membrane because of a localizing tail. The receptor binds to the protein transferrin, which itself binds to iron, and plays a key role in the transfer of this essential element into the cell cytoplasm.

3.1.1 Transferrin

The transferrins are a class of iron-binding molecules that are widely distributed among the vertebrates. They appear to have evolved as a means of transporting Fe^{3+} ions, which form extremely insoluble compounds under normal physiological conditions. Transferrin is a glycoprotein with a relative molar mass of about 80 000 Da and contains two high affinity binding sites (Figure 5). Plasma transferrin is involved in transporting iron from the alimentary tract to the tissues of the body, but its extremely high binding affinity (10^{22} dm^3 mol^{-1}) means that it can also outcompete many microorganisms for iron,[19] and it therefore also acts as a bacteriostat.[20] One of the characteristic properties of transferrin is its association with carbonate, and in the absence of these ions the iron transport system is inhibited. It appears that the anion acts as a bridging ligand between the protein and the iron, and in effect locks the metal in place by making a coordination site on the iron atom unavailable to water.[21] At the same time three protons are released for each iron bound and there is a change in shape of the transferrin molecule. The two iron-binding sites on transferrin are similar but not identical and

function independently. In the absence of iron transferrin is capable of binding aluminum, chromium, cobalt, copper, manganese, and vanadium as well as plutonium and europium ions *in vitro* and may be important in the *in vivo* uptake of these and similar ions.

3.1.2 Transferrin Receptors

The transferrin receptor is a disulfide-linked peripheral glycoprotein with a relative molar mass of approximately 180 000 Da. The molecule has two hydrophilic ends that extend into the extracellular fluid and a central hydrophobic region that is embedded in the membrane.[21] To that extent it is a typical peripheral protein, but it also has a tail that extends through the membrane and exits with a hydrophilic region with a phosphate-bound serine group (Figure 5).

The transferrin receptor binds to diferric transferrin with a dissociation constant of 5×10^{-9} mol dm^{-3}, but binds somewhat less tightly for the monoferric form. By binding the transferrin molecule to the cell membrane the receptor molecule clearly obtains access to iron atoms, but they must still cross the barrier of the cell membrane. This is achieved by the phenomenon of internalization by clathrin-coated pits.[22]

3.1.3 Clathrin-coated Pits and Endosomes

The 'iron transferrin–transferrin receptor complex' on the outside of the fluid mosaic membrane diffuses over the surface until it comes to a specialized coated pit region. The protruding tail region of the receptor may be involved in the accumulation of receptors at this site, where they are joined by another protein, clathrin. This is a three-legged molecule which is able to assemble into a lattice structure to form a membrane vesicle that buds off from the cell membrane (Figure 6).[23] This process of receptor-mediated endocytosis produces a fluid-containing sac that is membrane bound, with a clathrin lattice on the outside and transferrin receptors on the inside. The sac fuses with another vesicle to form an endosome, which is capable of pumping protons into its contents. The resulting acidification releases iron from its association with transferrin, probably via an effect on the carbonate anion and protonation of the binding sites. The iron that is released enters the cytoplasm while the transferrin, transferrin receptor, and clathrin molecules are recycled to the cell surface. In cells with a rapid iron metabolism there may be up to 800 000 receptors per cell, of which, at any one time, 60% are in the process of being recycled.[24] Normally, transferrin molecules are between 20 and 35% saturated with iron. Receptor molecules bind to all forms of transferrin, but because the di- and monoferric forms bind more strongly they are preferentially absorbed.[21] Although receptor-mediated endocytosis seems elaborate, it

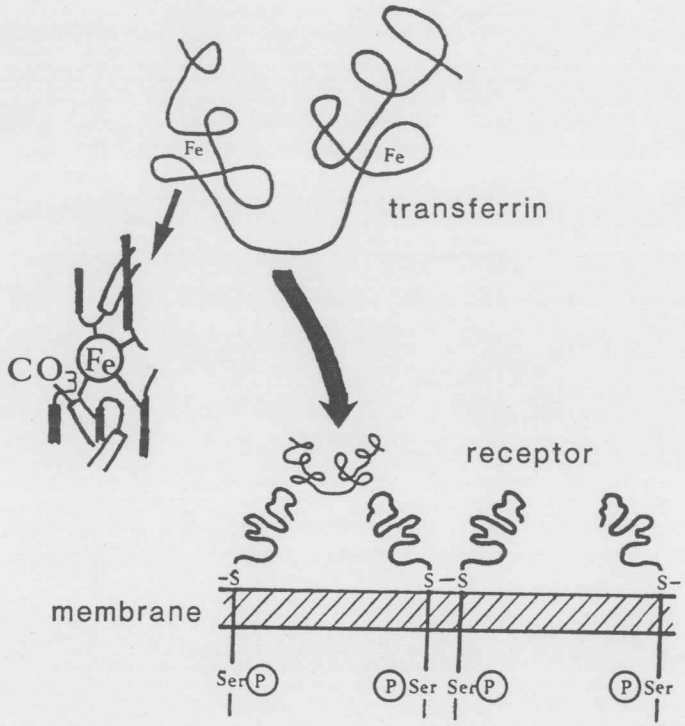


Figure 5. The transferrin molecule showing the carbonate associated with the iron atom (insert) and demonstrating how the molecule attaches to the membrane bound receptor. (Modified from Huebers, H. A., and Finch, C. A., *Physiol. Rev.*, **67**, 520, 1987.)

should be stressed that it is an extremely common process that is involved in the transport of a wide range of molecules across cell membranes. Because the receptors and the ligands that bind to them can be very specific in their binding properties receptor-mediated endocytosis can also be a very selective process.

3.1.4 Carrier Proteins, Ionophores, and Siderophores

Physiologists have tended classically to divide membrane transport systems into two types: carriers and pores. The distinction is either conceptual, in which a carrier is considered to be a binding site which is alternatively accessible to opposite sides of the membrane in a transient way, or it is kinetic, in which the distinction is made on the basis of selectivity, saturation kinetics, or stoichiometric coupling of transport molecules.[8,25] Neither set of criteria is satisfactory in isolating a specific process, but there is clearly a large set of peptides, cyclic molecules, and synthetic crowns that are capable of surrounding ions and carrying them across membranes. These compounds,

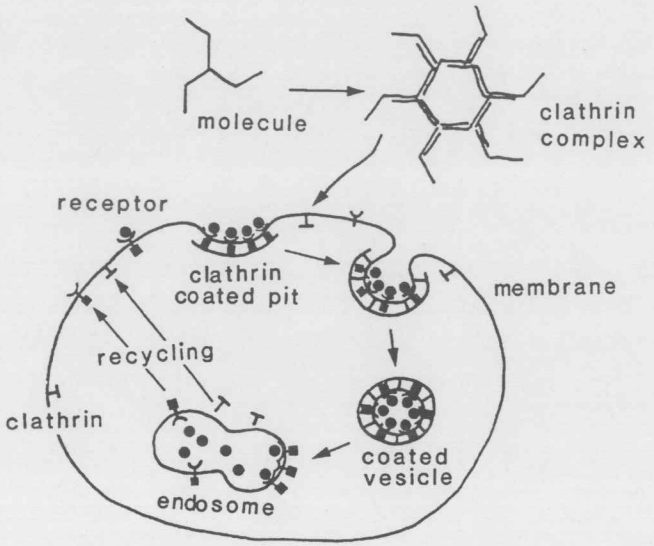


Figure 6. Clathrin molecules (top) are capable of forming a basket structure in which receptor molecules are internalized as coated vesicles at the cell surface. Endocytosis of these regions leads to enzyme and proton attack in the endosome and a recycling of the receptor and clathrin molecules. (Modified from Becker, W. M., and Deamer, D. W., *The World of the Cell*, Redwood, Ca: Benjamin Cummings, 1991.)

referred to collectively as ionophores, have been studied extensively in recent years. A number of ionophores are produced by microorganisms and often act as antibiotics, selectively enhancing the permeability of membranes to different cations. Depending on the ionophore they can transport cations by shuttling across the membrane or by forming a channel through the membrane.

The first discovered ionophore, valinomycin, selectively transports K^+ across membranes.[26] The neutral valinomycin binds cations by replacing the negative dipoles of water in the primary hydration shell of the cations with the negative dipoles of the backbone ester or ether oxygens in a stepwise, zipper-like mechanism. The cation selectivity is determined by size. The complex is soluble and stable in nonaqueous solvents and is thought to diffuse across the membrane. Its stability is lowered at the aqueous interface of membranes by interacting with water, which resolvates the cation. Some conformational fluctuations permit cations to enter and leave the cavities. Although it is no longer believed that macromolecules shuttle across membranes as carriers, small peptides may function in this way.

Microbial cells have developed specialized systems for the uptake of iron. In the environment iron occurs as insoluble hydrous ferric oxides, with a

solubility product, $K_s = 10^{-38}$, and is thus not readily available for assimilation by organisms. The microbes synthesize and export ligands called siderophores, which sequester the Fe^{3+} from the external environment.[27,28] Two important classes of siderophores exist, one based on hydroxamates such as the desferrioxamines, and the second based on catechols such as enterobactin. The mechanisms of this method of iron accumulation were studied most in Gram-negative bacteria such as *Escherichia coli*. The bacteria are surrounded by two membranes: an outer membrane and a cytoplasmic membrane, which is separated by the periplasmic space. Although transport of most small molecules through the outer membrane is through nonspecific porin protein channels, the ferri-siderophores bind to specific receptors on the outer membrane followed by an energy-dependent transport process into the periplasmic space. The periplasm consists of a rigid murein layer of peptidoglycan which stabilizes the shape of the bacterial cell. Transport of the ferri-siderophore across this space is believed to involve a specific periplasmic permease which then interacts with proteins on the cytoplasmic membrane. The mechanism of the translocation across the cytoplasmic membranes is not known, but the whole process from entry at the outer membrane to the cytoplasm is linked to the TonB and ExdB gene products. The Fe^{3+} is then released and reduced to Fe^{2+} by a ferri-siderophore reductase system.[29] Pathogenic organisms take up iron from eukaryotic cells via their host cognate iron chelates. Molybdenum can also be taken up as siderophore complexes in bacteria.[27]

3.2 INTRINSIC PROTEINS

Intrinsic proteins, which penetrate through the whole thickness of the cell membrane, provide the opportunity for the formation of a range of water-filled channels through which ions might permeate. In considering how such structures could be formed Singer[30] discussed the energetic advantages of forming the maximum number of C=O···HN hydrogen bonds and concluded that the interior domains of integral proteins may 'be largely in the α helix configuration'. The degree and type of interaction of individual amino acids with the lipid environment were calculated on a variety of hydrophobicity criteria[8] (Table 3), so that when the molecular sequences of intrinsic proteins became available, it was possible to suggest ways in which they might fit into the membrane. According to Singer[31] there are four types of topography (Figure 7) from single α helices with the amino end (type 1) or carboxyl end (type 2) on the external surface through to those where the polypeptide chain traverses the membrane several times (type 3) and where a central aqueous channel may be formed (type 4). In all cases the transmembrane sequence is thought to be formed of stretches of 15 to 25 amino acids uninterrupted by ionic residues.

Table 3. Properties of amino acids related to their insertion into the hydrophobic membrane core

Amino acid	A	B1	B2	B3	B4	C
Alanine	−1.75	−1.0	—	—	−1.0	1.56
Arginine	1.96	−3.7	7.5	7.5	11.3	0.45
Asparagine	2.97	−2.1	5.0	—	2.9	0.27
Aspartic acid	3.02	−1.9	5.0	4.3	7.4	0.14
Cysteine	−2.49	−1.5	—	—	−1.5	1.23
Glutamic acid	2.12	−2.9	5.0	3.8	5.9	0.23
Glutamine	2.49	−2.6	5.0	—	2.4	0.51
Glycine	0.00	0.0	—	—	0.0	0.62
Histidine	1.80	−3.0	5.0	1.4	3.4	0.29
Isoleucine	−6.68	−2.5	—	—	−2.5	1.67
Leucine	−7.00	−2.4	—	—	−2.4	2.93
Lysine	1.06	−3.1	2.5	4.8	4.2	0.15
Methionine	−0.599	−2.7	—	—	−2.7	2.96
Phenylalanine	−8.80	−3.4	—	—	−3.4	2.03
Proline	−2.28	−1.7	5.0	—	3.3	0.76
Serine	2.70	−1.0	2.5	—	1.5	0.81
Threonine	0.69	−1.6	2.5	—	0.9	0.91
Tryptophan	−6.31	−4.5	2.5	—	−2.0	1.08
Tyrosine	−5.57	−3.9	5.0	—	1.1	0.68
Valine	−4.77	−2.0	—	—	−2.0	1.14

Note: A, Free energy of transfer from water to *n*-octanol (kJ mol^{-1}); B, free energy transfer of a residue in an α-helix from water to an apolar solvent (kJ mol^{-1}); B1, hydrophobic contribution; B2, H-bond contribution; B3, charge contribution; B4, total; C, optimized preference parameter for submersion in membrane (in a linear relative scale).
From Kotyk, A. *et al. Biophysical Chemistry of Membrane Functions* (Chichester; Wiley, 1988). With permission.

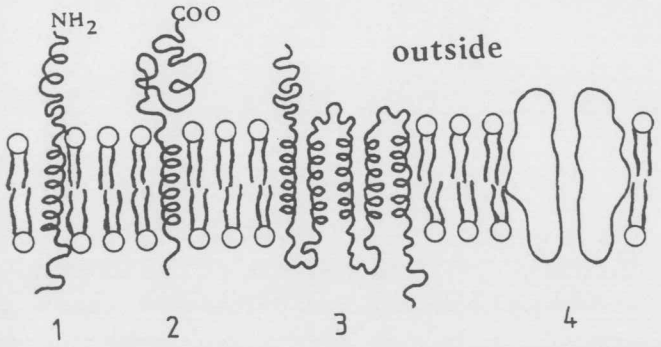


Figure 7. The four main types of integral proteins in membranes. Type 1 is amino terminal region to the outside, type 2 is carboxyl external, type 3 crosses the membrane several times, while type 4 is shown in cross-section to illustrate the presence of an aqueous transmembrane channel. (Modified from Singer, S. J., *Annu. Rev. Cell Biol.*, **6**, 247, 1990.)

The intrinsic protein, porin, which occurs in the outer membrane of mitochondria and many microorganisms, forms water-filled channels with a high permeability[32] to a wide range of molecules, with a relative molar mass of 600 Da. Different forms of the protein are found, some of which are weakly cation selective and others that are weakly anion selective. Characteristically, they are trimers of identical units which are resistant to dissociation by detergents. Each unit consists of a 16-strand antiparallel β barrel enclosing an aqueous pore.[33,34] X-ray crystallography of an osmoporin (OmpC) showed that the entrance to the pore is like a funnel that narrows at the mouth to an elliptical diameter of 1.1. and 1.9 nm, which is then constricted to 1.1×0.7 nm, but opens out abruptly just past the constriction to 2.2×1.5 nm at the smooth end. Thus, the initial screening with respect to charge and size occurs at the mouth end of the pore and any further selectivity occurs at the restriction zone, after which the molecule is released into the bulk solvent. The solutes are believed to pass through the pore in the hydrated form. The pores appear open most of the time.

3.2.1 Channel Proteins in Bacteria

Evidence demonstrates that membrane channels are formed from polypeptide chains that loop repeatedly back and forth across the lipid bilayer. The outer surface of these macromolecules are composed of hydrophobic residues that are stabilized within the lipid environment, while the inner surface provides an aqueous channel through which ions can permeate. It is easy to appreciate how such a system might function in ion transport, but it has been more difficult to imagine how such a complex molecule might evolve.[35] In discussing this problem an important principle has emerged. Thus, when many enzymes bind their ligands it is recognized that there is often an accompanying conformational change. What has tended to be overlooked is an associated change in the bound water, as most biological reactions involve hydrated species. It is therefore possible to envisage how such a molecule, which was electrostatically or hydrophobically associated with a membrane, could undergo a dramatic realignment during its catalytic activities, leading to the formation of a hydrated channel. With this as background it is possible to identify in bacteria a series of ion-permeating systems of increasing specificity that illustrate the general principles governing ion permeation in biological systems.

Most bacteria have a cell wall outside their plasma membrane and, in the so-called Gram-negative staining forms, there is a region, the periplasmic space between these two structures. All inorganic nutrients entering the bacterium pass across this structure, beginning with the outer cell wall which contains the porin protein channel.

As described above the screening of ions with respect to size and charge is initially done at the entrance to the porin channel, but permeating ions then

encounter a constriction zone where side chains from the walls of the channel determine the size limitation and ion selectivity of the pore.[34] This is relatively poor so that many small hydrophilic molecules are able to pass in a hydrated state through the porin channel into the periplasmic space. From there we find a much more selective system, and phosphate and potassium transport are considered as representative examples of these processes.

Phosphate ions that pass through the relatively unrestricting porin channel enter the periplasmic space of bacteria such as *E. coli*. Here, they meet a specific binding protein (PBP), which holds the anhydrous HPO_4^{2-} or $H_2PO_4^-$ ion in a deep cleft between two globular protein domains and carries it to two high affinity transporter molecules (PstA and PstC) and an adenosine triphosphate-(ATP) containing membrane protein (PstB), all of which are situated in the plasma membrane (Figure 8). This complex transports the phosphate across the membrane, apparently by using energy from ATP hydrolysis. This process is regulated by sensor-effector molecules situated in the membrane (PhoR) or cytoplasm (PhoB), which regulate the synthesis of the Pst phosphate transporter molecules.[29]

Potassium uptake by bacteria occurs via a similarly sophisticated system. Again, K^+ ions enter the periplasmic space through porin channels, but they then encounter two transport systems in the plasma membrane. One is a high V_{max} low affinity (high K_m) system (TrK) while the other is a low V_{max} high affinity (low K_m), positively regulated system (Kdp). Both systems depend upon forming phosphorylated intermediates using ATP; i.e., they are typical P type ATPases (Section 3.2.5). A considerable number of chromosomally based ion transport systems of this type are known in bacteria. They are usually

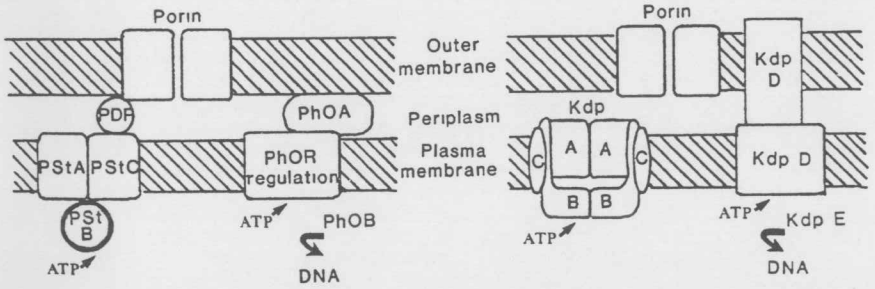


Figure 8. The outer membrane and periplasmic space outside the plasma membrane of a Gram-negative bacterium. The phosphate transport system (left) has a specific phosphate-binding protein (PDP) in the periplasm and a high affinity transporter system (PSt). The potassium uptake system (right) has two transport systems with different affinities. Both systems use ATP and probably have regulatory systems that influence the DNA. (Modified from Silver, S., and Walderhaug, M., *Microbiol. Rev.*, **56**, 195, 1992.)

involved with well-regulated ions such as phosphate and potassium. Other pumps such as the Cd^{2+}, Zn^{2+}, and Co^{2+} transporting systems are plasmid based (i.e., derived from nonchromosomal DNA), and often appear to act as toxic ion efflux systems. In most cases these ion transport systems are highly specific.

3.2.2 Channel Specificity

The basis of ion selectivity has attracted considerable interest because it appears to be a common aspect of most membrane transport systems. The problem is most clearly seen in the data of Diamond and Wright.[36] Taking the group I series of the periodic table (i.e., Li^+, Na^+, K^+, Rb^+, Cs^+) they compiled a table of the relative permeability of these ions across various membranes (Table 4). There are 120 possible sequences for these relative rates, but examples of only 11 such series could be found. Series 1 is easily explained because it is the sequence of nonhydrated ionic radii, whereas series 11 is the opposite, i.e., the sequence for hydrated ions (Table 4).

A more detailed explanation of these series was provided by Eisenman,[37] who was faced with similar results on the permeability of glasses to individual ions. By analyzing the energetics of the reaction between an ion in solution and its binding to a glass he was able to show that the relevant free energies were dominated by the electrostatic energy of attraction and the hydration energy of the ion. It was shown that the energy of interaction ΔG was inversely proportional to the sum of the radii of the binding site (r_e) and the cation (r_a).

$$\Delta G \propto \frac{1}{(r_e + r_a)} \tag{2}$$

Table 4. Series of alkali metal selectivity found in biological systems

Series		Example
1	$Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$	Black membrane with monoactin
2	$Rb^+ > Cs^+ > K^+ > Na^+ > Li^+$	Erythrocyte
3	$Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$	Gall bladder[a]
4	$K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$	Malpighian tubules
5	$K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$	Antibiotic nigericin
6	$K^+ > Na^+ > Rb^+ > Cs^+ > Li^+$	Blowfly salt receptor
7	$Na^+ > K^+ > Rb^+ > Cs^+ > Li^+$	Ionophore dianemycin
8	$Na^+ > K^+ > Rb^+ > Li^+ > Cs^+$	Squid axon[a]
9	$Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$	Squid action potential
10	$Na^+ > Li^+ > K^+ > Rb^+ > Cs^+$	Frog skin
11	$Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$	Cornea

[a]Incomplete series.
Modified from Diamond, J. M., and Wright, E. M., *Annu. Rev. Physiol.*, **31**, 581, 1969.