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# PERMEABILITY OF A MODEL LIPID MEMBRANE TO $T_4$

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SUMMARY

1. A model phospholipid membrane was measured for permeability to bacteriophage $T_4$ by diffusion.

2. The membrane was impermeable in distilled water or in NaCl (1.0–100 mM). In $MgCl_2$ (0.1–10 mM) it became permeable to $T_4$. The permeability was interface limited.

3. No diffusion lag time was demonstrated and the permeability coefficient was $6.9\ (\pm 3.1)\cdot 10^{-12}$ and $8.6\ (\pm 3.4)\cdot 10^{-12}$ cm/s for membrane areas of 0.03 and 0.06 $cm^2$, respectively. The difference was not significant.

4. Inactivation of $T_4$ particles by phospholipid sonicates occurred in the presence of 1 mM $MgCl_2$. The loss of infectivity was maximal at a phospholipid concentration of 1 mg/ml or greater.

5. Electronmicrographs showed interactions between phage tail and the membrane of model cells characteristic of the injection mechanism by which the phage infects bacterial host cells. The phospholipids used to form the membrane were mammalian in origin.

6. This method of introducing genomes into model cells may be of interest in studying important processes of the living cell.

## INTRODUCTION

The bacterial virus, bacteriophage $T_4$, normally infects its host cell by a three-step process[1]. The initial and intermediate stages involve the absorption of the long and short tail fibres, respectively, to the lipopolysaccharides of the bacterial cell wall. This interaction is thought to be chemically specific. The final stage of the injection process consists of shortening the tail sheath, penetration of the cell wall by the tail core, and transport of the deoxyribonucleic acid from the head of the virus through the tail core into the host cell.

The chemical specificity of the proposed injection mechanism of host cell infection has been placed in some doubt by results which have shown that $T_4$ particles infect bacterial cells under conditions where the chemically specific interaction cannot occur. Thus, it was observed that $T_4D$ phage deficient in gene 12 product (a baseplate gene) readily infects spheroplasts[2]. The result was interpreted to mean that the injection of the phage genome into the host cell occurs by means of the tail core but

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without any interaction between the baseplate short fibres and the spheroplast membrane.

Recent studies on the biochemical modification of human fibroblasts by transducing bacteriophage[3] appear to indicate that either the applicability of the injection mechanism must be more broadly defined or that other mechanisms of genome entry into host cells exist. In this report data are presented which show that the injection mechanism is activated by model lipid bilayer membranes, of mammalian origin, and that these membranes are also permeable to $T_4$ under certain conditions.

## EXPERIMENTAL PROCEDURE

### *Materials*

*Escherichia coli* B and bacteriophage $T_4$ were obtained from the American Type Culture Collection (ATCC 11303 and 11303-$B_4$, respectively). Phospholipids used for membrane formation were prepared from fresh beef brain as described previously[4]. On thin-layer chromatography the extract was ninhydrin negative in areas where protein was expected, so that any residual amount of protein was estimated to be less than 0.2 % by weight. Tetradecane was obtained from Eastman Kodak Co., Rochester, N.Y. All other chemicals were reagent grade.

### *Propagation and purification of $T_4$*

*E. coli* were grown aerobically at 37 °C in nutrient broth containing 0.8 % NaCl. At a cell density of about $5 \cdot 10^8$ cells/ml the culture was inoculated with $T_4$ at an input multiplicity of about 5 p.f.u. per cell. Following an additional 4-h incubation the broth was centrifuged (2000 × *g*, 10 min), filtered through a No. 02 Selas filter, and centrifuged again (25000 × *g*, 2 h). The pellet was resuspended in 0.8 % NaCl overnight at 4 °C and then further purified by two additional cycles of low and high speed centrifugation. The final $T_4$ suspension was then disinfected with chloroform (0.05 ml/ml of suspension) and separated from it by bubbling with $N_2$ under aseptic conditions. In later stages of the experiments high titre stocks of $T_4$ were prepared by the alternative plate method[5] and purified as in the foregoing. Titres of $10^{12}$–$10^{13}$ p.f.u./ml were obtained.

### *Permeability of phospholipid bilayer to $T_4$*

The permeability of the model phospholipid bilayer membrane to $T_4$ was measured by diffusion. The permeability cell used in the $T_4$ diffusion experiments has been described previously[6]. Its essential feature consisted of two approx. 3-ml compartments separated by a thin Teflon septum containing an aperture for membrane location. In addition it incorporated a flow-through system which permitted the solution in the compartment into which the $T_4$ diffused to be continually replaced with a fresh supply by a gravity-fed perfusion technique. During an experiment, therefore, virtually all the $T_4$ particles diffusing through the phospholipid membrane were collected in the perfusate.

The major components in the 2 % (w/v) phospholipid extract in chloroform–methanol (2:1, v/v) were phosphatidylcholine, phosphatidylethanolamine, cerebrosides and cholesterol. Sphingomyelin, phosphatidylserine and inositol were also

present in minor amounts. In our experience the formation of a durable membrane lasting up to 21 days required that cerebrosides, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine be present in the solution from which the membrane is made. It therefore seems likely that these components were present in the membrane as well. Membranes were made from phospholipid solutions to which 16 % (by vol.) of *n*-tetradecane was added.

Before the membrane was applied, however, the flow of the perfusate (distilled water, 1.0–100 mM NaCl, or 0.1–10 mM $MgCl_2$ in different experiments) was started and allowed to fill both compartments of the permeability cell. The solute composition of the aqueous medium in the two compartments was therefore the same initially. A fine sable hair brush was then used to apply the membrane solution to the aperture in the Teflon septum. After the membrane had fully thinned to a bimolecular thickness two 30-min control samples of perfusate were collected in new 20-ml bottles to establish the absence of $T_4$ in the perfusate prior to the addition of the particles to the non-perfused compartment. Following the addition of the $T_4$ suspension (100–200 $\mu$l) to the non-perfused compartment the perfusate was collected as hourly samples in separate bottles. The $T_4$ titre in the non-perfused compartment was monitored at 1 h after the addition and at the end of an experiment. No measurable loss in $T_4$ titre in the non-perfused compartment occurred during an experiment. All experiments were carried out at 37 °C in a controlled-temperature room. On completion of an experiment the permeability cell was sterilized along with the other equipment.

*$T_4$ assay*

The samples of perfusate collected every hour were assayed for $T_4$ by a method previously described[7]. It consisted of dividing each sample into 0.5 ml aliquots which, after warming to 45 °C, were then mixed with 3 ml of liquid agar (0.7 %) and seeded with *E. coli* (0.1 ml of broth culture containing $10^9$ cells/ml). The seeded samples were then poured onto separate petri plates of hardened nutrient agar and incubated overnight at 37 °C. The following morning a plaque count on each plate was made. The 10-$\mu$l samples of $T_4$ withdrawn from the non-perfused compartment were serially diluted and assayed in the usual manner. Doubtful plaques were confirmed by re-assay.

*Phospholipid sonicate*

Lipids may affect the infectivity of $T_4$ particularly in the presence of $Mg^{2+}$. To examine this possibility phospholipid sonicates were prepared from the phospholipid extract by removing the chloroform–methanol solvent through flash evaporation at 4 °C and, after the addition of distilled water (1 %, w/v), sonicating the lipid residue at 4 °C for 30 min at maximum power (Biosonik II, Bronwill Scientific Co., Rochester, N.Y.). $T_4$ was then added to the sonicated samples to a final titre of $10^9$–$10^{10}$ p.f.u./ml along with $MgCl_2$ (1.0–100 mM) in some cases.

*Preparation of spherical phospholipid membranes*

It was technically difficult to examine by electron microscopy the interaction of $T_4$ with the type of planar lipid membrane used in the permeability experiments. Spherically shaped phospholipid membranes, each enveloping an aqueous core, were considered to be more suitable for this type of study. Structures of this type

have been described[8] and in this instance were prepared by a modified version of a method used previously[9]. After removal of the chloroform–methanol solvent from the phospholipid extract by flash evaporation at 4 °C, 1 g of the dried lipid was suspended in 10 ml of 0.01 M Tris–HCl buffer (pH 7.0), homogenized for 5 min in a Potter–Elvehjem tissue homogenizer, and sonicated for 1 h as above under sterile conditions. The sonicate was centrifuged (105000 × *g*, 30 min) at 4 °C. The supernatant, containing the spherical phospholipid membranes of interest, was mixed at various dilutions with $T_4$ in the presence and absence of $Mg^{2+}$ and examined by electron microscopy.

*Electron microscopy*

Samples of the spherical phospholipid membrane–$T_4$ preparations were negatively stained with 2 % phosphotungstic acid at pH 7.4, applied to Formvar–carbon grids by spray or drop methods and examined. Other samples were freeze fractured, etched, and shadowed with Pt–C. Replicas were removed by flotation on distilled water and examined in a Philips EM 300.

## RESULTS

No infective $T_4$ permeated the phospholipid membrane when the perfusate consisted of distilled water or NaCl (1.0–100 mM). In the presence of $MgCl_2$, however, the permeability to $T_4$ was easily measurable and the results are shown in Fig. 1 for membrane areas of 0.03 and 0.06 $cm^2$. $Q(t)$ is the time integral of the number of p.f.u. in the perfusate and was calculated by summation of the number of p.f.u. per hourly sample over the total number of samples included at time $t$. $Q(t)$ is normalized to a $T_4$ titre difference across the membrane of $10^{11}$ p.f.u./$cm^3$. The vertical bars associated with the points represent the standard deviation. It is seen from the figure that $Q(t)$ increases linearly with time throughout. There is no apparent diffusion lag time.

From the slope of either line in Fig. 1 the number of $T_4$ permeating in a uni-

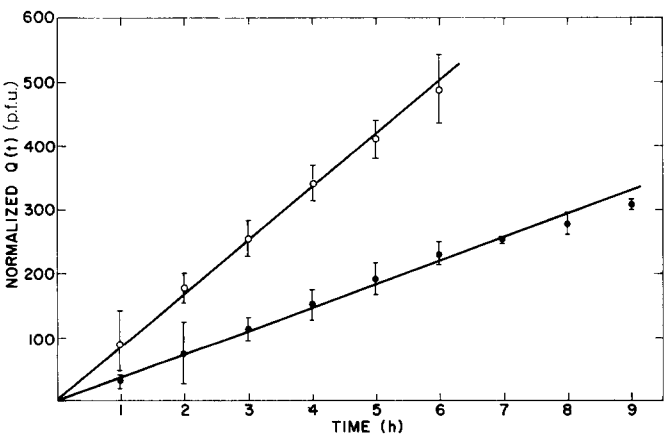


Fig. 1. $Q(t)$, the normalized permeability of a phospholipid membrane to $T_4$ *vs* time in 0.5 mM $MgCl_2$. Membrane area: ●—●, 0.03 $cm^2$; ○—○, 0.06 $cm^2$.

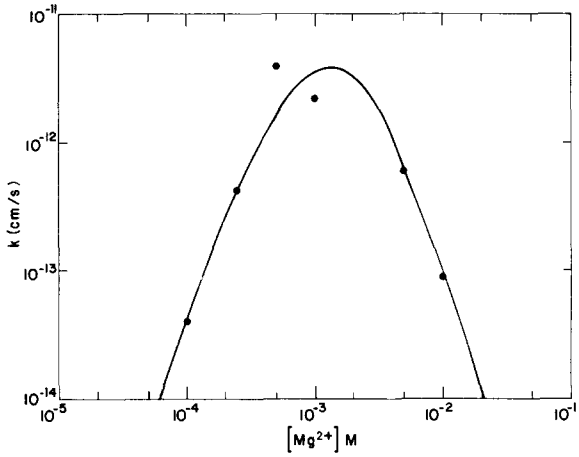


Fig. 2. Permeability coefficient $k$ *vs* $Mg^{2+}$ concentration. Membrane area 0.06 $cm^2$.

directional manner through the membrane per unit time, $dQ/dt$, was determined in p.f.u./s. The permeability coefficient $k$(cm/s) was then calculated from the equation

$$dQ/dt = k \cdot A \cdot \Delta c$$

where $A$ is the area of membrane (0.03 or 0.06 $cm^2$) and the $T_4$ titre difference across the membrane $\Delta c = c - c_p \simeq c$, since $c$ is the $T_4$ titre in the non-perfused compartment ($10^{10}$–$10^{11}$ p.f.u./$cm^3$) and $c_p$ is the $T_4$ titre in the perfused compartment (<3 p.f.u./$cm^3$). Values of $k$ for membrane areas 0.06 and 0.03 $cm^2$ were 8.6 ($\pm$3.4) $\cdot 10^{-12}$

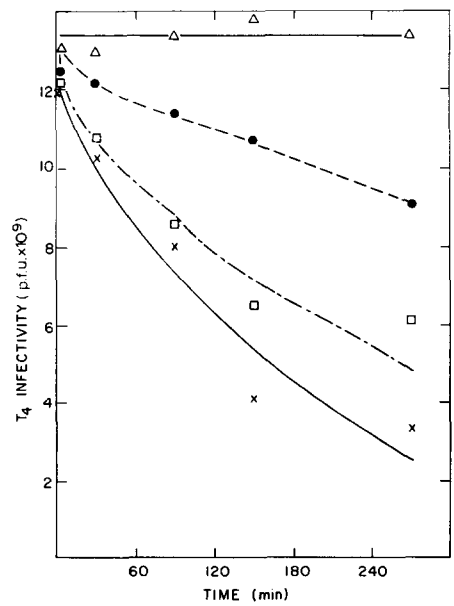


Fig. 3. $T_4$ inactivation by phospholipid sonicates (10 mg/ml) *vs* time. $MgCl_2$ concentrations: △—△, none; ●—●, 1.0 mM; □---□, 10 mM; ×---×, 100 mM. Temperature 37 °C.

and $6.9 (\pm 3.1) \cdot 10^{-12}$ cm/s, respectively. There is no significant difference between the two values.

The results shown in Fig. 1 were obtained with 0.5 mM $MgCl_2$ solution as the perfusate but typify the data at other concentrations of $MgCl_2$ as well. Only the slope of the line and hence $k$ changed as the $Mg^{2+}$ concentration varied. The result is shown in Fig. 2. It is seen that the maximum value for the permeability coefficient is obtained at 1.0 mM $Mg^{2+}$, the concentration of the ion in various physiological fluids[10]. At $Mg^{2+}$ concentrations below 0.1 mM or in excess of 10 mM membrane permeability to $T_4$ was not detectable. At 0.1–10 mM the $Mg^{2+}$ exceeded the number of infective $T_4$ particles by many orders of magnitude so that stoichiometric considerations do not seem important.

Since the measurement of membrane permeability to $T_4$ was based on an infectivity assay the remarkable dependence of the permeability coefficient on the $Mg^{2+}$ concentration might be related to an ion effect on the $T_4$ particles, especially in the presence of phospholipid. Lipid sonicates (10 mg/ml) were mixed with high titres of $T_4$, both in the presence and absence of $Mg^{2+}$ and assayed for $T_4$ at regular periods thereafter. The results on infectivity are shown in Fig. 3. In the absence of $Mg^{2+}$ the $T_4$ particles are not inactivated by the phospholipid over the 270 min period. The observed impermeability of the phospholipid membrane to $T_4$ in the absence of $Mg^{2+}$ is therefore not due to loss of infectivity. An essential role for $Mg^{2+}$ is indicated. On increasing the $Mg^{2+}$ concentration from 1.0 mM to 100 mM a progressive loss of infectivity of the $T_4$ particles developed. However, the magnitude of the loss (less than 4-fold at 100 mM $Mg^{2+}$) is insufficient to account for the dependence of the permeability coefficient on $Mg^{2+}$ as shown in Fig. 2.

The stimulative effect of $Mg^{2+}$ on membrane permeability to virus particles has been observed previously[4,16] and contrasts with their inhibitory influence on the flux of diffusing $Na^+$ and ATP molecules[6,11].

The possibility that increasing amounts of phospholipid stimulated loss of $T_4$ infectivity was examined by mixing sonicated samples of the dried phospholipid preparation, at various concentrations, with a constant titre of $T_4$ and 1.0 mM $Mg^{2+}$

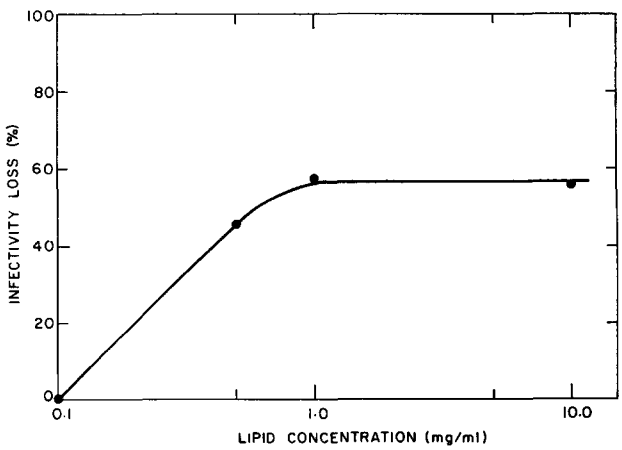


Fig. 4. $T_4$ inactivation after 300-min exposure to phospholipid sonicates. $[MgCl_2] = 1.0$ mM. Temperature 37 °C.

and checking the $T_4$ titre 300 min later. From the results shown in Fig. 4 it is seen that at a phospholipid concentration of 0.1 mg/ml there was no loss of $T_4$ infectivity. With larger amounts of phospholipid, phage inactivation occurred to a maximum of

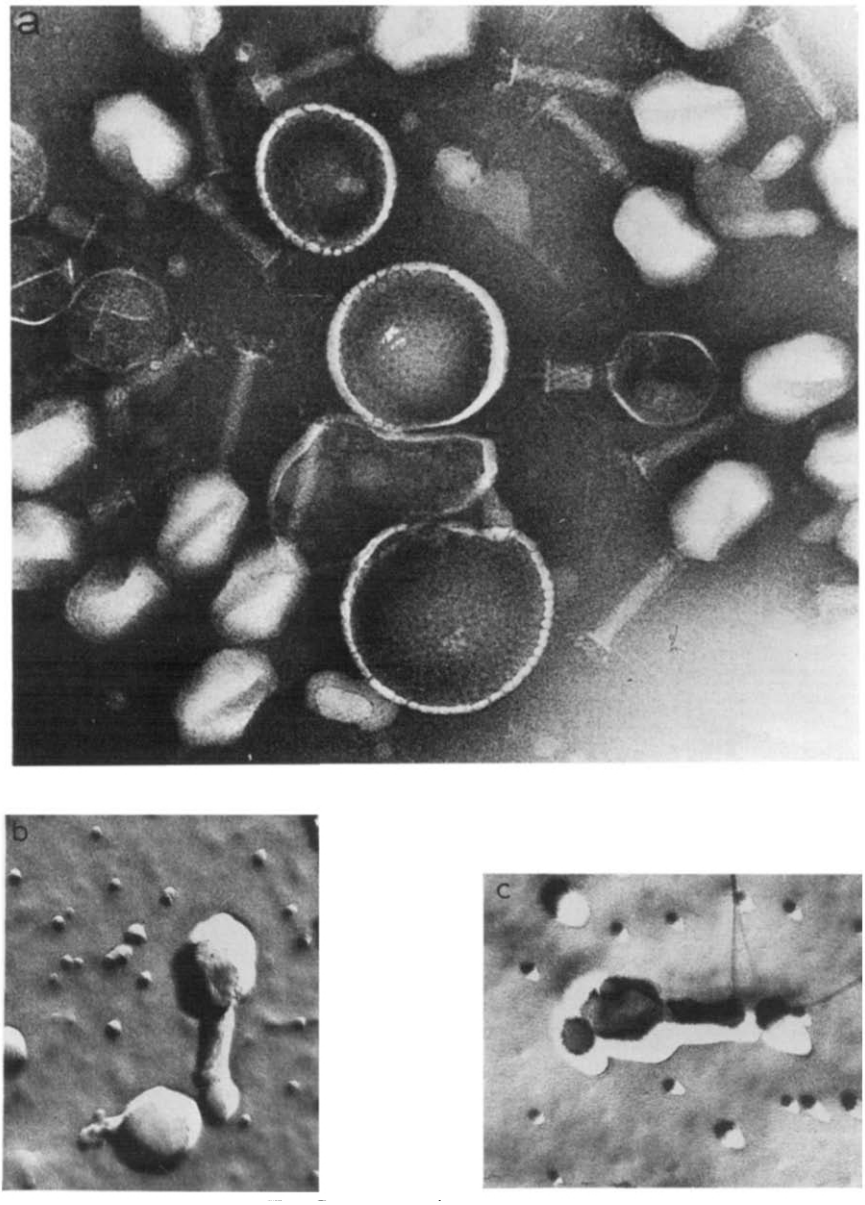


Fig. 5. Interaction of $T_4$ with phospholipid membrane of model cell in 1.0 mM $MgCl_2$: (a) $T_4$ particle in the last stage of the injection process in relation to a spherical phospholipid membrane. Phosphotungstic acid stained. Magnification 256500×. (b) $T_4$ tail interacting with spherical phospholipid membrane. Freeze etched 1 h. Pt–C shadowed replica. Magnification 153900 ×. (c) Interaction of spherical phospholipid membranes with tail and head of $T_4$. Freeze etched 1 h. Pt–C shadowed replica. Magnification 153900×.

57 % at a lipid concentration of 1 mg/ml or higher. The result does not account for the impermeability of the membrane to $T_4$ at $Mg^{2+}$ concentrations of 10 mM or greater.

The loss in infectivity of $T_4$ in the presence of phospholipid and $Mg^{2+}$ was further examined by electron microscopy. There was no general evidence of empty phage heads when the $T_4$ particles were exposed either to sonicated phospholipids or 1.0 mM $Mg^{2+}$. In the presence of both for an hour, however, approximately a third of the phage particles exhibited empty heads and shortened sheaths. The electron-micrograph shown in Fig. 5a illustrates these findings along with other interesting details. It shows a number of $T_4$ particles in the presence of three artificially constructed model cells represented by the ring structures in the centre of the picture. The cells consist simply of an aqueous core bounded by a bimolecular phospholipid membrane. It is noted that the model cell in the centre of the illustration has a $T_4$ particle correctly attached to it for DNA injection to occur. It is also seen that the tail sheath is contracted, the $T_4$ head is empty, and the tail core can be traced on either side of the membrane. This type of $T_4$–membrane interaction increased in number with time and was not observed in the absence of $Mg^{2+}$. While the electron micrograph illustrates the accepted injection mechanism of $T_4$ as far as the mechanics of the process are concerned it is of interest to note the difference. In the illustration as well as in Figs 5b and 5c, which were obtained by freeze etching, the interaction of the $T_4$ involves a bimolecular membrane composed of phospholipids of mammalian origin, rather than the lipopolysaccharides of a bacterial cell wall[12].

It is therefore probable that in the permeability experiments with $Mg^{2+}$ some of the $T_4$ particles ejected their DNA upon, or following, their adsorption to the phospholipid membrane. The assay procedure, however, would not have detected any of these $T_4$ particles, inactivated on adsorption, that may have passed through the membrane. While this possible process may have reduced the value of the permeability coefficient at the higher concentrations of $Mg^{2+}$ its magnitude was not estimated for technical reasons.

## DISCUSSION

The $T_4$ assay procedure used normal cells of *E. coli* which are not infected by subviral particles or their nucleic acid[13]. Therefore, the data presented are a measure of the permeability of the phospholipid membrane to intact $T_4$ particles.

The permeability data clearly indicate a dependence on $Mg^{2+}$. A role for $Mg^{2+}$ is apparent in terms of their effect on the interfacial properties of phospholipid membranes[4]. Upon sorption to the polar heads of the molecules in a phospholipid membrane[14] they influence the positive interfacial potential as well as the thickness of the counterion double layer in the adjacent aqueous phase. Negatively charged $T_4$ particles[15], as well as other viruses[4,16] are electrically attracted to the membrane and bound to it by the divalent cations. It is of interest to note that the maximum value of the permeability coefficient for $T_4$ occurred at 1.0 mM $Mg^{2+}$, the concentration of divalent cation at which the positive interfacial potential has been estimated to be at near maximum, and that the range over which it extends into the aqueous phase is substantial[17]. At concentrations of $Mg^{2+}$ of 10–100 mM the range of the potential is progressively reduced and the interfacial region of sorbed $Mg^{2+}$ becomes saturated[17]. Divalent cations are strongly sorbed to a phospholipid membrane

interface[14] and at saturation inhibit sorption of additional charge into the region. Adsorption of the charged $T_4$ particles is consequently reduced and loss in permeability follows concomitantly.

The foregoing account of the $Mg^{2+}$ effect emphasizes that the permeability to $T_4$ is interface limited rather than bulk limited. To further substantiate this point permeability measurements were made on cholesterol membranes. It was found that at least $10^3$ p.f.u./h passed through a 0.06 $cm^2$ membrane irrespective of the presence or absence of ions (uni- or divalent). The rate is approximately ten times that for phospholipid membranes of similar area at the optimum $Mg^{2+}$ concentration and indicates additionally the extent to which $T_4$ particles partition into a lipid phase.

The physical dimensions of a $T_4$ particle[1] are large compared to the thickness of a bimolecular phospholipid membrane. Therefore absorption into the membrane phase by phagocytosis, invagination, or encapsulation does not seem attractive while passage through the membrane by thermal diffusion along the concentration gradient does. In the process many ionic bridges are probably formed and broken between the $T_4$ particle, $Mg^{2+}$, and the polar groups of the phospholipid molecules irrespective of the orientation of the particle with respect to the plane of the membrane. A drag effect, arising from the formation of ionic bridges, would account for the lower $T_4$ permeability rates calculated for phospholipid, as compared with cholesterol, membranes.

Entry into mammalian cells of $T_4$ particles on the basis of permeability through exposed phospholipid and/or cholesterol regions of the cytoplasmic membrane must be limited to cells of adequate physical size. This limitation does not need to apply to the injection mechanism, however, and electron micrographic, as well as other, evidence of its occurrence in relation to mammalian cells should be demonstrable.

The illustration in Fig. 5 of the injection mechanism in relation to the phospholipid membrane of a model cell is believed to be the first of its kind. Because it simulates the biological mechanism of genome injection closely it is probable that the genetic material can be transferred without harm. With prior loading of the model cells with minimal supportive biochemical machinery it may be possible to examine in relative isolation some important processes of a living cell.

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