ORGANISATION OF PHOSPHOLIPIDS IN BIOMEMBRANES

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ABSTRACT

Phospholipids consist of a short polar group (α) and two comparatively long hydrocarbon chains (β and γ) connected to a glycerol residue. Molecular orbital calculations indicate that the possible conformations of phospholipids are highly restricted. When considering conformations relevant to structures in cell membranes, a further selection is possible because of the fact that in aqueous solutions hydrophobic interactions stabilise an arrangement where the β and γ chains are packed parallel to one another leading to a bilayer structure. Various models which satisfy these conditions have been compared and it has been found that only four are favoured by energy considerations. These arrangements differ from one another in the orientation of β and γ chains, close to its linkage with the glycerol group. Similarly, the polar group (α chain) can exist in four possible conformations. A low energy pathway connects these conformations and thus a phospholipid molecule can easily flip from one preferred conformer to the other.

The proposed model provide explanations to a number of dynamic and static properties of cell membranes in addition to a theoretical basis for lipid bilayers. More interesting however, is the fact that based on the conformational freedom of phospholipids, it is possible to postulate a model for Na^+ and K^+ channels and their passive movement across biomembranes.

Key words: Biomembranes, Phospholipids, Conformation.

1. INTRODUCTION

Biomembranes are the vital bioorganic systems which are responsible for some of the most important functions in living systems [1–4]. Existence of cytoplasmic membranes which act as boundaries of cells, forms the basic foundation of modern cellular biology. Cytoplasmic membranes are responsible for the biological organisation within the cell at the expense of disorganisation in their environment. However, the diversity and functions of biomembranes are much more widespread. They surround almost all the cell organelles like the cell nucleus, mitochondria, Golgi apparatus and also exist inside the cell as endoplasmic reticulum.

The cytoplasmic membranes act as selective gateways for transport of substances and thus control the osmotic and ionic balance between the interior of the cell (cytoplasm) and the exterior [5]. For example, the concentration of aminoacids inside the cell is almost ten times larger than that outside. Many of the chemically modified aminoacids are selectively left out during this transport. Within the cell, one finds a high K^+ concentration and a low Na^+. concentration while the extradellular medium has a much higher concentration of Na^+. Cytoplasmic membranes also selectively transport other metabolites such as sugars, sulphates, etc. A transport against the normalt hermodynamic behaviour is termed as active transport for which the energy is supplied by the hydrolysis of ATP.

The ionic gradient across the nerve cells plays a crucial role in the conduction of impulses. The difference of K^+ and Na^+ concentrations across the membranes gives rise to an electrochemical potential difference. The excitation of these cells occurs through a brief controlled movement of alkali ions caused by a highly cooperative alteration of membrane structure under the influence of stimulus [6].

The mitochondrial membrane system is the site of oxidative phosphorylation. During this process, the food molecules are oxidised and ATP it synthesised. Likewise, photosynthesis in plants take place at chloroplast membranes. Thus, the whole thermodynamics of living system is controlled by these two membrane systems [7].

To a certain extent biomembranes are self assembling systems. Organelles such as mitochondria and chloroplasts contain a protein synthesising system seperate from the classical one consisting of ribosomes and nucleus.

In this review, we have discussed some of the important physiochemical properties of biomembranes. The molecular organisation of one of the components—phospholipids has been considered in detail and the possible biological consequences of such an organisation have been pointed out.

2. CHEMICAL COMPOSITION OF BIOMEMBRANES

In recent years, it has become feasible to isolate membranes free of other cellular components and characterise them. It is now known that the major components of membranes are proteins and lipids. In mammalian cells, a small amount of carbohydrate is also present in the form of glycoprotein or glycolipid. The relative concentrations of lipid and protein depends on the type of membrane system and is closely linked with its biological function. The mitochondrial membranes, for example, contain a

fairly large amount of enzymes involved in ATP synthesis. Nerve cells (axon) on the other hand are largely composed of lipids.

The most familiar form of lipid in animal fat which is a triglyceride. The cell membranes however contain mostly phospholipids where only two of the hydroxyl groups in glycerol are esterified with fatty acid chains β and γ (Fig. 1) containing 15 to 18 carbon atoms. The β chain sometimes contains an unsaturated olefinic linkage. The third hydroxyl group of glycerol is esterified with a phosphoric acid derivative leading to compounds such as phsophatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), etc. The choline version usually called lecithin (Fig. 1) is the most common one and makes up more than 50% of the total phospholipid content of the cells.

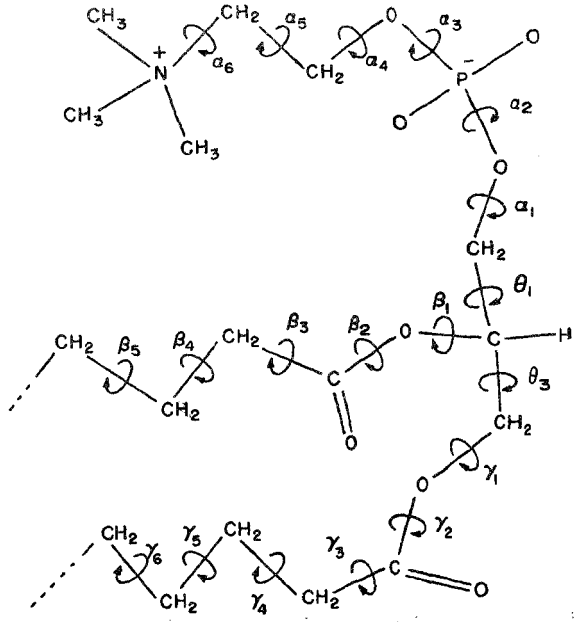

FIG. 1. A molecule of phosphatidyl choline (Lecithin) depicting the α, β and γ chains. The figure also shows the torsional angles which determine the geometry of the molecule.

3. MODELS OF BIOMEMBRANES

There are two important properties of phospholipids which distinguish them from other biomolecules such as proteins, nucleic acids or polysaccharides: lipids are amphiphatic and they exhibit fairly rapid intra and intermolecular motions when dissolved in water. Phospholipids have a large polar head consisting of the α-chain, and acylester groups of the β and γ chain which tend to dissolve in water while the nonpolar hydrocarbon chains try to organise in such a way so as to make minimum contact with water. A direct consequence of this is that phospholipid-water mixtures

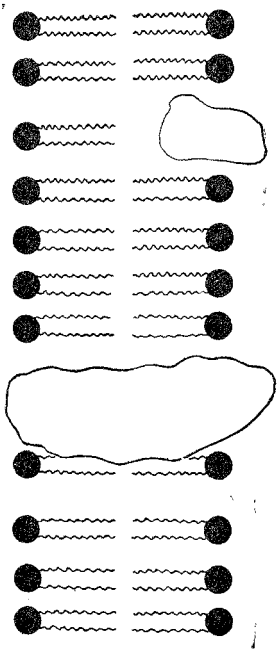

FIG. 2. Fluid mosaic model of biomembranes showing the extrinsic and intrinsic proteins embedded in a matrix of phospholipids.

show a large number of phases depending on the relative concentration of water. When water is present in excess, phospholipids acquire a laminar structure such that the hydrocarbon chains are aligned parallel to one another (Fig. 2) in the form of a bilayer with the polar heads submerged in water and the hydrophobic tails keeping very little contact with it. Such a bilayer structure is an important feature of phospholipids [8, 9]. X-ray diffraction studies of phospholipid bilayers reveal that the thickness of bilayer is around 50 Å and the cross sectional area near the polar head group is — 60 Å^2. The tails are aligned at an angle of about 62° with the water surface and the spacing between hydrocarbon chains is around 4 Å [9].

Since the molecular weight of phospholipids is in the vicinity of 800 while the proteins have molecular weights of few thousands, the latter are present as largely compact masses embedded in a matrix of phpsoholipid bilayers (Fig. 2). Basically, it has been possible to distinguish between proteins which are loosly bound on the surface of lipid bilayers (extrinsic proteins) and those which are deeply embedded in the lipid matrix (intrinsic proteins). The complete molecular architecture of biomembranes is stabilised by lipid-lipid, lipid-protein and protein-protein interactions. The extrinsic proteins can be removed by mild treatments such as high salt concentrations or chelating agents. The intrinisic proteins however require a much stronger treatment for seperation and when completely freed from lipids, these are usually highly insoluble. On the average, a considerable amount of protein fraction is present in the form of α-helical structure.

4. Dynamic Nature of Biomembranes

In the last few years, there has been a fundamental change in ideas on models of membrane organisation with the realisation that they have a highly fluid structure. The molecular motions have been investigated quantitatively, in pure lipid bilayers, model membranes and natural membrane system using ESR spin label technique and ^{1}H, ^{2}H and ^{13}C nuclear magnetic spin lattice relaxation times [9, 10]. Basically three types of molecular motions have been detected:

(i) An intramolecular chain motion involving rotations about single bonds in the α, β and γ chains of phospholipids. Experimental evidences indicate that the motion is more restricted near the glycerol backbone and the freedom increases as one moves towards the terminal groups [9].

(ii) The lateral diffusion of lipids and macromolecules on each side of the lipid bilayer. Again such a diffusion is fairly fast [10].

(iii) The rate of exchange of phospholipids between the two surfaces of bilayers (flip-flop exchange) is relatively slow. The diffusion rates for flop-flip are almost 10^{10} times slower than that for lateral diffusion [10].

One of the most significant observation is that there is a 1:1 correspondence between the degree of fluidity of biomembranes and the rate of transport of ions or molecules across it [10]. Thus, the transport properties are directly linked with their fluidity.

A direct consequence of the fluidity of biomembranes is that it severely limits the application of electron microscopy and X-ray diffraction to the study of molecular organisation of such systems. In particular, X-ray diffraction on oriented multilayers of membranes yields only a one dimensional electron density diagram which consist of contributions both from lipids and proteins. It is therefore of some importance to investigate the detailed structure of the molecules forming the matrix, before an understanding of the structure of biomembranes can be reached.

The phospholipid bilayers exhibit a characteristic temperature (called the freezing point) at which the liquid crystalline bilayers go into a rigid structure with the 'freezing' of molecular mentions discussed above.

5. POSSIBLE CONFORMATIONS OF PHOSPHOLIPIDS IN BIOMEMBRANES

In recent years, there have been several attempts to understand the conformational structure of phospholipids. Basically, three types of approaches have been followed:

(i) Single crystal X-ray diffraction studies of the constituents of phospholipids. The three dimensional structures of several molecules relevant to this discussion have been determined and the data has been recently discussed by Sundaralingam [11].

(ii) Three bond (*vicinal*) NMR coupling constants have been measured in lecithin [12] and throw valuable light on the conformational structure of this molecule.

(iii) Energy minimisation techniques using both classical potential functions—CPF [13, 14] and molecular orbital (MO) theories like EHT and CNDO [15, 16] enable one to predict the stable conformers of phospholipids. We shall discuss below the results based on the three techniques.

For the purpose of discussion, it is convenient to lable the various forsional angles in α, β and γ chains by $\alpha_1, \alpha_2 \ldots \alpha_n$, $\beta_1, \beta_2 \ldots \beta_n$, $\gamma_1, \gamma_2 \ldots \gamma_n$ with the suffix index increasing as one moves away from the glycerol moiety (Fig. 1). The relative orientation of the α, β and γ chains is fixed by the angles θ_1 and θ_3 which determine the gemometry of the glycerol group. The sense of rotation and convention used in measuring various torsional angles is shown through Newman projection diagrams in Fig. 3. This notation and nomenclature is identical with the one suggested by Sundaralingam [11].

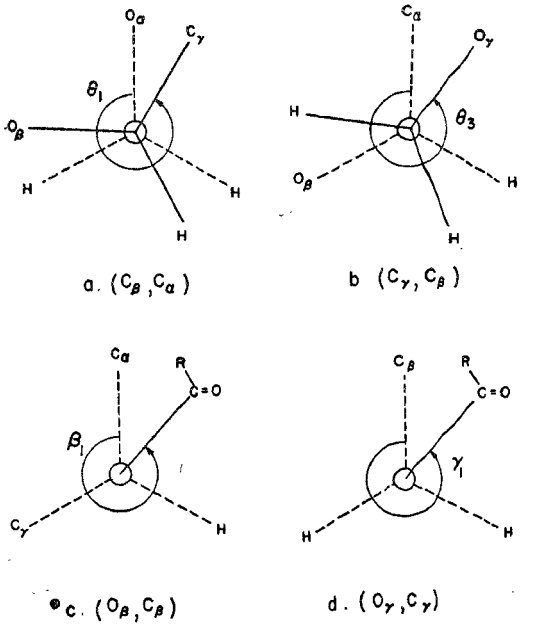

FIG. 3. Newman projection diagrams illustrating the convention followed in measuring some of the torsional angles in phospholipids.

The structure of phospholipid consists predominantly of what may formally be called single bonds. In a classical sense, one may think of three stable conformations corresponding to the torsional angles of 60° (*gauche*), 180° (*trans*) *and* 300° (*gauche'*). With these fully staggered arrangements, the possible rotamers of a molecule like phospholipid become very large when the possibility of rotations about all the bonds is considered. MO calculations rule out many of these possibilities and in some cases predict torsional angles different from those corresponding to fully staggered arrangements. Another selection criterion can be used when models pertinent to biological membranes are considered. The unfavourable entropy change which results from the reordering of the water molecules around nonpolar hydrocarbon chains, causes the fatty acid chains to aggregate together with the total exclusion of water from their vicinity.

The number of possible models for phospholipids become surprisingly small, when the criterion of bilayer lipid structure is coupled with the energy calculations. On the basis of MO calculations, Gupta, Govil and Mishra [16] have predicted four possible arrangements for β and γ chains which are characterised by torsional angles listed in Table I. In models A and B the two hydrocarbon chains β and γ are found to be completely parallel to one another, Model A has a θ_3 angle of 180°, β_1 of 100° and γ_3 of 280°, while the corresponding angles for model B are 60°, 150° and 80° respectively. The value of β_3 is 280° in both the models and the remaining torsional angles are 180°. The major part of the long hydrocarbon chains is characterised by a zig-zag (all trans) conformation as is the case in chains of polythene. The potential energy curve with respect to β_1, (Fig. 4) shows in interesting behaviour. Both CNDO and EHT calculations show very sharp increases in energy beyond the ranges of 50°–190° and 80°–170° respectively. One finds two energy minima corresponding to β_1 value around 100° and 150°, separated by a low energy barrier. We thus get another set of models C and D, which differ from A and B only in values of β_1. However in these cases the β and γ chains form a V shape and may be excluded in membrane structures. The low energy barriers between the four models in Table I, along with the fact that the barrier to internal rotations around the C–C bonds in hydrocarbon chains are also low (3–5 kcal mole^{-1}) explains the observed intramolecular motions in lipid bilayers. The α-chain likewise can exist in four conformations (Table II) P, Q, R, S with almost equal energies. These arrangements arise from two possible *gauche-gauche* arrangements with respect to O–P bonds coupled with a value of $\pm$ 60° for α_5. As in the case of arrangements of β and γ chains, the predicted energies of the four conformers are very similar.

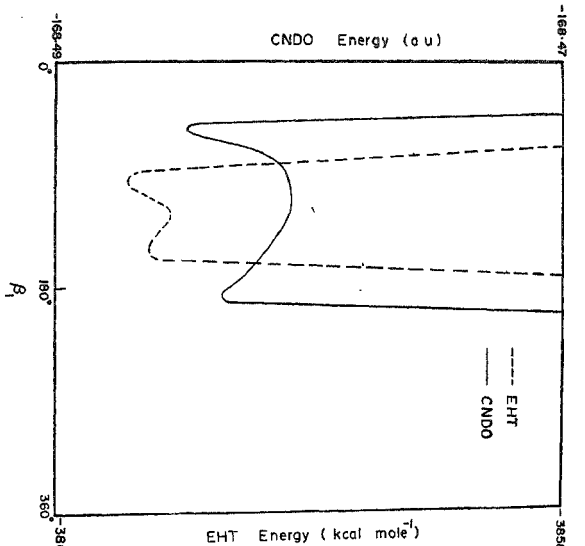

FIG. 4. Potential energy curve for β.

TABLE I

Proposed models for and chains of Phospholipid molecule

Model	θ_3	1	3	3
A	180	100	280	280
B	60	150	280	80
C	180	150	280	280
D	60	100	280	80

All other torsional angles are 180°.

The CPF calculations [13, 14] predict somewhat similar models for phospholipid conformations.

TABLE II

Proposed models for the chain of phospholipid molecule

Model	1	2	3	4	5
P	180	60	60	180	300
Q	180	60	60	180	60
R	180	300	300	180	300
S	180	300	300	180	60

We thus see that the theoretical calculations predict eight possible conformations pertinent to phospholipids in organised bilayers. These arise from the combination of A and B with P, Q, R or S. The theoretical findings are fully supported by the available experimental measurements. Basically such evidences come from ^{1}H and ^{13}C NMR of dipalmitoyl lecithin [12] in nonaqueous solvents and single crystal X-ray diffractions on molecules related to phospholipids. Table III compares the relative population of conformers with $\theta_3 = 60°$, 180° and 300° as estimated from NMR experiments. It is found that the population of the rotammers with $\theta_3 = 300°$, where the two hydrocarbon chains run in opposite direction, is negligible even in $CDCl_3$ and CD_3OD. In such solvent, structure A seem to be somewhat preferred over B. X-ray diffraction studies on values of the torsional angles in secondary esters indicate that β_1 values generally lie in the range 80–155° [11] which is the predicted low energy region. The rotation around the ester C–O–C = O bond (angle β_2) is known to behave like a highly hindred double bonded system with β_2 and γ_2 values of 180° [17, 18]. The polar group in glycerol phosphoryl choline is found to crystallise in various sets of predicted structures given in Table II depending upon the conditions of crystallisation [11]. In solutions, NMR results based on $^{1}H - {}^{1}H$, $^{1}H - {}^{31}P$ and $^{13}C - {}^{31}P$ confirm that a similar situation prevails. There is still no direct verification available for the values of β_3, γ_3 and θ_1. Further, the available experimental results are either in solid state or in nonaqueous solutions. It is not possible to say at this stage whether in lipid-bilayers there exist all the possible structures

listed in Tables I and II, or the lipid-lipid and lipid-water interactions selectively rule out some of the possible structures. As discussed below, it is very likely that some of the confomational degress of freedom are preserved in lipid bilayers and may be important in biological activity.

TABLE III

The relative population of conformers with $\theta_3 = 60°$, $180°$ *and* $300°$ *as obtained from NMR measurements and MO theories*

	p (60°)	p (180°)	p (300°)
(1) EHT theory	0·10	0·78	0·12
(2) CNDO theory	0·40	0·42	0·18
(3) Dipalmitoyl-lecithin			
(*a*) in CD_3OD	0·38	0·56	0·06
(*b*) in $CDCl_3$	0·27	0·63	0·10
(4) Sn-1, 2-dipalmitoyl glycerol in $CDCl_3$)	0·43	0·40	0·17

6. ORGANISATION OF PHOSPHOLIPIDS

In this section we consider the various possible modes of phospholipid organisation in the light of its conformational structure and physical properties of lipid bilayers discussed earlier. The lipid bilayers of the reported dimensions can be formed by taking β and γ chains in either of the conformations (A or B) and coupling it with α chain in conformations P or Q. Thus structures abbreviated as A–P, A–Q, B–P or B–Q may be found in the regular arrangements of lipid bilayers. For example Fig. 5 shows a pictorial view of the lipid bilayers arrangement with A–P conformation where the phospholipid molecules have been laced at closest contact distances and the hydrocarbon chains parallel to one another. The coordinate system is chosen in such a way that the X–Z plane forms the surface of the membrane, and the view is of the Y–Z plane. If one calculates the distances between the intramolecular and intermolecular hydrocarbon chains, then both turn out to be about 4·2–4·4 Å. This explains why only one reflection corresponding to a repeat distance of 4·2 Å is obtained in

the X-ray analysis of both lipid bilayers and soaps. For maximum hydrophilic interactions the polar part of the lipid bilayer has to be completely immersed in water and this is achieved for an angle of tilt of 60°. Such a model also explains the observed thickness and the observed area occupied by polar head groups. If the polar chain is taken in conformation R or S the distance between the lateral phospholipid molecules will have to be increased considerably from the observed value of 4·2 Å.

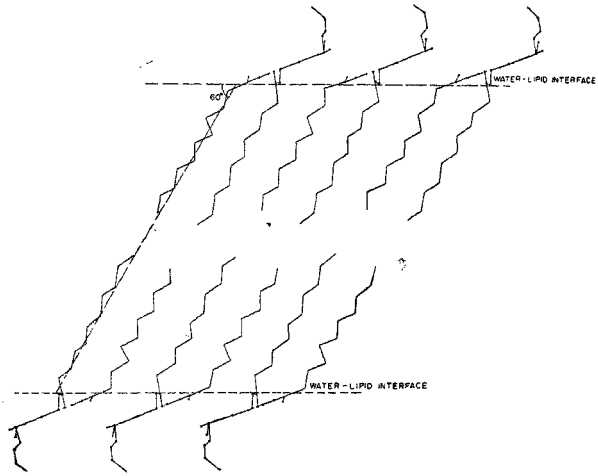

FIG. 5. Shows the bilayer formed by putting the phospholipid molecules in *A–P* conformation in a regular array. The hydrocarbon chains are tilted at an angle of 60° from the surface and are parallel to each other.

In addition to the regular structure permeability studies [19] have shown that membranes have pores having radii varying between 4·5 — 6 Å. These pores can be formed by arranging polar ends of the phospholipid molecules in a circular fashion. A pore of the above size can be formed by organising three phospholipid molecules in conformation A–R in the way mentioned above. For example Fig. 6 shows such a repeated arrangement when viewed from the surface of the membrane. Only the polar part has been shown in the diagram. The hydrocarbon chains project down the

plane of projection at an angle of 60°. One of the oxygen from each phospholipid molecule carrying negative charge protrudes out of surface of the membrane. The positive charge that is indicated is actually distributed over the length of the polar group. The nitrogen atom lies at about 4 Å below the surface. This pore has a lining of positive charge on the surface. A conformational transition to the other possible structures (for example, B–S) leads to a decrease in the effective size of the pore (Fig. 7). There is an equilibrium between these two structures. In this arrangement 3 phospholipid molecules form one unit. Repetition of such units leads to another interstitial pore which has a different size Fig. (8) and (9). The interstitial pore has a lining of negative charges. The dimensions of both the holes are governed by the equilibrium

$$A - R \rightleftharpoons B - S$$

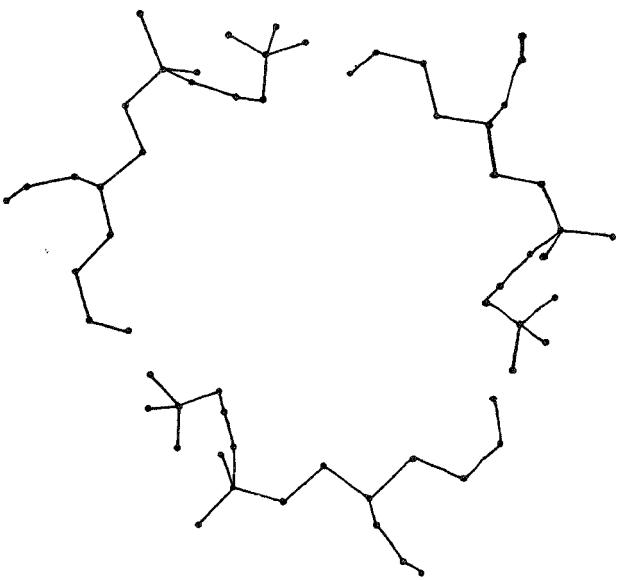

FIG. 6. Shows the pore that is formed by organising three phospholipid molecules that are in *A–R* conformation. This is the postulated open K^+ channel.

which is postulated to exist in this arrangement. At the surface, both the holes have a radius of 4·5 – 5 Å but, the above mentioned equilibrium alters the effective size of the pore. Arrangements such as *A–S* and *B–R* lead to intermediate situations. However structures involving polar groups in *P* and *Q* conformations cannot be organised in the manner above to give pores of above dimensions.

In short, the proposed model of structure of phospholipids in biomembranes involves two types of arrangments. The majority of the molecules lie in the set of conformations *A–P*, *B–P*, *A–Q* and *B–Q* and lead to regular bilayer structures. In certain localised regions, the other set of molecular conformations (*A–R*, *B–R*, *A–S* and *B–S*) organise in another bilayer structure with the formation of pores of around 5 Å radius.

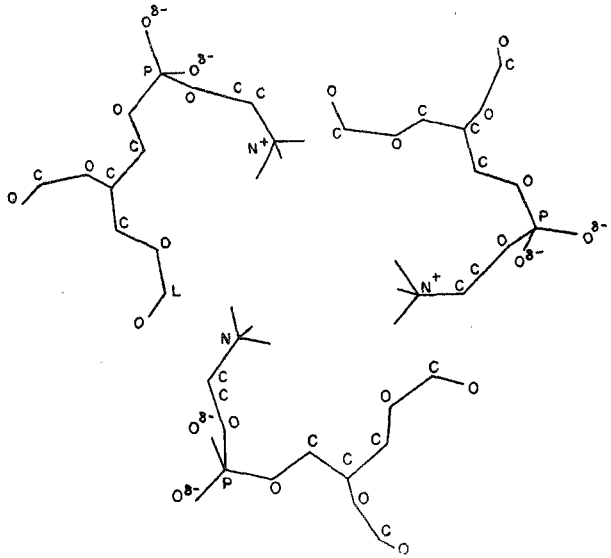

FIG. 7. Shows the pore that is formed by organising three phospholipid molecules in *B–S* conformation. This is the postulated closed K^+ channel.

We have also made some preliminary investigations on the lipid-lipid and lipid-water interactions which stabilise the two classes of arrangements discussed above. The lipid-lipid interactions are mainly electrostatic in nature and arise because of the partial charges in the polar part of the molecule. The lipid-water interactions lead to the aggregation of hydrocarbon chains. Water may also be involved in bridging phospholipid molecules together through lipid-water-lipid hydrogen bonds. Quantitative estimates of both the type of interactions are presently being made.

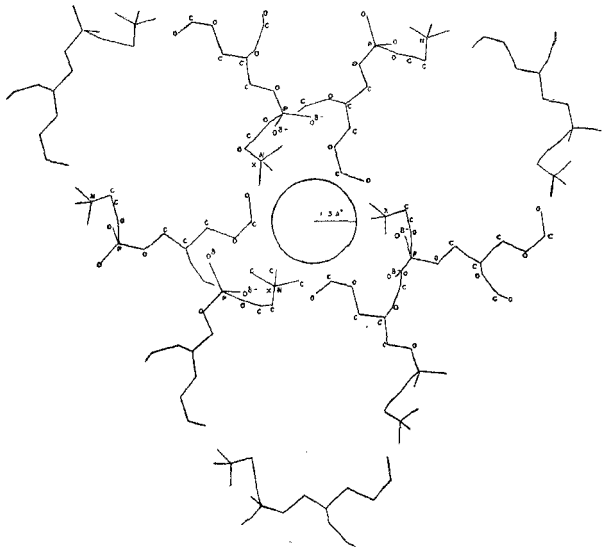

FIG. 8. Shows the interstitial pore that is formed when three units of Fig. 6 are put together. This is the postulated closed Na^+ channel.

7. BIOLOGICAL IMPLICATION OF PHOSPHOLIPID ORGANISATION

One of the important functions of biomembranes is to conduct information about external stimuli. The membranes (axon) which perform such a function are usually very rich in phospholipids. In the resting state the

inner surface of the membrane has a negative electrical potential with respect to the outer surface of about 70 mV.

The external stimuli are conducted along the axon in the form of an electrical wave known as action potential. The action potential consists of a rapid rise in the membrane potential to about + 40 mV (inside becoming positive) and then a rather slow return to its original condition. There is also a slight undershoot (Fig. 10). Hodgkin and Huxley [6] have shown that this can be explained on the basis of passive movement of Na^+ and K^+ ions, which is controlled by the membrane potential itself. The molecular mechanism of interdependence of membrane potential and conductance has not been given previously. We have made have an attempt in this direction using the model discussed in the previous section.

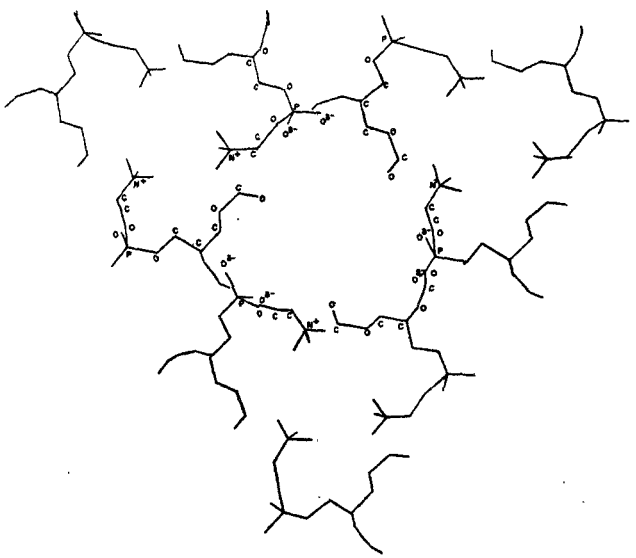

FIG. 9. Shows the interstitial pore that is formed when three units of Fig. 7 are put together. This is the postulated open Na^+ channel.

We postulate that the interstitial pore is the site of Na^+ transfer and the pore at the centre of each unit is a site for K^+ transfer, in the axon membrane These will be called as Na^+ and K^+ channels respectively. It is clear that K^+ channels can exist independent of Na^+ channels but the converse is not true. It is found experimentally that there are about 13Na^+ channels per ^{2} on the membrane surface. Since the area occupied by these channels is very small, these are very sparsely distributed on the membrane. In the K^+ channel if all the three phospholipid molecules have *A–R* conformation, the channel is open but otherwise it is closed. Similarly the Na^+ channel (which is surrounded by 3K^+ channel) is closed if all the three K^+ channels are open but otherwise it is open. It is clear that 6 conformational changes should occur simultaniously for the opening of a K^+ channel while only two are sufficient for the opening of a Na^+ channel. Consequently there is a greater time lag between the potential change and the opening of a K^+ channel as compared to that of Na^+ channel. Further, the conductance of Na^+ channel is about 400 times that of K^+ channel the reason being that Na^+ channel has a lining of negative charges on the surface while the K^+ channel has a lining of positive charges. Consequently the rate of change of Na^+ conductance is greater than that of K^+ conductance.

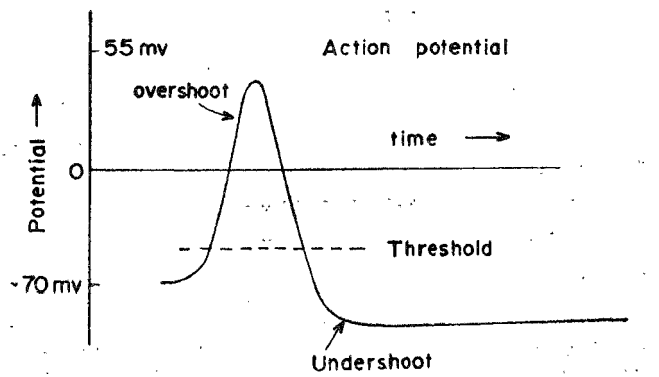

Fig. 10. Shows the action potential.

The conformation of the polar parts of phospholipid molecules and their organisation is stabilised mainly by electrostatic lipid-lipid interactions. Further there will be an interaction between the electric field arising from the potential difference between the two sides of the membrane and the dipoles of the phospholipid molecules. This will influence the distribution of molecular conformations (because of difference in dipole moments of different conformers) and hence the number of open and closed channels of either type on the two sides of the membrane will differ. Since the dipole moments of the phospholipid molecules are oriented in opposite directions, the effect of electric field will be opposite on the two sides. Any change in the membrane potential is expected to cause a shift in the distribution of open and closed channels. In the resting state the equilibrium constants are postulated to be such that, on the outer surface most of the Na^+ channels are closed while on the inner surface most of the K^+ channels are closed. This implies that most of the K^+ channels on the outer surface are open and most of the Na^+ channels on the inner surface are open. The depolarisation of membrane tends to open up K^+ channels on the inside and closes the K^+ channels on the outside resulting in opening of Na^+ channels on the outside and their closing on the inside.

For the sake of argument let us suppose that in the resting state the distribution of open and closed channels is as follows:

Outside	30% K^+ closed	70% K^+ open
Inside	90% K^+ closed	10% K^+ open.

The probability that a K^+ channels is open throughout is 7%.

Now suppose a slight depolarisation causes a 5% shift in the distribution. The new distribution becomes

Outside	35% K^+ closed	65% K^+ open
Inside	85% K^+ closed	15% K^+ open.

The new probability that a K^+ channel is open throughout is 9·75%. A phenomenon of this type results in an increase in the K^+ permeability. The process can continue until a distribution of 50% closed and 50% open is reached though it might happen that peak of the action potential is reached even before the 50:50 distribution is reached. However since change in K^+ permeability has time lag with potential, the K^+ conductance continues to increase even beyond the peak of the action potential.

It is obvious that depolarisation causes opening of Na^+ channels which in turn causes further depolarisation resulting in a cooperative phenomenon. Since there is hardly any time lag between the opening of a Na^+ channel and the potential change, the peak of the Na^+ conductance is at the peak of the action potential.

Once the distribution of Na^+ channels is disturbed due to excitation, the thermal processes will tend to drive the system back to equilibrium. The rate of such a deactivation process will depend on the total number of open channels. The deactivation will dominate after the excitation processes have stopped.

Using these principles it is possible to explain the action potential qualitatively. The raising phase is due to increase in Na^+ conductance while the falling phase is partly due to deactivation of Na^+ channels and partly due to increase in K^+ permeability. Because of the time lag between K^+ activation and potential change, K^+ permeability is not at the resting value when the membrane potential has reached its resting value. But Na^+ permeability has reached its resting value. As a result K^+ outflow continues for sometime and thus causes an undershoot.

6. Conclusions

In spite of the tremendous technological advances in the power of theoretical and experimental techniques in Molecular Biophysics, our knowledge of biomembranes is far from completes. At present, even the phospholipid organisation is not completely understood. We can hope however for some significant advances in this area which will pave way for explaining the molecular mechanisms of the biological functions which take place at membrane surfaces.

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