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PERMEABILITY OF A THIN PHOSPHOLIPID MEMBRANE TO IONS AND TOBACCO MOSAIC VIRUS

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SUMMARY

1. The electrical resistance of a thin phospholipid membrane decreases appreciably if solutions of NaCl, or $MgCl_2$ are added to the aqueous medium in which it is formed. The dielectric breakdown voltage of the membrane is increased under these conditions.

2. Tobacco mosaic virus passes through this membrane in the presence of Mg^{2+}. When the membrane is modified by a protein obtained from a thioglycollate broth culture of *Aerobacter cloacae* its ion permeability is increased but its permeability to tobacco mosaic virus is little affected. A d.c. potential applied across either membrane appears to enhance its permeability to the virus.

INTRODUCTION

Many biologically important substances cross the membranous structures of the living cell. In recent years considerable interest has been focused on these structures in order to determine their properties, especially their permeability properties. Ostensibly these membranes are permeable to ions, molecules, macromolecules, and even particles. Different mechanisms are probably involved.

The study of the properties of membranes, of biological dimensions, has been facilitated by recent developments in the formation of a 60–90 Å thick phospholipid membrane separating two aqueous media[1]. With these membranes it is possible to study their permeability to various permeant moieties under controlled conditions. In this report the permeability of such a thin phospholipid membrane to certain ions and tobacco mosaic virus is described.

EXPERIMENTAL PROCEDURE

Lipid extraction

Membranes were formed from a phospholipid fraction extracted from fresh white brain matter of cattle by a slightly modified version of a method previously

reported[2]. The procedure was carried out in a temperature-controlled room at 4°. 100 g of brain material were placed in a Waring-Blendor with 500 ml of de-aerated chloroform–methanol (2:1, v/v) solvent to which 0.10 ml α-tocopherol had been added as an antioxidant. The tissue was homogenized for 2 min at 4° under N_2. The homogenized mixture was centrifuged at 5000 rev./min for 10 min at 4° in a refrigerated Spinco Model L preparative ultracentrifuge. Both the rotor (No. 21) and tubes were pre-cooled before use. The supernatant was filtered through Whatman No. 3 filter paper that had previously been pre-cooled and washed by running 300 ml of chloroform–methanol (2:1, v/v) solvent through it in three equally divided portions. The filtrate was emulsified with deionized, deoxygenated, distilled water (20 ml/l) and then brought to dryness at 4° under N_2 in a flash evaporator. Most of the residue was then brought into solution by the addition of 50 ml of chloroform. This solution was again centrifuged at 5000 rev./min as before for 5 min. The supernatant was filtered through Whatman No. 3 as before. To the filtrate one-half volume of methanol was added. To reduce the residual protein, the filtrate was recycled 4 times through this procedure starting with the water emulsification. 6 ml of water were added to the final chloroform–methanol solution. After centrifuging, this solution separated into 2 phases. The upper phase was discarded and the lower phase was filtered 3 times through Whatman No. 3, as before. One-third volume of methanol was added to the final filtrate. The solids content of the extract was then adjusted to 2% (dry wt.) by adding the required volume of chloroform–methanol (2:1, v/v) solvent. The solution was stored under N_2 in a refrigerator.

Composition of the extract

A qualitative analysis of the phospholipid extract was carried out by thin-layer chromatography[3]. The chromatogram was run under N_2 on silica gel G (Merck) layers, 250 μ thick, prepared with a Desaga applicator and dried at 110° for 30 min. The solvent used was chloroform–methanol–30% NH_3 (14:6:1, v/v/v). Sample application was by microsyringe. The amount applied is given in Fig. 2.

Detection of the spots was carried out with iodine vapour, by their absorption or fluorescence in ultraviolet light, and their reaction with ninhydrin. The ninhydrin reaction was determined by spraying the plates with a 0.2% solution of ninhydrin in 95 ml butanol and 5 ml 10% acetic acid.

Identification of the individual components in the extract was made by concurrently chromatographing on the same plate known solutions of phosphatidyl inositol, phosphatidyl serine, sphingomyelin, α-lecithin, phosphatidyl ethanolamine, cerebrosides, phosphatidic acid and cholesterol (Fig. 2). The phospholipids were obtained from Koch-Light Laboratories, Colnbrook, Bucks., England. The cholesterol was purchased from Fisher Chemical. The phospholipid extract was also chromatographed on silica gel G (Merck) layers, prepared as before, but developed in chloroform–cyclohexane (1:1, v/v). The amount of extract applied was about 240 μg and the spray reagent consisted of 20 ml conc. H_2SO_4 in 100 ml of abs. methanol.

A quantitative determination of the total nitrogen content of some of the extracts was made using a micro-Kjeldahl and distillation procedure[4]. A titrimetric estimate of the ammonia in the distillate was made by the boric acid method.

The method of FISKE AND SUBBAROW was used to estimate the inorganic

phosphorus content of some of the extracts[4]. The colour intensity of the solution was measured in a Coleman photoelectric colorimeter at 660–720 mμ.

Membrane formation

The membrane was formed in a 2 mm diameter hole in the side of a 5 ml polyethylene cup. The wall of the cylindrical cup was turned down to a thickness of 0.13 mm. This cup was placed in a larger lucite compartment which formed the outer chamber of the two-compartment vessel (Fig. 1). Both the inner and outer

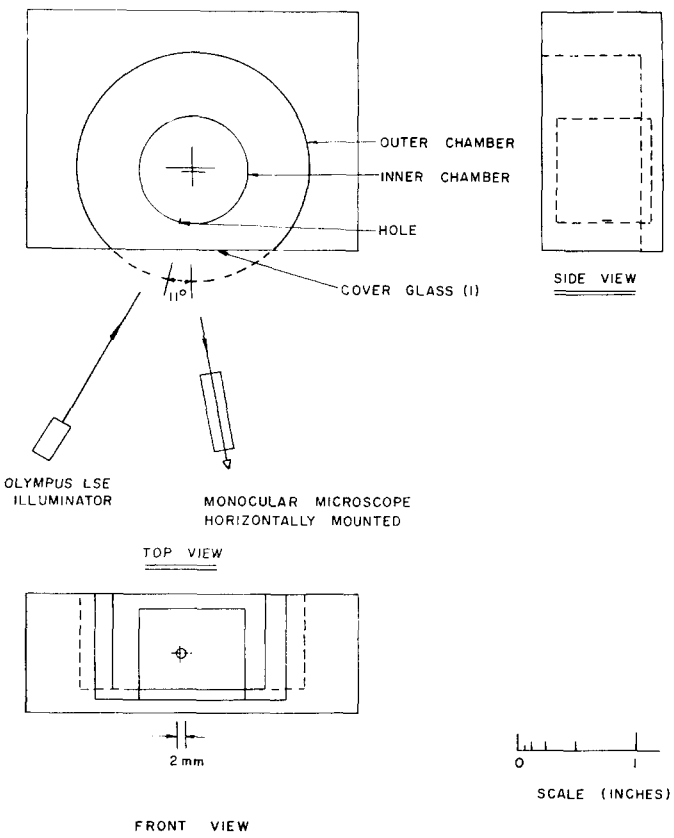

Fig. 1. Schematic representation of cell in which membranes were formed.

chambers were filled to equal depth with deionized, deoxygenated, distilled water to a level sufficient to bring the hole well below the surface of the water. The volumes of water in the inner and outer chambers were 4 and 12 ml, respectively.

Membrane formation and the subsequent permeability studies were carried out at 37° in a temperature-controlled room. To make the membrane from the phospholipid extract it was necessary to add to it 15–25% by vol. of *n*-tetradecane (Eastman Kodak P2221, m.p. 2–4°). The amount added was determined by the rate at which the membrane thinned. As the amount of *n*-tetradecane was increased the rate dropped. A formation time of 15–75 min was considered satisfactory. The membrane

solution was applied under water to the 2 mm diameter hole with a fine sable-hair brush. The thinning process and the accompanying variations in the interference colour patterns have been described[5]. They were observed through the monocular microscope. The electrical resistance measurements and permeability studies were carried out after the whole membrane had progressed to the stable 'secondary black' stage. Only membranes with no visible torus were used.

Membrane resistance measurements

When the membrane had fully thinned, its resistance was measured with a Model 600A Keithley electrometer using 0.38 mm diameter platinum electrodes. Platinum electrodes were used because of their low impedance and the mismatch was satisfactorily low (2–3 mV). During measurements the electrometer supplied a constant current to the membrane placed across its input terminals. The membrane resistance was determined by measuring the voltage drop across it.

The resistance measurements were made discontinuously with the current shut off between measurements. Consequently no polarization effects between measurements were expected and none were observed. During the measurements, however, small polarization effects were observed and corrected for. In general they were less than 5% of the corrected value.

The resistance of the unmodified membrane in water was measured at regular intervals over a period of several hours in order to determine its constancy. In addition its dependence on the ionic composition of the aqueous media was measured over a period of about 5 h. Solutions of NaCl, or $MgCl_2$, were added to both chambers; 300 μl to the outside chamber and 100 μl to the inside chamber.

The time at which the solutions were added is indicated in the legends and by the arrows accompanying the graphs of Figs. 4 and 5. Column 2 of Table I gives the final concentration of the ions in each chamber as well as the time at which

TABLE I

VARIATIONS IN MEMBRANE RESISTANCE FOLLOWING EXPOSURE TO DIFFERENT ELECTROLYTES

The figures give the time required for the resistance of the membranes to drop to one-tenth the value they had just prior to the addition of the electrolyte given in Column 2 of the table. The figures in parentheses refer to the time (min) at which the electrolytes were added to the aqueous media. The ionic strength is expressed in terms of the final concentration in each chamber.

Graph	*Type of membrane and electrolyte added*	*Time required for resistance drop (min)*
A	Unmodified	>300
B	Modified	>300
C	Unmodified, 150 mM NaCl (180)	60
D	Unmodified, 150 mM NaCl (30)	90
E	Modified, 150 mM NaCl (150)	21
F	Modified, 150 mM NaCl (30)	33
G	Unmodified, 0.50 mM $MgCl_2$ (30)*	>300
H	Unmodified, 0.50 mM $MgCl_2$ (30)	>300
I	Unmodified, 250 mM $MgCl_2$ (90)	135
J	Modified, 0.50 mM $MgCl_2$ (60)*	>300
K	Modified, 250 mM $MgCl_2$ (45)	35

* The 0.5 mM $MgCl_2$ was added to the inner chamber only.

they were added. The membrane resistance measurements were then continued at regular intervals for the duration of the experiment. Similar resistance measurements were made on membranes modified as described below.

The dielectric breakdown voltage of some membranes was measured by applying an increasing d.c. potential to the membrane. The variable potential was obtained from a precision potentiometer energized by a 1.5 V dry cell.

Permeability measurements

In this study tobacco mosaic virus particles were used. The tobacco mosaic virus solution was prepared by a method which yields a suspension in which the virus particles are of fairly uniform size[6]. Of this suspension 300 μl were added to the outer chamber after the applied membrane had fully thinned to 'secondary black'. To the inner chamber 100 μl of a $MgCl_2$ solution was added at the same time such that the final concentration of $MgCl_2$ in the inner chamber was 0.50 mM. The additional water in the inner chamber equalized the hydrostatic pressure on both sides of the membrane. The reason for introducing Mg^{2+} into the inner chamber will be discussed later.

The solutions in both chambers were periodically sampled for tobacco mosaic virus by withdrawing 50 μl from the inner chamber and 150 μl from the outer chamber. A number of samples from both chambers was taken during each sampling. The sampling times are detailed in Column 2 of Tables III and IV. The solutions in the two chambers were simultaneously stirred with a set of small glass rods prior to sampling. Between samplings both chambers were covered by a glass cover to minimize water loss through evaporation.

The permeability experiment was repeated in the presence of an electric field applied across the membrane. The objective was to have the tobacco mosaic virus particles move down their electric field gradient as they diffused towards, and penetrated through, the membrane. The isoelectric point of the ordinary strain of tobacco mosaic virus is 3.5 ± 0.1 (see ref. 7). The pH of the tobacco mosaic virus solution and the water in the chambers was about 7.0. Since this pH is on the alkaline side of the isoelectric point the tobacco mosaic virus particles should have a negative net charge and be anionic. The positive electrode was, therefore, placed in the inner chamber.

Sample assay

Nicotiana glutinosa leaves were used to assay the samples for tobacco mosaic virus. Prior to inoculation the leaf was injured by rubbing it with a smooth glass rod. Immediately after injury the same glass rod was used to inoculate the whole leaf with a sample. Only one leaf per sample was used and *vice versa*. The inoculum consisted of 50 μl of sample.

The local lesions on the leaves were counted on the 7th day after inoculation. In order to verify the infective nature of these lesions, some of the local lesions were excised and used to inoculate some fresh leaves of *Nicotiana glutinosa*.

Membrane modification

The thin phospholipid membrane was modified by the adsorption of protein[8]. The modifying protein was obtained from a thioglycollate broth culture of *Aerobacter*

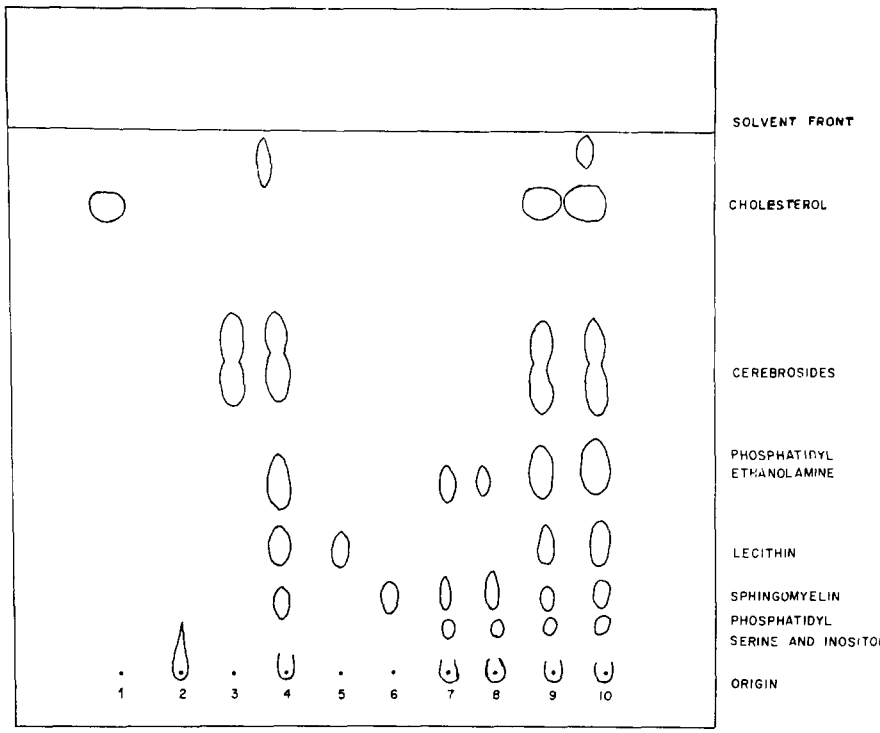

Fig. 2. Thin-layer chromatogram of phospholipids on silica gel G plates. Developing solvent: chloroform–methanol–30% NH_3 (14:6:1, v/v/v). Developing time: 48 min. Chromogenic reagent: iodine vapours. 1, cholesterol (20 μg); 2, phosphatidic acid (20 μg); 3, cerebrosides (30 μg); 4, phosphatidyl ethanolamine (30 μg); 5, L-α-lecithin (20 μg); 6, sphingomyelin (20 μg); 7, phosphatidyl serine (20 μg); 8, phosphatidyl inositol (20 μg); 9, membrane-forming phospholipids (240 μg); 10, mixture of lipids: 1 (40 μg), 3 (40 μg), 4 (40 μg), 5 (20 μg), 6 (20 μg), 7 (15 μg), 8 (15 μg).

cloacae (P. MUELLER, personal communication). An aqueous solution of the protein was made such that when 150 and 50 μl of this solution were added to the outer and inner chamber, respectively, its concentration in the aqueous medium of both chambers was 1000 ppm. The permeability of the modified membrane to tobacco mosaic virus was examined using the procedure described for the unmodified membrane.

RESULTS

Typical chromatograms of a fresh sample of the phospholipid extract are shown in Figs. 2 and 3. Fig. 2 is a chromatogram on silica gel G developed in a chloroform–methanol–30% ammonia (14:6:1, v/v/v) solvent. It indicates the presence of a phosphatidyl serine and inositol fraction, sphingomyelin, lecithin, phosphatidyl ethanolamine, several cerebroside components, and cholesterol. Additional components were shown to be present in the extract by developing the silica gel G plates in chloroform–cyclohexane (1:1, v/v) and then spraying them with the H_2SO_4 in methanol reagent. The chromatogram obtained is shown in Fig. 3. It shows the

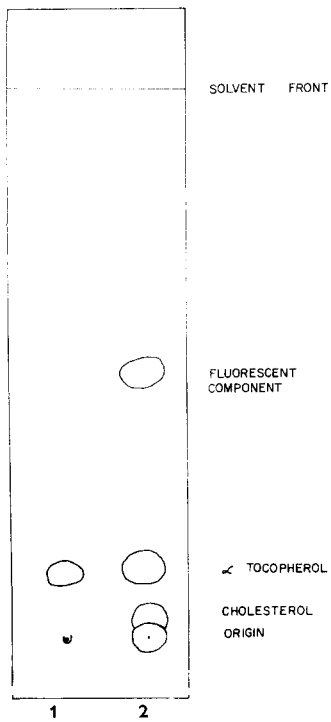

Fig. 3. Thin-layer chromatogram of phospholipids on silica gel G plates. Developing solvent: chloroform–cyclohexane (1:1, v/v). Developing time: 30 min, detection by spraying with conc. H_2SO_4 acid in methanol (1:5, v/v). 1, α-tocopherol (20 μg); 2, membrane-forming phospholipids (50 μg).

presence of cholesterol, α-tocopherol, and one as yet unidentified component. In this chromatogram the phospholipids remain at the origin. The unidentified component fluoresces after being sprayed with the H_2SO_4 in methanol reagent. This observation suggests that it might be a glycoside, an alkaloid, an amine, a cholesterol ester, or an oxidized fatty acid.

The membranes formed from the phospholipid extract need not contain all of the components present. In fact membranes have been made from lipid preparations having simpler compositions. However, to obtain a membrane with similar characteristics as the one made from the phospholipid extract it was necessary to include cholesterol, cerebrosides and/or the phosphatidyl serine–inositol fraction in approximately equal proportions. α-Tocopherol was also present in minute amounts because of its addition during the initial stage of the extraction procedure. It is not essential, however. The physical appearance of the thinned membrane under obliquely incident light and its duration in water are two characteristics by which the membranes were assessed. Membranes lasting 6 h or more have only been made from the phospholipid extract and from the combination of components referred to earlier. The use of membrane durability as a criterion by which different membranes were assessed was dictated by the expectation that tobacco mosaic virus particles would penetrate membranes slowly if at all.

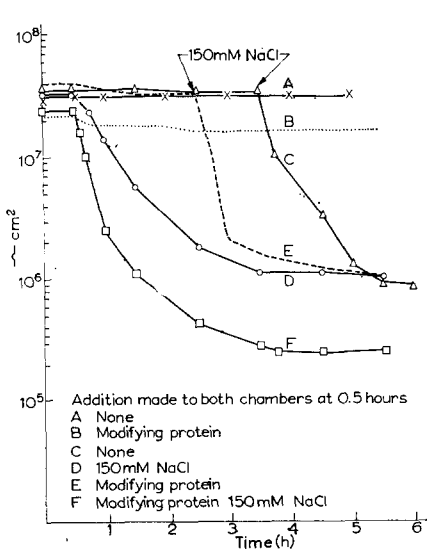

Fig. 4. Membrane resistance changes induced by NaCl.

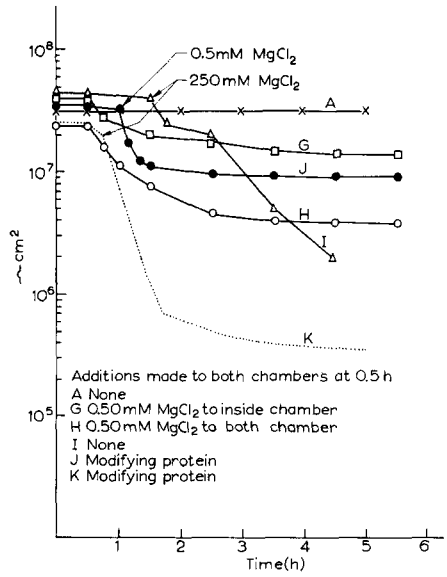

Fig. 5. Membrane resistance changes induced by $MgCl_2$.

The average nitrogen and phosphorus content of the three phospholipid extracts in which they were determined was P 1.72%, N 1.2%, N/P ratio = 1.54:1. This suggests that the amount of residual protein in the extract is low, since a N/P ratio greater than unity is expected from consideration of the phospholipids present in the extract. A value of 0.15% residual protein after 3 purification cycles has previously been reported[2].

The results of the electrical resistance measurements on the unmodified and modified membranes are given in Figs. 4 and 5. In Fig. 4 it is shown that the electrical resistance of the unmodified membrane in water is constant (Graph A); that a slight drop in resistance of the membrane occurs immediately after being modified by the adsorption of protein (Graph B); that a more than 10-fold drop in resistance of the unmodified membrane occurs within 1.5–3 h of adding 150 mM NaCl to the aqueous media (Graphs C and D); and that the time required to obtain the 10-fold drop in resistance upon the addition of 150 mM NaCl is significantly shortened by first modifying the membrane (Graphs E and F).

In Fig. 5 it is shown that the electrical resistance of the unmodified membrane stabilizes at a level that decreases with increasing concentration of $MgCl_2$ (Graphs G, H, and I). A similar but more marked relationship is observed for the modified membrane (Graphs J and K).

Comparing the graphs of Figs. 4 and 5 the impression is gained that the drop in electrical resistance of the phospholipid membrane is less sensitive to $MgCl_2$ than to NaCl. The data presented in Column 3 of Table I support this impression. The figures in this column refer to the time required for the resistance of the membranes to drop to one-tenth the value they had just prior to the addition of the electrolyte mentioned in Column 2 of the table. The data also show that for a given electrolyte

TABLE II

DIELECTRIC BREAKDOWN VOLTAGE OF UNMODIFIED AND MODIFIED MEMBRANES IN THE PRESENCE OF DIFFERENT ELECTROLYTES

Type of membrane	*Electrolyte concn. (mM)*	*Dielectric breakdown voltage ($mV \pm \sigma$)*
Unmodified	Deionized H_2O	208 ± 20
Unmodified	150 NaCl	220 ± 20
Unmodified	0.5 $MgCl_2$	253 ± 7
Unmodified	250 $MgCl_2$	285 ± 5
Modified	Deionized H_2O	213 ± 41
Modified	150 NaCl	290 ± 10
Modified	0.5 $MgCl_2$	268 ± 18
Modified	250 $MgCl_2$	520 ± 25

the resistance of the modified membrane drops more rapidly than in the unmodified membrane. Assuming the increased conductivity to be ionic in nature it follows that the modified membrane is more permeable to NaCl and $MgCl_2$ than the unmodified membrane.

TABLE III

PERMEABILITY OF THE UNMODIFIED MEMBRANE TO TOBACCO MOSAIC VIRUS

Expt. No.	*Sampling time (min after TMV added)*	*$MgCl_2$ added (mM)*	*Electric field applied* (mV)*	*Number of lesions per leaf/50 µl sample from inside chamber***	*Proportion of samples positive for TMV after time 0*
1	0	0.5	None	0, 0	4/16***
	240	0.5	None	0, 1	
2	0	0.5	None	0, 0, 0	
	60	0.5	None	0, 0	
	180	0.5	None	0, 0, 0	
3	240	0.5	None	5, 0, 1, 0	
4	240	0.5	None	0, 1, 0, 0, 0	
5	0	0.5	50	0, 0, 0	4/10***
	60	0.5	50	0, 0, 0	
	180	0.5	50	0, 1, 0	
6	0	0.5	150	0, 0	
	120	0.5	150	0, 1	
	240	0.5	150	3, 1	
7	0	None	None	0, 0, 0	0/6
	240	None	None	0, 0, 0	
	300	None	None	0, 0, 0	

Abbreviation: TMV, tobacco mosaic virus.

* Positive electrode in inside chamber.

** The mean number of lesions per leaf/50 µl sample from the outside chamber sampled throughout the course of the experiments was 81.

*** Significantly different at 5% level from proportion of samples positive for tobacco mosaic virus at time 0 in Expts. 1–6 and after time 0 in Expt. 7.

The possibility exists that trans-membrane potentials resulting from inequalities in the ionic composition of inner and outer chambers could lead to apparent changes in the membrane resistance. These potentials were small compared to the range of test voltages applied (50–200 mV d.c.). Normally at least 2 test voltages were used to make the resistance measurements. For a given membrane the 2 values were in agreement suggesting that the effect of the trans-membrane potentials on membrane resistance was small.

The data in Table II show that the value of the dielectric breakdown voltage depends, for a particular membrane, on whether it is modified, as well as on the ionic composition of the media. The relatively higher values obtained for the modified membrane upon exposure to 150 mM NaCl or 250 mM $MgCl_2$ may be due to (1) polarization of the membrane dielectric, (2) a change in the thickness of the membrane, or (3) an increase in the electric field strength of the membrane.

Consistent with the latter is the observation that in some cases the modified membrane could be made to last in excess of 48 h. This feature was not fully utilized in the tobacco mosaic virus permeability experiments because thermal inactivation of the virus particles was appreciable after 4 h.

Both the unmodified and modified membranes were found to be significantly permeable to tobacco mosaic virus. In Table III the data for the unmodified mem-

TABLE IV

PERMEABILITY OF THE MODIFIED MEMBRANE TO TOBACCO MOSAIC VIRUS

Expt. No.	*Sampling time (min after TMV added)*	*$MgCl_2$ added (mM)*	*Electric field applied* (mV)*	*Number of lesions per leaf/50 µl sample from inside chamber***	*Proportion of samples positive for TMV after time 0*
8	0	0.5	None	0, 0	
	300	0.5	None	0, 0	
9	0	0.5	None	0, 0	2/6†
	120	0.5	None	0, 0	
	240	0.5	None	1, 1	
10	0	0.5	50	0, 0	
	120	0.5	50	0, 1	
	240	0.5	50	2, 1	
11	0	0.5	50***	0, 0	4/9†
	240	0.5	50	0, 0	
	480	0.5	50	1, 0, 0	
12	0	None	None	0, 0, 0	
	240	None	None	0, 0, 0	
13	0	None	None	0, 0, 0	0/6
	300	None	None	0, 0, 0	

Abbreviation: TMV, tobacco mosaic virus.

* Positive electrode in inside chamber.

** The mean number of lesions per leaf/50 µl sample from the outside chamber sampled throughout the course of the experiments was 75.

*** Positive electrode in outside chamber.

† Significantly different at 5% level from proportion of samples positive for tobacco mosaic virus at time 0 in Expts. 8–11 and after time 0 in Expts. 12 and 13.

branes are given. They suggest that $MgCl_2$ is necessary for the tobacco mosaic virus to penetrate the membrane and that an applied electric field perhaps facilitates the penetration.

In Table IV the data for the modified membranes are given. Modification of the membrane does not appear to alter its permeability to tobacco mosaic virus. However, the results point out again the necessity of having $MgCl_2$ in the aqueous media and suggest that an electric field applied across the membrane may be beneficial. In one experiment (No. 11) the effect of reversing the direction of the applied potential is shown. To penetrate the membrane the negatively charged virus particles had to diffuse against the electric field. Under this condition permeation through the membrane was diminished but not prevented.

DISCUSSION

Mg^{2+} was introduced into the medium of the inner chamber for several reasons. It is known that, in water, phospholipids will spontaneously produce myelin forms consisting of concentric tubes of bimolecular leaflets[9]. This suggests that in water saturated with phospholipid the bimolecular leaflet is the lowest free energy configuration for the undissolved lipid.

Since the polar heads of the phospholipids against water form an interface of considerably lower energy than that of hydrocarbon against water the latter contacts will be as small and the former as large as possible. The fatty acid hydrocarbon chains will be effectively close packed[10]. Since the cross-sectional area of the polar head of a phospholipid molecule is approximately the same as that of the two hydrocarbon chains the molecules should therefore not be significantly wedge-shaped and in the absence of any other forces acting in the system, the continuous bimolecular leaflet is expected to be the most favoured structure.

In the event that some of the molecules forming the membrane are ionized, a mutual repulsion between the polar heads of the molecules is introduced. The hydrocarbon chains do not share this repelling force and therefore a tendency will exist for the bimolecular leaflet to break up into aggregates of radially oriented molecules. This transition will proceed until the decrease in free energy, resulting from the separation of the charged heads, is approximately counterbalanced by the increase in free energy caused by the contacts which can then occur between water and the outer ends of the hydrocarbon chains[10]. For the phospholipid membrane to maintain a more or less flat bimolecular leaflet structure it is necessary to have electroneutrality at the interface. The net fixed charge of phospholipids at a lipid–water interface at physiological pH values is negative[11]. The electroneutrality requirement can then be satisfied by the absorption of cations such as Mg^{2+}. This ion was therefore introduced into the aqueous medium of the inner chamber.

Introducing Mg^{2+} into the inner chamber also relates to the lipid–water interface potential. The sign of this potential is determined by the sign of the fixed head charges of the phospholipid membrane[12]. The latter must be negative if the phosphatidyl serine and inositol fraction is present in some form in the membrane. The lipid–water interface potential should then be positive and attract the negatively charged virus particles. The magnitude of this potential increases with decreasing electrolyte concentration[12] and for this reason 0.5 mM $MgCl_2$ was used. The effect

of this attractive force in drawing the virus to the membrane interface may be considerable in view of the rather large negative surface charge on the tobacco mosaic virus particle.

Divalent cations are absorbed more closely than monovalent cations, entering the Stern layer in relatively greater number[11]. The diffuse counterion atmosphere is compressed. The potential in the counterion atmosphere then falls off more rapidly with distance from the interface, leaving the phase boundary potential of the double-layer unchanged. Compression of the double-layer may not only draw the negatively charged virus particles more closely to the membrane but also increase the close packing of the head groups of the phospholipid molecules. The membrane might then have greater strength. The results on the dielectric breakdown voltage appear to support this.

The role of Mg^{2+} in facilitating the diffusion of the virus particles through either membrane may be that it binds simultaneously to negative sites on the phospholipid molecules and on the virus particles. It is noteworthy that Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} are equally effective in promoting phage infection[13].

While the number of lesions obtained from samples withdrawn from the inner chamber was small their infective nature was verified. It appears that only 1 out of 10 tobacco mosaic virus particles is infectious[14] and that it takes at least 5×10^4 particles to initiate the formation of one lesion[15]. It is possible that only infectious tobacco mosaic virus particles penetrated the membranes. The permeability rate would then be reduced appreciably but it implies a certain specificity to the permeability property of the membrane. No information on this point is available. In either case the permeability of the membranes to tobacco mosaic virus appears to be appreciable.

The possibility exists that the virus entered the inner chamber by penetrating around the membrane. A satisfactory way of ruling out peripheral leakage in these experiments would be to demonstrate that the number of virus particles entering the inner chamber per unit time is a linear function of the membrane area. While it is technically possible to increase the membrane area by a factor of 2, with some loss of membrane stability, this increase is not likely to lead to a significant difference in the assay results. Peripheral leakage did not occur in the experiments in which $MgCl_2$ was excluded, however.

It was also expected that the number of lesions produced per sample would increase linearly with time. The data do not really show this. The reason for the failure would appear to lie in the fact that the infectious tobacco mosaic virus particles are thermally inactivated to a significant degree during the course of the experiment. Experiments of longer duration were therefore precluded. The experiments were repeated on membranes formed from the same, as well as different, phospholipid extracts. No differences in the results were apparent.

The tobacco mosaic virus particles have been measured to be 3000 Å long and 149 Å in diameter[6]. It is unlikely that isolation of the tobacco mosaic virus protein from the nucleic acid takes place under the conditions of the permeability experiment[16]. The conclusion is therefore drawn that the membrane is permeable to the whole tobacco mosaic virus particle.

A description of the mechanism by which the tobacco mosaic virus particles penetrate the phospholipid membrane must involve a description of the membrane

at the molecular level. This is still lacking. However, if the membrane is in fact a bimolecular lipid leaflet, some molecules within it will be subjected to temporary displacement when the tobacco mosaic virus particles penetrate it. During these events the intermolecular attractive forces of the Van der Waals–London type may prevent the rupture of the membrane. The effect of an applied electric field on the permeability of the membrane to tobacco mosaic virus particles may in part be due to it counteracting these forces. This may lead to a certain degree of structural reorientation within the membrane. The fact that the membrane can be shattered by an applied electric field suggests that the intermolecular forces of attraction are put under stress by it.

NOTE ADDED IN PROOF (Received September 18th, 1967)

The experiments in Table III, in which no electric field was applied, have been repeated. The data confirm the present results.

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