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# PERMEABILITY OF A THIN PHOSPHOLIPID MEMBRANE TO COXSACKIE B2 VIRUS

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## SUMMARY

A thin phospholipid membrane is impermeable to infective particles of Coxsackie B2 virus, both in the absence and presence of an applied electric field. When the membrane is modified by the adsorption of a protein, obtained from a thioglycollate broth culture of *Aerobacter cloacae*, it becomes permeable to the virus in the presence of $MgCl_2$ and an applied electric field.

## INTRODUCTION

It has been shown elsewhere[1] that a thin phospholipid membrane is permeable to a rod-shaped virus, the tobacco mosaic virus. It is of interest, therefore, to know whether this effect is peculiar to a virus of this shape and size or whether the membrane is similarly permeable to a spherical virus such as Coxsackie B2.

## EXPERIMENTAL PROCEDURE

The procedures used in this work have been described[1]. The permeability studies were carried out in a temperature-controlled room set at 37°. The membrane, separating 2 aqueous media, was formed from the phospholipid extract as previously described[1].

The virus suspension was prepared by infecting primary human amnion cell cultures[2] with a strain of Coxsackie B2 virus (OH10-1) and harvesting the fluid 5 days later when cell degeneration was complete. The tissue culture fluid was collected and centrifuged at 1500 rev./min to remove the cell debris. 180 ml of the supernatant fluid was placed in a Visking dialysis bag and dialyzed overnight at 4° against polyethylene glycol (Carbowax PEG 20 000, Union Carbide of Canada). 10.0 ml of Hanks' balanced salt solution was introduced into the dialysing bag and 'milked' by running Hanks' balanced salt solution up and down the interior wall of the bag

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to remove virus from the wall. The virus suspension was then aspirated and, after being divided into 2.0-ml aliquots, frozen until required.

The permeability of the membrane to the virus was determined by adding 150 $\mu$l of the Coxsackie B2 virus suspension to the outer chamber of the two-compartment vessel after the membrane had fully thinned. At the same time, in some cases, 50 $\mu$l of a $MgCl_2$ solution was added to the inner chamber such that its final concentration in the inner chamber was 0.5 mM. The chambers were then covered with a glass plate to minimize water loss through evaporation.

At regular intervals thereafter the solutions in both chambers were manually stirred and then sampled. The sampling periods varied from one experiment to another and are given in Tables I and II. Samples withdrawn from the inner chamber were always 50 $\mu$l and from the outer chamber 150 $\mu$l. 50-$\mu$l samples were retained for assay in each case. The samples were immediately frozen and kept at $-20°$ until assayed.

The samples were assayed for infective Coxsackie B2 virus by the terminal-dilution method using whole log serial dilutions. All dilutions were made in Hanks' balanced salt solution and 0.2 ml of each dilution was inoculated into each of 3 monkey-kidney cell cultures. The titres were calculated by the 50% endpoint method of

TABLE I

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| *Expt. No.* | *Sampling time (min after virus added)* | *$MgCl_2$ added (mM)* | *Electric field applied (mV)* | *Calculated T.C.I.D.$_{50}$ titre of sample from* | | *Proportion of inner chamber samples virus positive after time 0* |
|---|---|---|---|---|---|---|
| | | | | *Outer chamber* | *Inner chamber* | |
| 1 | 0 | 0.5 | None | $10^{-6.1}$ | 0 | |
| | 90 | 0.5 | None | $10^{-6.0}$ | 0 | |
| | 180 | 0.5 | None | $10^{-5.2}$ | 0 | |
| 2 | 0 | 0.5 | None | $10^{-5.2}$ | 0 | |
| | 120 | 0.5 | None | $10^{-6.0}$ | 0 | |
| | 240 | 0.5 | None | $10^{-4.8}$ | 0 | |
| 3 | 0 | 0.5 | None | $10^{-7.5}$ | 0 | 0/8 |
| | 240 | 0.5 | None | $10^{-5.5}$ | 0 | |
| 4 | 0 | 0.5 | None | $10^{-7.5}$ | 0 | |
| | 120 | 0.5 | None | $10^{-5.2}$ | 0 | |
| | 420 | 0.5 | None | $10^{-2.8}$ | 0 | |
| 5 | 0 | 0.5 | None | $10^{-7.3}$ | 0 | |
| | 300 | 0.5 | None | $10^{-5.2}$ | 0 | |
| 6 | 0 | None | None | $10^{-3.8}$ | 0 | |
| | 180 | None | None | $10^{-3.8}$ | 0 | 0/3 |
| | 300 | None | None | $10^{-2.5}$ | 0 | |
| | 360 | None | None | $<10^{-2.0}$ | 0 | |
| 7 | 0 | None | 50 | $10^{-4.5}$ | 0 | |
| | 180 | None | 50 | $10^{-3.5}$ | 0 | 0/3 |
| | 300 | None | 50 | $10^{-2.0}$ | 0 | |
| | 360 | None | 50 | $<10^{-2.0}$ | 0 | |
| 8 | 0 | 0.5 | 50 | $10^{-4.7}$ | 0 | |
| | 300 | 0.5 | 50 | $10^{-3.5}$ | 0 | 0/2 |
| | 360 | 0.5 | 50 | $10^{-4.5}$ | 0 | |

KÄRBER[3] and were expressed in terms of the dose required to infect 50% of the tissue cultures ($T.C.I.D._{50}$).

The permeability experiments were performed on the unmodified phospholipid membrane in the absence and presence of an applied potential of 50 mV. The positive electrode was placed in the inner chamber since preliminary paper electrophoresis of the Coxsackie B2 virus suspension at pH 7.1 indicated the virus to be anionic. Identical experiments were also performed on the modified membrane. The procedure for modifying the phospholipid membrane has been described elsewhere[1].

## RESULTS

The data for the unmodified and modified membrane are tabulated in Table I and II, respectively. It is noted that the titre of samples withdrawn from the outer chamber in most experiments decreased with time. This is probably due to thermal inactivation of the virus since a similar effect was observed in control samples.

Usually 3 control samples were prepared and titrated for virus. The first sample (Control No. 1) consisted of 50 $\mu$l of undiluted virus suspension which was withdrawn prior to the experiment and immediately frozen. The second sample (Control No. 2) was made up by diluting 150 $\mu$l of virus suspension in 12 ml of water prior to the experiment and freezing a 50-$\mu$l aliquot. From the remaining portion of the diluted suspension a 50-$\mu$l sample was withdrawn and kept at 37° for the duration of the experiment and then frozen (Control No. 3). The control samples were titrated along with the test samples. Typical $T.C.I.D._{50}$ titres for the three control samples were as follows: Control No. 1 ($10^{-6.8}$), Control No. 2 ($10^{-3.5}$), and Control No. 3 ($10^{-2.5}$). It is noted that Control No. 3 has a lower titre than Control No. 2. This drop is attributed to thermal inactivation.

In Table I it is noted that all the samples withdrawn from the inner chambers showed no infective virus. Since it is assumed that only one infective virus particle is necessary to initiate a cytopathogenic effect on monkey-kidney cells the conclusion is drawn that, as far as could be determined, no infective virus particles penetrated the phospholipid membrane with or without an electric field applied across the membrane. It is known that the Coxsackie B2 virus is ether insensitive[4], suggesting it is non-soluble in lipids. The results presented here support this conclusion.

From the data in Table II it is observed that only some of the samples withdrawn from the inner chamber give a positive titre for virus. The positive samples were withdrawn in the later stages of experiments in which $MgCl_2$ was added to the water and a potential of 50 mV was applied across the modified membrane. Thus, the effect of the electric field, either on the membrane or on the virus, was to make the membrane permeable to infective Coxsackie B2 virus. Similar permeability measurements made on the modified membrane in the absence of $MgCl_2$ in the water (Expts. 17 and 18) suggest that the membrane is then relatively impermeable to the virus.

Regarding the samples that were positive for virus a $T.C.I.D._{50}$ titre of $10^{-0.5}$, which is less than the experimental values obtained, is generally regarded as being significant. Obtaining a higher experimental titre, in samples withdrawn from the inner chamber over the same period of time, is difficult to achieve for several reasons. A significant dilution factor (80) is introduced in adding the concentrated virus to

the outer chamber, resulting in an initial titre that is less than optimum. The virus undergoes thermal inactivation during the experiment and this probably lessens the virus concentration gradient across the membrane as well as diminishing the ambient titre in either chamber. It was therefore not expected that a linear increase in the concentration of virus in the inner chamber would be demonstrated. In similar experiments with the bacteriophage T4, where higher initial titres can be achieved and thermal inactivation is less of a problem, the concentration of bacteriophage in the inner chamber increases with time (work in progress).

TABLE II

PERMEABILITY OF THE THIN MODIFIED PHOSPHOLIPID MEMBRANE TO COXSÁCKIE B2 VIRUS

| *Expt. No.* | *Sampling time (min after virus added)* | *$MgCl_2$ added (mM)* | *Electric field applied (mV)** | *Calculated T.C.I.D.$_{50}$ titre of sample from* | | *Proportion of inner chamber samples virus positive after time 0* |
|---|---|---|---|---|---|---|
| | | | | *Outer chamber* | *Inner chamber* | |
| 9 | 0 | 0.5 | None | $10^{-4.8}$ | 0 | |
| | 120 | 0.5 | None | $10^{-2.5}$ | 0 | |
| 10 | 0 | 0.5 | None | $10^{-3.8}$ | 0 | |
| | 120 | 0.5 | None | $10^{-3.8}$ | 0 | |
| | 240 | 0.5 | None | $10^{-4.1}$ | 0 | |
| 11 | 0 | 0.5 | None | $10^{-2.0}$ | 0 | |
| | 120 | 0.5 | None | $<10^{-2.0}$ | 0 | |
| | 240 | 0.5 | None | $<10^{-2.0}$ | 0 | 0/10 |
| 12 | 0 | 0.5 | None | $10^{-4.6}$ | 0 | |
| | 60 | 0.5 | None | $10^{-3.5}$ | 0 | |
| | 120 | 0.5 | None | $<10^{-2.0}$ | 0 | |
| | 180 | 0.5 | None | $<10^{-3.0}$ | 0 | |
| | 240 | 0.5 | None | $<10^{-3.0}$ | 0 | |
| | 300 | 0.5 | None | $<10^{-3.0}$ | 0 | |
| 13 | 0 | None | None | $10^{-4.2}$ | 0 | |
| | 60 | None | None | $10^{-2.0}$ | 0 | |
| | 120 | None | None | $<10^{-2.0}$ | 0 | |
| | 240 | None | None | $<10^{-2.0}$ | 0 | 0/5 |
| 14 | 0 | None | None | $10^{-3.5}$ | 0 | |
| | 60 | None | None | $10^{-2.8}$ | 0 | |
| | 120 | None | None | $<10^{-2.0}$ | 0 | |
| 15 | 0 | 0.5 | 50 | $10^{-4.1}$ | 0 | |
| | 120 | 0.5 | 50 | $10^{-3.5}$ | 0 | |
| | 240 | 0.5 | 50 | $10^{-3.5}$ | 0 | |
| | 300 | 0.5 | 50 | $10^{-3.5}$ | $>10^{-0.7}$ | 3/5** |
| 16 | 0 | 0.5 | 50 | $10^{-4.7}$ | 0 | |
| | 300 | 0.5 | 50 | $10^{-4.5}$ | $>10^{-0.5}$ | |
| | 360 | 0.5 | 50 | $10^{-4.7}$ | $>10^{-0.5}$ | |
| 17 | 0 | None | 50 | $10^{-3.8}$ | 0 | |
| | 360 | None | 50 | $10^{-3.2}$ | 0 | |
| | 420 | None | 50 | $10^{-3.5}$ | 0 | 0/5 |
| 18 | 0 | None | 50 | $10^{-4.2}$ | 0 | |
| | 360 | None | 50 | $10^{-4.5}$ | 0 | |

* Positive electrode in inside chamber.

** Significantly different at 5% level from proportion of samples positive for virus at time 0 in Expts. 15 and 16 and after time 0 in Expts. 9–14 and 17 and 18.

## DISCUSSION

The results suggest that the combined effects of modification of the membrane together with the application of an electric field across the membrane are associated with making the membrane permeable to the spherical Coxsackie B2 virus. The presence of $MgCl_2$ in the aqueous medium is also necessary since the observed permeability is eliminated if the addition of the $MgCl_2$ solution is omitted. The role of the $MgCl_2$ has been discussed elsewhere[1].

Modification of the membrane by the protein obtained from the thioglycollate broth culture of *Aerobacter cloacae* is presumed to take place by adsorption of the protein on the free surfaces of the bimolecular lipid membrane. One way in which protein may become attached to a lipid membrane is by the formation of ionic or hydrogen-bond type of linkages between the head groups of the lipids and the polar groups of the protein. On this basis there would be no association between the fatty acid chains of the membrane and the adsorbed protein, leaving the structure of the lipid interior unchanged. The adsorbed protein might be expected to improve the stability of the membrane. This was observed since it was possible to maintain the modified membrane in excess of 48 h whereas the unmodified membranes shatter within 12 h.

Another mechanism for attachment of protein to the lipid membrane is for the protein chain to lie along the surface of the membrane in such a way as to intersperse its short non-polar side chains between the fatty acid chains of the lipids[5]. Because of the short length of the protein side chains (approx. 4 Å) relative to the length of the polar heads (approx. 8.5 Å) the interaction of the side chains with the lipid interior will only be appreciable if the polypeptide chain of the protein lies between the polar heads of the lipid molecules. The interaction would be achieved at the expense of separating the lipids adjacent to the polypeptide chain and might have the effect of exposing a certain amount of the hydrocarbons of the lipid chains to water[6].

The three major forces holding the phospholipid molecules together then result from (1) electrostatic interactions between polar groups of lipids and oppositely charged groups in adjacent proteins, (2) hydrogen bonding between oxygen and nitrogen atoms in lipids and adjacent proteins, and (3) Van der Waals–London dispersion forces between $CH_2$ pairs in fatty acid chains of adjacent lipid molecules[7].

The major force holding the phospholipid molecules together may be that resulting from interactions between $CH_2$ pairs in adjacent hydrocarbon chains. The total force due to $CH_2$-pair interactions is sizable since such interactions are additive and the number of $CH_2$ pairs is large. It is estimated that the maximum Van der Waals–London force arising between a lecithin molecule containing saturated 18-carbon fatty acids and two identical molecules on either side of it in closest possible contact, is approx. −18 kcal (ref. 7). On the other hand, the total electrostatic interaction between the ionically charged phosphoryl-choline groups of a lecithin molecule and the oppositely charged groups of an adjacent protein in closest contact is approx. −10 kcal (ref. 7). From energy considerations it appears, therefore, that the hydrocarbon interior of the bimolecular phospholipid membrane is the effective permeability barrier to the Coxsackie B2 virus.

To account for the observed permeability of the modified membrane to Coxsackie

B2 virus in the presence of $MgCl_2$ and the applied potential it is suggested that: (1) The side chains of the adsorbed protein penetrate the hydrocarbon interior of the phospholipid membrane to some extent. This may have the effect of reducing the Van der Waals–London forces between $CH_2$ pairs in fatty acid chains of adjacent lipid molecules. (2) $Mg^{2+}$ ions interact with the negatively charged sites on the virus particles and with the protein that modifies the membrane. (3) The applied electric field may polarize the polar heads and/or the C–C bonds of the fatty acid chains of the lipid molecules in a manner that results in a reduction of the attractive forces between these molecules. Some change in the molecular structure of the membrane may then occur allowing the virus particle to pass through by virtue of the various forces acting on it, such as ion–ion, ion–dipols, Coulombic, chemical and electrical gradients.

Another function of the adsorbed protein may be to facilitate the diffusion of the Coxsackie B2 virus by providing a necessary structural relationship between the membrane and the virus. In this connection it would be of interest to know whether dissociation of the virus into its nucleic acid and capsid occurs before it penetrates the membrane.

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