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The Permeability of Liposomes to Nonelectrolytes

II. The Effect of Nystatin and Gramicidin A

B. Eleazar Cohen *

Biophysics Unit, Agricultural Research Council,
Institute of Animal Physiology, Babraham, Cambridge CB 2 4 AT, England

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Summary. The process of selective permeation of nonelectrolytes across liposomes of different lipid composition and amount of cholesterol has been studied. The extent of the selectivity for diffusion within the membranes has been found to be related to the physical state of the hydrocarbon chains. It has also been found that incorporation of cholesterol into egg-lecithin membranes decreases the overall permeability by affecting the dehydration step more than the subsequent diffusion of the solute. The incorporation into liposomes of the antibiotics nystatin and gramicidin A produces changes in the selective permeation of nonelectrolytes that are consistent with the formation by these molecules of aqueous pores of fixed dimensions. Finally, comparisons are made between the process of permeation in biological membranes and in liposomes with and without antibiotics.

The idea that a continuous lipid barrier is a main route for the nonspecific permeation (passive permeability) of nonelectrolytes across biological membranes, is not immediately compatible with observations that water and other small hydrophilic molecules penetrate cells more rapidly than would be expected on the basis of their solubility properties in a lipid solvent. Furthermore, the introduction of an alkyl group into the first member of a homologous series of nonelectrolytes decreases the permeation rate in many cells instead of increasing it as might be expected from a consideration of their increased partition into a bulk lipid solvent (Collander & Bärlund, 1933; Collander, 1949; Diamond & Wright, 1969; Sha'afi, Gary-Bobo & Solomon, 1971). To account for this "sieve-like" behavior of biological membranes, many authors have postulated the presence of "aqueous pores" in the hydrocarbon matrix as being a fundamental characteristic of cell membranes.

* *Present address:* Departamento de Biologia Celular, Facultad de Ciencias, Universidad Central de Venezuela, Apartado 10098, Caracas, Venezuela.

But some consideration must be given to whether a continuous lipid bilayer can be expected to behave either as a membrane having intermolecular channels or pores or as a bulk lipid solvent. For, it can be argued that if the hydrocarbon chains are in a liquid state, the selectivity to the diffusion of molecules would then not be very different to that in a bulk solvent, whereas if they assume a solid-like condition, the rigidity of the hydrocarbon chains could determine that a "sieve-like" behavior would result.

The analysis of the nature of the permeability process across biological membranes is particularly complicated because no direct measurements of pure diffusion through the membrane have been made and, for most of the solutes used, measurements of partition coefficients in the membrane are not available. Recently, Lieb and Stein (1969) used a double regression analysis to estimate the best partition coefficient in a lipid solvent for adjusting a set of permeability values to the molecular weights of the solutes. They found that values for olive oil provide a good estimation of the partition properties of the membranes of *Chara* cells studied by Collander and Bärlund (1933) and they were able to calculate the probable values for diffusion through these membranes. The mass dependence of such calculated diffusion values was found to be greater than that corresponding to diffusion in bulk liquids and rather similar to the mass dependence for diffusion across polymeric materials such as rubber. Lieb and Stein (1969) concluded that there was no need to postulate the existence of aqueous pores in biological membranes if their "sieve-like" properties could be explained by treating the membranes as a homologous polymer network. This hypothesis has been tested using liposome membranes (smectic mesophases of phospholipids) which have a structure and composition which is simple and well defined (Cohen & Bangham, 1972). These authors found that the barriers to transfer of nonelectrolytes appear to have the same fundamental structure in polymeric networks, smectic mesophases and biological membranes such as *Chara* membranes.

However, the presence of "aqueous pores" in certain specialized membrane systems – such as the human red cell membrane – cannot be completely ruled out. The main evidence in support of this hypothesis comes from the observation that the ratio between the water permeability coefficient measured by tracers and by osmotic flow is greater than one (Paganelli & Solomon, 1957; Solomon, 1968) and from the finding that the activation energy for water permeation is, in human red cell membranes (Vieira, Sha'afi & Solomon, 1970), lower than the corresponding activation energy for permeation across egg-lecithin membranes which have a rather disordered hydrocarbon region (Cohen, 1975).

A difference between the diffusional and the osmotic coefficient for water permeation has also been found in thin lipid films in the presence of nystatin (Holz & Finkelstein, 1970) and this ratio has been used to estimate an "equivalent pore radius" of 4.6 Å (Solomon & Gary-Bobo, 1972). This has made the nystatin pore rather interesting since an "equivalent pore radius" of 4.3 to 4.5 Å has been calculated for human red cell membranes (Solomon, 1968).

In the present work, a systematic study of the selectivity to nonelectrolytes of liposomes of different lipid composition, including cholesterol, is reported. In addition, the nature of the alterations in permeability induced by the presence of nystatin in cholesterol-containing liposomes and by gramicidin A in liposomes prepared with saturated phospholipids below their transition temperature is studied. The permeability measurements have been made with the same set of nonelectrolytes that have been used to characterize "aqueous pores" in biological membranes.

Materials and Methods

Egg-lecithin was extracted from egg yolks by alumina and silicic acid chromatography, according to the procedure of Papahadjopoulos and Miller (1967). Cholesterol, 1,2-dipalmitoyl-L-3-lecithin, 1,2-dimyristoyl-L-3-lecithin and cerebrosides were obtained commercially (Koch-Light Lab. Ltd. and Sigma Chemical Co.). Phosphatidic acid was prepared from egg-lecithin and dipalmitoyl-lecithin by enzymatic hydrolysis (Papahadjopoulos & Miller, 1967).

New batches of lecithins and phosphatidic acid were routinely examined for purity on activated silica gel plates using chloroform/methanol/7 N aqueous ammonia (230:90:15, v/v) (Abramson & Blecher, 1964). Oxidation of the lipids was examined by the method of Klein (1970). All lipid solutions in chloroform were stored at −20 °C under an atmosphere of nitrogen, until required.

Nonelectrolytes were obtained from British Drug House Ltd. (Poole, England), Eastman Kodak Co. (Rochester, N.Y.), Hopkin and Williams Ltd. (Essex), May and Baker Ltd. (Dagenham, England), Pfaltz and Bauer Inc. (Flushing, N.Y.), and Koch-Light Lab. (Colnbrook, England). Gramicidin A was obtained from Koch-Light, nystatin and dicetylphosphate from Sigma Chemical Co. All reagents were analytical grade and were used without further purification. Water used to prepare the solution of nonelectrolytes was twice distilled, the second time from $KMnO_4$ in borosilicate apparatus.

Permeability coefficients to nonelectrolytes were measured by the osmotic method developed by Hill and Cohen (1972). A full description of the experimental method is given in the previous paper (Cohen, 1975). Briefly, liposomes were prepared in a 20 mM KCl solution and 0.18 ml of this suspension (adjusted to a constant optical density) were rapidly mixed with 2.5 ml of 100 mM nonelectrolyte solution. The time course of the optical changes were monitored by light scattering at 450 nm, graphically and by digital print-out of the voltage analogue ψ of the transmittance at 1-sec time intervals or slower. Permeability coefficients were calculated from the linear portion of the volume

changes following the minimum volume point by using the sequence of voltage readings corresponding to the portion where $d\psi/dt$ is a constant.

The permeability coefficients reported are referred to as P values. They are the average values of the measurements of the slope in voltage units per second, for different liposome preparations but having the same initial optical density.

Since the surface areas of different liposome preparations are not known, no attempt has been made to calculate the absolute value for the permeabilities. Instead, relative permeability values are calculated using urea as a reference solute; hence P_{rel} for urea is equal to one.

Finally, it is necessary to point out that when nystatin and gramicidin A are incorporated into liposomes the coupling term between water and those solutes that cross the membranes via these pathways can no longer be neglected as is done for the homogeneous membrane systems. In consequence, the measured permeability values for such solutes cannot be considered as true permeability coefficients.

Results

The permeability of liposomes for a range of nonelectrolytes was measured in the following systems (the lipid compositions are given in molar ratios):

(a) 96% egg-lecithin/4% phosphatidic acid (PA).

(b) 81% egg-lecithin/15% cholesterol/4% phosphatidic acid.

(c) 66% egg-lecithin/30% cholesterol/4% phosphatidic acid.

(d) 48% egg-lecithin/48% cholesterol/4% phosphatidic acid.

(e) 48% dipalmitoyl-lecithin/48% cholesterol/4% dipalmitoyl-phosphatidic acid (DPL-PA).

(f) 48% cerebrosides/48% cholesterol/4% dipalmitoyl-phosphatidic acid.

(g) 48% cerebrosides/24% egg-lecithin/24% cholesterol/48% dipalmitoyl-phosphatidic acid.

(h) 24% cerebrosides/24% egg-lecithin/48% cholesterol/4% dipalmitoyl-phosphatidic acid.

The permeability measurements in systems (a) to (d) were carried out at 10 and at 30 °C in systems (e) to (h). The slopes of the swelling curve after the minimum volume in voltage units per second ($d\psi/dt$) are tabulated in Tables 1 and 2 for each of the above systems. Relative permeabilities were calculated with respect to urea. The number of determinations for each solute is in parentheses.

The results in Tables 1 and 2 indicate that the order of permeation of different nonelectrolytes in the various liposome systems investigated is the same, regardless of the lipid composition, the amount of cholesterol or the temperature. This order of permeation follows the corresponding partition coefficients of the solutes in a lipid solvent such as olive oil. When the

Table 1

Solute	96% Egg-lecithin/ 4% phosphatidic acid		81% Egg-lecithin/ 15% cholesterol/ 4% phosphatidic acid		66% Egg-lecithin/ 30% cholesterol/ 4% phosphatidic acid		48% Egg-lecithin/ 48% cholesterol/ 4% phosphatidic acid	
	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}
Urea	0.335 ±0.05 (11)	1.0	0.20 ±0.05 (5)	1.0	0.1 ±0.01 (6)	1.0	0.032 ±0.005 (16)	1.0
Methylurea	0.82 ±0.1 (10)	2.4	0.53 ±0.05 (6)	2.7	0.3 ±0.05 (3)	3.0	0.11 ±0.01 (12)	3.3
Ethylurea	1.17 ±0.2 (9)	3.5	0.74 ±0.1 (5)	3.7	0.36 ±0.05 (3)	3.6	0.19 ±0.01 (7)	5.9
Propylurea	1.23 ±0.2 (3)	3.7	0.84 ±0.1 (5)	4.2			0.25 ±0.04 (4)	7.8
Isopropylurea			0.88 ±0.2 (3)	4.4			0.20 ±0.04 (4)	6.3
Butylurea	1.75 ±0.2 (10)	5.2	1.11 ±0.2 (6)	5.6			0.38 ±0.05 (3)	11.9
1,3-Dimethylurea	1.32 ±0.2 (11)	3.9	0.98 ±0.1 (5)	4.9			0.32 ±0.05 (7)	10.0
1,1-Diethylurea	1.66 ±0.2 (8)	5.0	1.33 ±0.2 (4)	6.7			0.40 ±0.05 (7)	12.5
Tertbutylurea	0.68 ±0.1 (5)	2.0	0.50 ±0.1 (4)	2.5			0.21 ±0.01 (4)	6.6
Thiourea	0.40 ±0.05 (5)	1.2			0.12 ±0.05 (3)	1.2		
Hydroxyurea	0.12 ±0.02 (5)	0.36					0.008 ±0.001 (4)	0.25
Malonamide	0.12 ±0.02 (5)	0.36	0.053 ±0.01 (4)	0.27	0.027 ±0.005 (3)	0.27	0.006 ±0.001 (3)	0.2
Formamide					0.63 ±0.1 (6)	6.3	0.52 ±0.05 (10)	16.2
Acetamide					0.53 ±0.05 (7)	5.5	0.32 ±0.05 (14)	10.3
Propionamide			1.13 ±0.2 (5)	5.7	0.72 ±0.05 (7)	7.2	0.45 ±0.05 (12)	14.0
Butyramide					0.67 ±0.1 (7)	6.7	0.59 ±0.05 (10)	18.4
Valeramide			1.19 ±0.2 (4)	6.0	0.94 ±0.1 (7)	9.4	0.75 ±0.05 (12)	23.4
Succinimide							0.45 ±0.05 (5)	14.1
Dimethylformamide					0.68 ±0.1 (5)	6.8	0.65 ±0.05 (10)	20.3
Ethyleneglycol			0.93 ±0.1 (5)	4.7	0.48 ±0.1 (3)	4.8	0.35 ±0.04 (8)	11.2
1,2-Propanediol							0.51 ±0.05 (9)	16.0
Glycerol	0.25 ±0.02 (5)	0.75	0.15 ±0.03 (5)	0.75			0.015 ±0.002 (3)	0.47
Lactamide			0.58 ±0.1 (5)	2.9	0.20 ±0.1 (3)	2.0	0.09 ±0.01 (4)	2.9
Monacetin							0.12 ±0.01 (7)	3.8
Diacetin							0.27 ±0.03 (5)	8.4
Trimethylcitrate							0.36 ±0.05 (5)	11.2
Erythritol	0.035 ±0.005 (5)	0.10						

All the measurements in this Table were carried out at 10 °C.

Table 2

Solute	48% Dipalmitoyl-lecithin/ 48% cholesterol/ 4% DPL−PA		48% Cerebrosides/ 48% cholesterol/ 4% DPL−PA		48% Cerebrosides/ 24% egg-lecithin/ 24% cholesterol/ 4% DPL−PA		24% Cerebrosides/ 24% egg-lecithin/ 48% cholesterol/ 4% DPL−PA	
	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}
Urea	0.040 ± 0.002 (4)	1.0	0.011 ± 0.002 (2)	1.0	0.19 ± 0.01 (3)	1.0	0.081 ± 0.01 (3)	1.0
Methylurea	0.20 ± 0.02 (3)	5.0	0.044 ± 0.1 (2)	4.0	0.87 ± 0.1 (2)	4.6	0.23 ± 0.03 (2)	2.8
Ethylurea	0.27 ± 0.05 (4)	6.7	0.068 ± 0.1 (2)	6.2	1.22 ± 0.05 (2)	6.4	0.37 ± 0.05 (2)	4.6
Propylurea	0.33 ± 0.05 (3)	8.3	0.117 ± 0.1 (2)	10.6	1.9 ± 0.1 (2)	10	0.61 ± 0.05 (2)	7.5
Butylurea	0.46 ± 0.05 (3)	11.5	0.185 ± 0.1 (2)	16.8	2.95 ± 0.1 (2)	15.5	0.70 ± 0.1 (2)	8.6
1,1-Dimethylurea	0.46 ± 0.05 (3)	11.5	0.15 ± 0.1 (2)	13.6	2.3 ± 0.1 (2)	12.1	0.48 ± 0.05 (2)	6.0
1,3-Dimethylurea	0.48 ± 0.1 (3)	12.0	0.137 ± 0.1 (2)	12.4	1.5 ± 0.1 (2)	8.0	0.73 ± 0.1 (2)	9.0
1,1-Diethylurea	0.46 ± 0.05 (3)	11.5	0.12 ± 0.1 (2)	10.9	2.8 ± 0.1 (2)	14.7	0.78 ± 0.1 (2)	9.6
Formamide	0.57 ± 0.05 (3)	14.3	0.21 ± 0.03 (2)	19.1				
Acetamide	0.41 ± 0.05 (3)	10.3	0.145 ± 0.1 (2)	13.2	1.88 ± 0.1 (2)	10.0	0.69 ± 0.1 (2)	8.5
Propionamide	0.44 ± 0.01 (3)	11.0	0.165 ± 0.1 (2)	15.0	2.0 ± 0.1 (2)	10.5	0.83 ± 0.1 (2)	10.2
Butyramide	0.49 ± 0.05 (3)	12.3	0.23 ± 0.1 (2)	21.0	2.4 ± 0.1 (2)	12.6	0.88 ± 0.1 (2)	10.9
Valeramide	0.57 ± 0.1 (3)	14.3	0.275 ± 0.1 (2)	25.0	2.7 ± 0.1 (2)	14.2	1.1 ± 0.2 (2)	13.6
Ethyleneglycol	0.24 ± 0.02 (3)	6.0	0.067 ± 0.1 (2)	6.1				
1,2-Propanediol	0.32 ± 0.05 (3)	8.0						
Succinimide	0.27 ± 0.05 (2)	6.8						
Lactamide	0.073 ± 0.01 (2)	1.8	0.033 ± 0.1 (2)	3.0				
Trimethylcitrate	0.20 ± 0.02 (2)	5.0						

All the measurements in this Table were carried out at 30 °C.

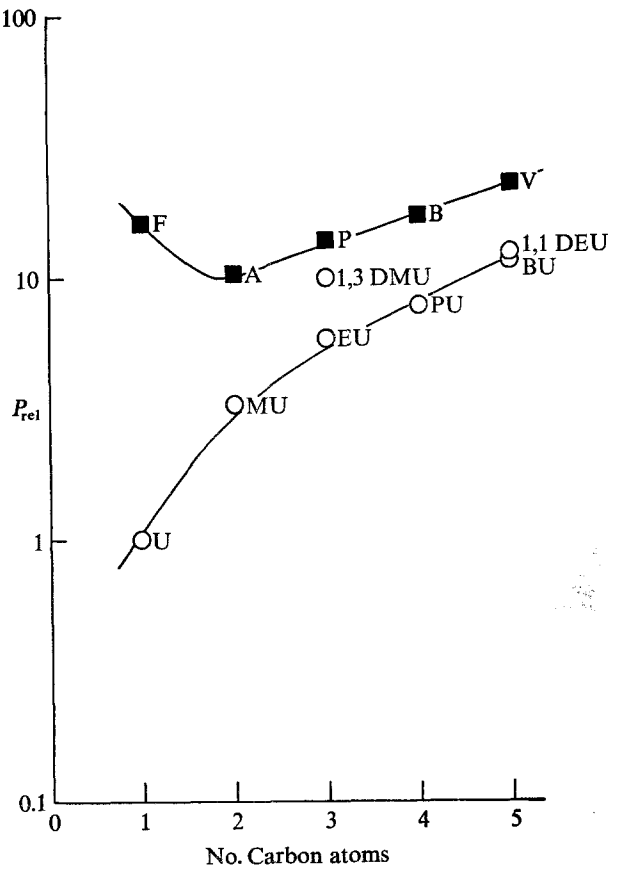

Fig. 1. Relative permeabilities for nonelectrolytes across egg-lecithin/cholesterol/PA (48:48:4) liposomes. Temperature = 10 °C. Ordinate: P_{rel} ($P_{urea} = 1$). Abscissa: number of carbon atoms. Alkyl-ureas: urea (U), methylurea (MU), ethylurea (EU), propylurea (PU), 1,3-dimethylurea (1,3 DMU), butylurea (BU), 1,1-diethylurea (1,1 DEU). Aliphatic amides: formamide (F), acetamide (A), propionamide (P), butyramide (B) and valeramide (V)

relative permeabilities obtained for the homologous series of alkyl-ureas and aliphatic amides in egg-lecithin liposomes containing 48% cholesterol (system d) are plotted (Fig. 1) against their number of carbon atoms, it is observed that there is an increase in the permeability in both series even though this increase tends to become smaller with each additional CH_2 group. The only exception is formamide which in all the systems investigated permeates faster than acetamide.

To establish how large the variations in water permeability are as a result of changes in the lipid composition, the initial rate of shrinkage of liposomes when mixed with various hypertonic KCl solutions were measured

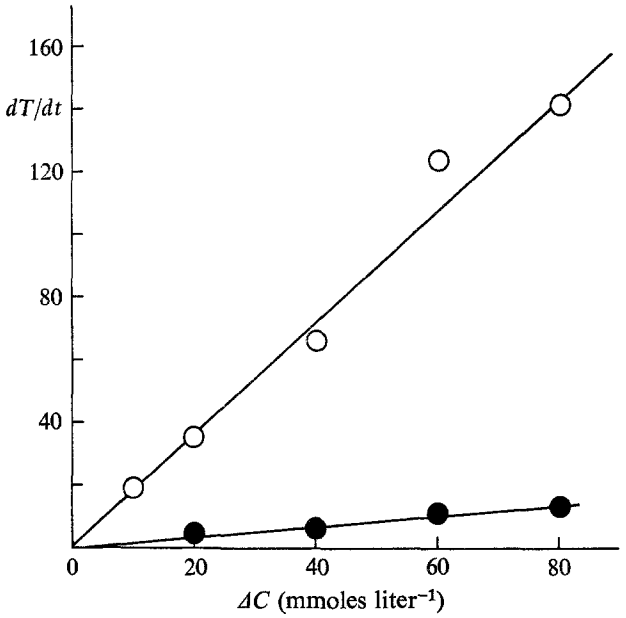

Fig. 2. The effect of the lipid composition of the liposomes on their water permeability. Temperature = 10 °C. Ordinate: the initial rate of change in the transmittance dT/dt of a liposome suspension when put into a hypertonic KCl solution. Abscissa: the difference in KCl concentration between the outside and the inside of the liposomes. ○ egg-lecithin/PA (96:4) liposomes. ● cerebrosides/cholesterol/egg-lecithin/PA (24:48:24:4) liposomes

(Fig. 2). The slopes in Fig. 2 (proportional to the water permeability) indicate that at 10 °C there is a 10-fold difference in the water rate of permeation between egg-lecithin liposomes (system a) and liposomes prepared from a mixture of cerebrosides, egg-lecithin and cholesterol (system h). The lipid composition of this latter system simulates that found in biological membranes – such as the bladder luminal plasma membrane – which also has associated a low water permeability (Ketterer, Hicks, Christodoulides & Beale, 1973).

The Effect of Nystatin on the Permeabilities to Nonelectrolytes of Liposomes Containing Cholesterol

When nystatin is added to the external aqueous phase, in a concentration of 5 μg/ml, no significant change in the rate of permeation or in the relative permeabilities takes place. If, alternatively, an equivalent amount of nystatin, dissolved in methanol, is added to the lipid phase before the liposomes are formed, there is a change in the relative permeabilities of permeation for the

Table 3. The effect of nystatin on the permeabilities of egg-lecithin/48% cholesterol liposomes (Temperature = 10 °C)

Solute	$d\psi/dt \pm$ SD (sec^{-1})	P_{rel}	$(P_{nys} - P_0)/P_0$
(a) Concentration of nystatin in the lipid phase = 0.48×10^{-2} moles/mole of lipid			
Urea	0.20 ±0.05 (21)	1.0	5.25
Methylurea	0.38 ±0.05 (12)	1.9	2.45
Ethylurea	0.26 ±0.05 (9)	1.3	0.58
Propylurea	0.62 ±0.1 (4)	3.1	
1,3-Dimethylurea	0.79 ±0.3 (7)	1.84	2.5
1,1-Diethylurea	0.68 ±0.2 (7)	3.4	1.03
Butylurea	0.86 ±0.2 (3)	4.3	
Formamide	1.33 ±0.3 (7)	6.7	1.88
Acetamide	0.58 ±0.3 (12)	2.9	0.88
Propionamide	0.6 ±0.2 (8)	3.0	0.78
n-Butyramide	1.12 ±0.1 (7)	5.6	1.20
n-Valeramide	1.76 ±0.1 (7)	8.8	1.53
Lactamide	0.24 ±0.05 (3)	1.2	1.35
Ethyleneglycol	0.95 ±0.1 (3)	4.8	1.17
1,2-Propanediol	1.22 ±0.1 (3)	6.1	1.02
Monacetin	0.27 ±0.05 (3)	1.4	0.64
Diacetin	0.5 ±0.05 (3)	2.5	0.50
Dimethylformamide	1.64 ±0.1 (3)	8.2	
Succinimide	1.52 ±0.1 (3)	7.6	1.1
Trimethylcitrate	0.42 ±0.05 (3)	2.1	−0.16
Glycerol	0.095 ±0.005 (12)	0.47	5.33
Thiourea	0.24 ±0.05 (4)	1.2	5.32
Malonamide	0.05 ±0.1 (3)	0.25	6.69
(b) Concentrations of nystatin in the lipid phase = 0.96×10^{-2} moles/mole of lipid			
Urea	0.23 ±0.05 (4)	1.0	6.2
Methylurea	0.40 ±0.05 (4)	1.7	2.6
Ethylurea	0.15 ±0.05 (4)	0.7	−0.21
1,3-Dimethylurea	0.50 ±0.05 (4)	2.2	0.56
1,1-Diethylurea	0.29 ±0.05 (3)	1.3	0.28
Formamide	1.5 ±0.2 (3)	6.5	1.9
Glycerol	0.087 ±0.01 (3)	0.40	4.8

The number of permeability measurements for each solute is in parentheses.

different solutes. Table 3 gives the values of the permeabilities obtained in an egg-lecithin/48% cholesterol system when 0.48×10^{-2} moles of nystatin per mole of lipid is present in the lipid phase. It can be seen in Table 3 that the order of permeation of different solutes is changed. Thus, the relative permeabilities for homologous series of alkylureas now show a minimum at ethylurea. An analysis of the relative increase in the permeabilities induced by nystatin shows some characteristics of this pathway. Thus, if $(P_{nys} - P_0)/P_0$

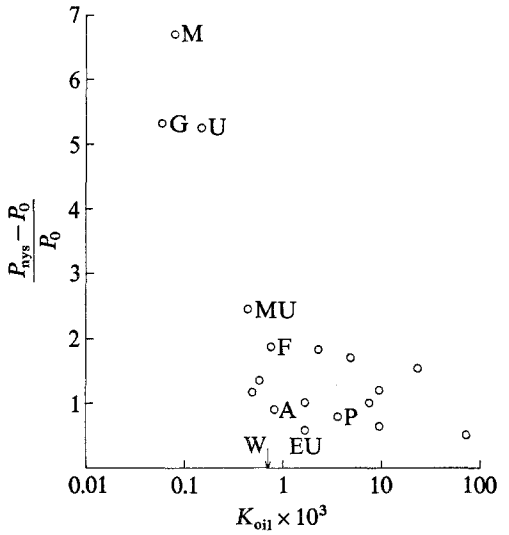

Fig. 3. The effect of nystatin on the permeability of nonelectrolytes across egg-lecithin/cholesterol/PA (48:48:4) liposomes. Temperature = 10 °C. Ordinate: relative increase in the permeability $(P_{nys} - P_0)/P_0$ induced by nystatin. Abscissa: the olive oil/water partition coefficients of the solutes. The partition coefficient of water in olive oil (Collander, 1954) is indicated by an arrow. Malonamide (M), glycerol (G), urea (U), methylurea (MU), formamide (F), acetamide (A), propionamide (P), ethylurea (EU). Thiourea was not plotted because the partition coefficient of this solute in olive oil appears to be too high (*see* Table 6)

where P_0 is the permeability of nystatin-free liposomes (Table 3), are plotted against the corresponding olive oil/water partition coefficients for the different solutes, it is observed that only the more hydrophilic solutes, i.e. those having a partition coefficient in olive oil less than the K_{oil} for water, are substantially using the route of permeation provided by nystatin (Fig. 3). These solutes are malonamide, glycerol, urea, thiourea and methylurea. The same pattern of relative permeabilities was obtained when the amount of nystatin in the lipid phase was doubled (Table 3).

Finally, similar alterations in the relative permeabilities to different nonelectrolytes was obtained when nystatin was added to the dipalmitoyllecithin system containing 48% cholesterol (Table 4). In this case, the concentration of nystatin in the lipid phase was 1.4×10^{-2} moles of nystatin per mole of lipid, but the measurements were carried out at 30 °C. It has been seen in Table 4 that in spite of the relatively large concentration of antibiotic used, the ratios $(P_{nys} - P_0)/P_0$ are smaller than in the egg-lecithin systems.

It is important to point out that all these nystatin-treated liposomes are impermeable to glucose.

Table 4. The effect of nystatin on the permeabilities of dipalmitoyl-lecithin liposomes containing 48% cholesterol (Temperature = 30 °C)

Solute	$d\psi/dt \pm$ SD	P_{rel}	$(P_{nys} - P_0)/P_0$
Urea	0.09 ± 0.01 (7)	1.0	1.25
Methylurea	0.25 ± 0.01 (5)	2.8	0.25
Ethylurea	0.26 ± 0.01 (5)	2.9	0
Propylurea	0.30 ± 0.02 (2)	3.3	0
1,3-Dimethylurea	0.49 ± 0.02 (3)	5.4	0
1,1-Dimethylurea	0.43 ± 0.02 (3)	4.7	0
Formamide	0.95 ± 0.1 (3)	10.6	0.66
Acetamide	0.67 ± 0.1 (3)	7.4	0.63
Propionamide	0.41 ± 0.05 (3)	4.6	0
Valeramide	0.58 ± 0.05 (3)	6.4	0

Concentration of nystatin in the lipid phase = 1.4×10^{-2} moles/mole of lipid.

Relative Permeabilities to Nonelectrolytes in Gramicidin-Containing Liposomes

Liposomes prepared with saturated phospholipids and gramicidin A are osmotically sensitive structures below their transition temperature in contrast to such liposomes free of gramicidin A (Cohen, 1975). In consequence, the selectivity of this system to the permeation of nonelectrolytes was thought to be of some interest. For this purpose, liposomes were prepared from a mixture of 96% dimyristoyl-lecithin (DML) and 4% dicetylphosphate (DCP) or 96% dipalmitoyl-lecithin (DPL) and 4% dipalmitoyl-phosphatidic acid (DPL-PA). Gramicidin A was added to the lipid phase, before the liposomes were formed, in a concentration of 100 μg per moles of lipid dissolved in methanol. The liposomes were made in a 20 mM KCl solution. DPL liposomes were prepared at 50 °C and DML liposomes at 30 °C.

The values of the relative permeabilities to different solutes across DML-gramicidin A liposomes at 10 °C are tabulated in Table 5 (P_{rel} for urea is equal to 1) and it can be seen that the permeability for formamide is 12.9 times greater than acetamide in contrast to only 1.6 times in the egg-lecithin systems containing 48% cholesterol at the same temperature (Table 1). Furthermore, the homologous series of alkyl-ureas now have a minimum at methylurea.

When the relative permeabilities for the homologous series of alkyl-ureas and aliphatic amides are plotted against the number of carbon atoms (Fig. 4) it will be observed that they tend to increase with the introduction

Table 5. Relative permeabilities of nonelectrolytes across liposomes containing gramicidin A below their transition temperature

Solute	96% Dimyristoyl-lecithin/ 4% DCP (T=10 °C) (P_{rel})	96% Dipalmitoyl-lecithin/ 4% DPL−PA (T=30 °C) (P_{rel})
Urea	1.0 ±0.1 (19)	1.0 ±0.1 (8)
Methylurea	0.86±0.1 (12)	1.9 ±0.3 (3)
Ethylurea	0.9 ±0.1 (11)	3.0 ±0.5 (3)
Propylurea		6.2 ±0.5 (2)
1,1-Dimethylurea	1.5 ±0.1 (3)	
1,3-Dimethylurea	1.2 ±0.2 (3)	
1,1-Diethylurea	1.9 ±0.2 (3)	
Butylurea	2.1 ±0.2 (3)	8.4 ±0.5 (3)
Formamide	24.0 ±5.0 (10)	90.0 ±10 (6)
Acetamide	1.95±0.3 (14)	6.2 ± 2.0 (9)
Propionamide	2.6 ±0.5 (3)	10.5 ± 1.0 (10)
Butyramide	2.9 ±0.5 (3)	16.1 ± 2.0 (11)
Isobutyramide		10.7 ± 1.0 (2)
Valeramide	7.9 ±1.0 (4)	28.5 ± 3.0 (9)
Isovaleramide		7.9 ± 0.5 (3)
Ethyleneglycol	1.5 ±0.1 (3)	5.8 ± 0.5 (2)
1,2-Propanediol	3.0 ±0.2 (3)	11.9 ± 0.5 (2)
Monacetin	0.69±0.1 (3)	4.7 ± 0.5 (3)
Diacetin	0.55±0.1 (3)	4.3 ± 0.5 (3)
Trimethylcitrate	0.54±0.1 (2)	5.7 ± 0.5 (2)
Erythritol	0.08±0.01 (2)	
Thiourea	0.48±0.15 (6)	1.3 ± 0.1 (2)
Dimethylformamide	10.4 ±2.0 (2)	41.9 ± 5.0 (5)
Dimethylsulphoxide	1.7 ±0.1 (2)	21.0 ± 3.0 (5)
Malonamide	0.21±0.05 (5)	
Glycerol	0.23±0.05 (6)	0.47± 0.05 (2)

of CH_2 groups instead of decreasing as in the previous systems studied (*see* Fig. 1).

The values for the relative permeabilities in the DPL-gramicidin A liposomes at 30 °C are also listed in Table 5. At this temperature, formamide remains the most permeable solute but no minimum in the urea series is observed.

It is also interesting to note that such liposomes show a substantial discrimination for branched solutes. Thus, the permeabilities of valeramide and butyramide are 3.6 and 1.5 times larger than for isovaleramide and isobutyramide, respectively (Table 5). Such discrimination was not observed in egg-lecithin liposomes.

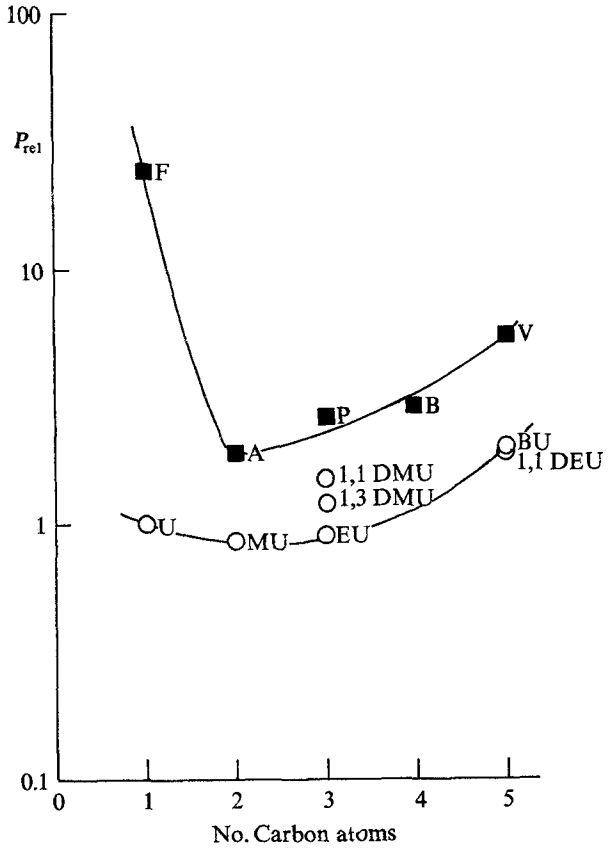

Fig. 4. Relative permeability values for nonelectrolytes across dimyristoyl-lecithin/DCP (96:4) liposomes containing gramicidin A. Temperature = 10 °C. Ordinate: P_{rel} (P_{urea} = 1). Abscissa: number of carbon atoms. ○ alkylureas, ■ aliphatic amides. 1,1-dimethylurea (1,1-DMU). The code for the other solutes is the same as in Fig. 1

Analysis of the Permeability Data

The free energy of activation for the process of permeation across a continuous lipid bilayer can be separated into the following number of events (Cohen, 1975),

$$\overset{+}{\Delta G} = \overset{+}{\Delta G_{ads}} + N_H \overset{+}{\Delta G_H} + \overset{+}{\Delta G_{dif}}$$

where $\overset{+}{\Delta G_{ads}}$ and $\overset{+}{\Delta G_{dif}}$ are the free energy contributions to the adsorption at the lipid membrane/water interphase and to the diffusion through the hydrocarbon chains, respectively, that are not due to hydrogen bonds. N_H is the number of potential hydrogen bonds that a solute can form in water and ΔG_H is the free energy of activation per H-bond.

Table 6. Physical-chemical properties of nonelectrolytes

Solute	Number of hydrogen bonds (N_H)	Cylindrical[a] radius (r)	Olive oil/water[b] partition coefficient $\times 10^3$	Density[c]	Molar[d] volume (cm^3 $mole^{-1}$)
Urea	5	2.41	0.15	1.335	45.0
Methylurea	4	2.60	0.44	1.204	61.5
Ethylurea	4		1.7	1.213	72.6
1,3-Dimethylurea	3	2.70		1.142	77.1
1,1-Dimethylurea	3		2.3	1.255	70.1
1,1-Diethylurea	3		7.6	1.155	100.5
Thiourea	5	2.86	1.2	1.405	54.2
Hydroxyurea	6				
Formamide	3	2.07	0.76	1.134	40.0
Acetamide	3	2.38	0.83	0.998	59.2
Propionamide	3	2.61	3.6	1.042	70.1
Butyramide	3	2.68	9.5	1.032	84.4
Isobutyramide	3	2.97		1.013	86.0
Valeramide	3	2.75		1.023	98.8
Isovaleramide	3	3.08	23.0	0.965	104.8
Lactamide	5		0.58	1.138	78.3
Malonamide	6	2.78	0.08		69.3
Succinimide	3		4.9	1.412	70.2
Dimethylformamide	2		4.9	0.948	77.1
Ethyleneglycol	4	2.38	4.9	1.126	55.1
1,2-Propanediol	4	2.80	1.7	1.038	73.3
Glycerol	6	3.03	0.07	1.261	73.0
Monacetin	5		9.5	1.206	111.2
Diacetin	4		71.0	1.178	149.6
Triacetin	3		440.0	1.16	188.1
Trimethylcitrate	2		47.0		
Erythritol	8		0.03		98.4[e]

[a] Taken from Soll (1967) and Sha'afi *et al.* (1971).
[b] Taken from Collander and Bärlund (1933) and Collander (1954).
[c] Handbook of Chemistry and Physics, The Chemical Rubber Co., 51st Edition, 1971; and International Critical Tables.
[d] Calculated by dividing the molecular weights of the solutes by their density.
[e] Calculated from atomic volumes (Partington, 1951).

The rate of permeation will be proportional to the product of the probability of occurrence of the successive events,

$$P=\lambda^2 \frac{kT}{hl} \exp\left(-\Delta G^{+}_{ads}/RT\right) \exp\left(-N_H \Delta G^{+}_{H}/RT\right) \exp\left(-\Delta G^{+}_{dif}/RT\right) \qquad (1)$$

where λ is the distance between two successive equilibrium positions in the membrane (Zwolinski, Eyring & Reese, 1949), l is the thickness of the membrane, R is the gas constant, T is the absolute temperature, k is the Boltzman constant and h is the Planck constant.

Since the total rate of permeation has been the only parameter that has been measured in the present work, the relative contribution of each of these steps cannot be evaluated separately. This difficulty can be partially overcome by the use of a model system that simulates the first two events, the so-called partitioning step. An alternative procedure is to analyze the changes in permeability produced by changes in the lipid composition of the membranes, i.e. cholesterol, or by changes in the capability of the solutes to form hydrogen bonds in water (Table 6).

The Dependence of the Permeability Values on the Number N_H of Hydrogen Bonds

The logarithms of the measured permeabilities for permeation across egg-lecithin liposomes with different amounts of cholesterol (Table 1) are correlated with the number N_H of H-bonds per molecule (Fig. 5). According to Eq. (1), the slopes of such correlation give an average value for the free energy change per H-bond, $\overset{+}{\Delta G_H}$. Table 7 gives the calculated values for each of those systems obtained by the least-squares method. It can be observed that $\overset{+}{\Delta G_H}$ increases slightly with the amount of cholesterol in the membrane.

The presence of cholesterol in an egg-lecithin bilayer can modify the permeability of a solute by changing any of the parameters in Eq. (1). Thus,

$$\frac{\delta \log P}{\delta[\mathrm{CHOL}]} = \frac{\delta \overset{+}{\Delta G}_{ads}/RT}{\delta[\mathrm{CHOL}]} + \frac{N_H \delta \overset{+}{\Delta G_H}/RT}{\delta[\mathrm{CHOL}]} + \frac{\delta \overset{+}{\Delta G}_{dif}/RT}{\delta[\mathrm{CHOL}]}. \tag{2}$$

In fact, the average values of measured permeabilities across egg-lecithin membranes show an exponential decrease with increasing amounts of cholesterol (Fig. 6). It can be noted in Fig. 6 that the rate of decrease in $\log P$ with the cholesterol concentration in the membrane varies with the type of solute. The magnitude of the slopes for some solutes are tabulated in Table 8.

The slopes for different solutes in Table 8 are linearly correlated with the number N_H of H-bonds that a solute can form in water (Fig. 7). The data were adjusted by the least-squares method. For 13 solutes, the regression coefficient was 0.93.

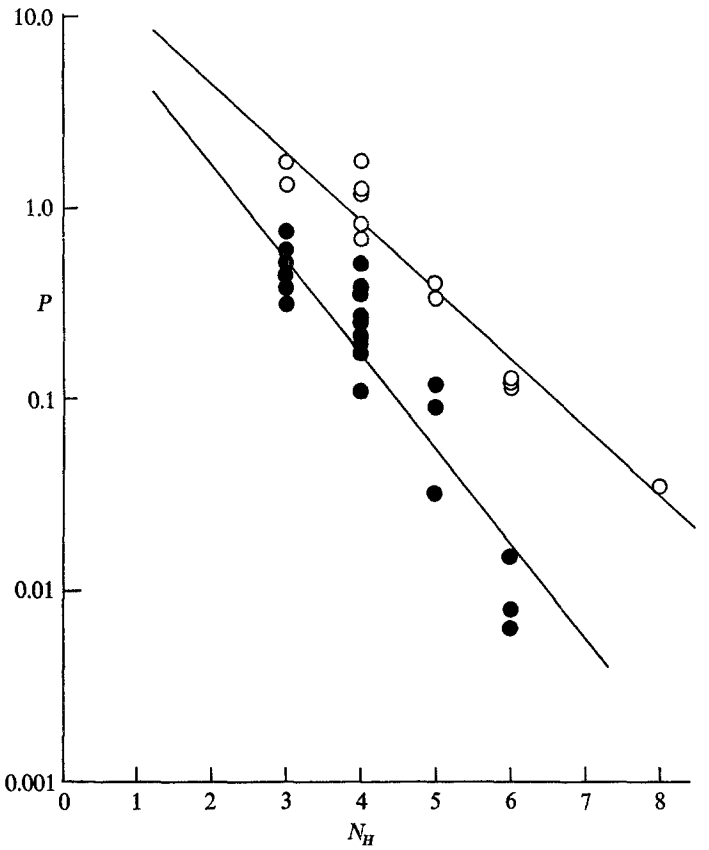

Fig. 5. The relation between the permeability values of nonelectrolytes across liposomes and the capacity of the solutes to form hydrogen bonds in water. Ordinate: permeability values. Abscissa: number of hydrogen bonds. ○ egg-lecithin/PA (96:4) liposomes. ● egg-lecithin/cholesterol/PA (48:48:4) liposomes

Table 7. Activation free energy changes per hydrogen bond across different membrane systems

System	n[a]	$\Delta G_H^{\ddagger}$ (cal/mole)
96% Egg-lecithin/4% PA	13	468.4±37
81% Egg-lecithin/4% PA/15% cholesterol	14	485.3±79
66% Egg-lecithin/4% PA/30% cholesterol	12	539.3±54
48% Egg-lecithin/4% PA/48% cholesterol	23	643.5±64

[a] n is the number of solutes used in the calculation.

According to Eq. (2), the slope in Fig. 7 represents the change in free energy per H-bond when cholesterol is introduced into an egg-lecithin membrane. From this slope it can be claculated that when 48% cholesterol

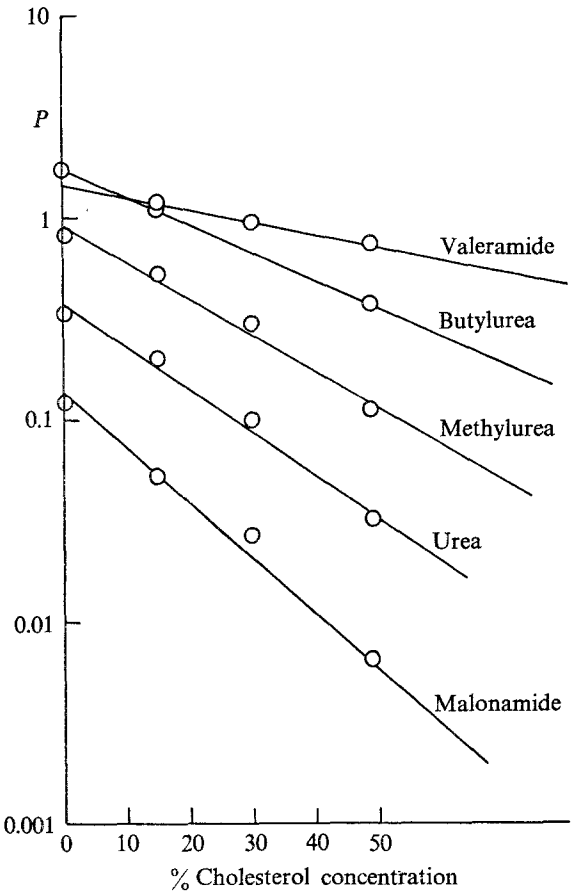

Fig. 6. The effect of cholesterol on the permeability of nonelectrolytes across egg-lecithin liposomes. Ordinate: logarithms of the permeability values. Abscissa: concentration of cholesterol (in moles %) in the membrane

Table 8. Rate of change of the logarithm of the permeabilities with the cholesterol concentration in the membrane

Solute	N_H	$\delta \log P/\delta$ [CHOL]
Glycerol	6	−0.0273
Malonamide	6	−0.0265
Lactamide	5	−0.0243
Urea	5	−0.0212
Methylurea	4	−0.0181
Ethylurea	4	−0.0168
Propylurea	4	−0.0146
Butylurea	4	−0.0138
Ethyleneglycol	4	−0.0126
1,3-Dimethylurea	3	−0.0131
1,1-Diethylurea	3	−0.0133
Propionamide	3	−0.0120
Valeramide	3	−0.0060

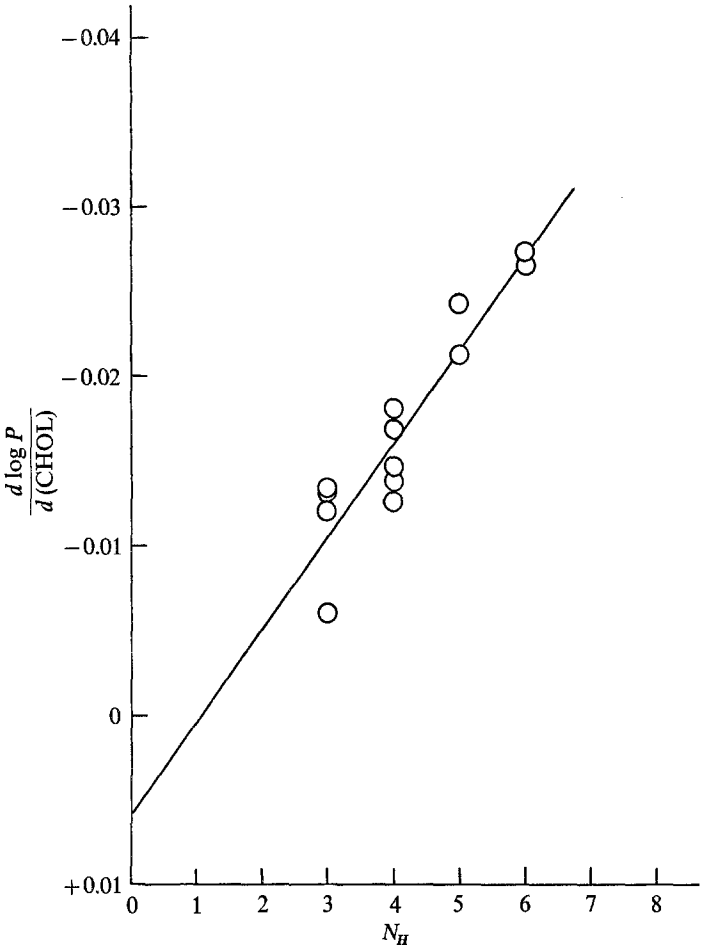

Fig. 7. The relation between the rate of change in the logarithms of the permeabilities with cholesterol concentration in the membrane and the capacity of the solutes to form hydrogen bonds in water. Ordinate: $d \log P/d(\mathrm{CHOL})$. Abscissa: number of hydrogen bonds

is incorporated, there is a change of about 370 cal/mole per H-bond. Since the enthalpy change per H-bond does not appear to change with cholesterol (Cohen, 1975), the resulting entropy change is about 1.3 entropy units per H-bond at 10 °C.

The value of the intercept of Fig. 7 indicated the extent by which the free energy for the other permeation steps are affected by cholesterol. The small value obtained suggests that the reported increase in the activation energy for permeation with cholesterol (Cohen, 1975) is nearly compensated by a concomitant change in entropy. On the other hand, for a molecule

that does not make hydrogen bonds in water, such as a hydrocarbon, this intercept predicts a small but positive increase in permeability with cholesterol. Thus, when 48% cholesterol is introduced into egg-lecithin membranes, the permeability to a hydrocarbon may increase by 1.9 times. However, it is uncertain if this increase is statistically significant.

The Use of Olive oil/Water Partition Coefficients as an Estimation of the Partition Properties of Solutes in Lipid Membranes of Different Composition

Partition coefficients of solutes in lipid solvents are generally used to simulate the corresponding values in lipid membranes, since these are not known. To evaluate the extent in which the partition data in a solvent system is adequate for a given membrane, the following test can be carried out (Lieb & Stein, 1969; Stein & Nir, 1971).

The permeability coefficient is written as

$$\log P = \log P_0 + S_k \log K_{\text{est}} - S_m \log M \tag{3a}$$

or

$$\log P = \log P_0 + S_k \log K_{\text{est}} - S_m M \tag{3b}$$

where K_{est} is the partition coefficient of a solute in a solvent system, S_m is the mass coefficient for diffusion across the membrane, S_k is a coefficient which measures the extent of the deviation of K_{est} from the real K in the membrane, M is the molecular weight of the solute. S_k will be equal to one when the partition in the solvent system is the same as that of the membrane.

The magnitude of S_k and S_m for a given set of permeability data, partition coefficients in a solvent system K_{est} and molecular weights, can be obtained by the least-squares method. Table 9 gives the values of S_k and S_m obtained for the present permeability data. The olive oil/water partition coefficients obtained by Collander and Bärlund (1933) and Collander (1954) are used.

It can be seen in Table 9 that S_k is 0.8 for egg-lecithin membranes containing 48% cholesterol but decreases to 0.6 in the other cholesterol-containing systems. For membranes treated with nystatin and gramicidin A, the value of S_k is even lower suggesting the presence of a more hydrophilic phase than olive oil.

It can be considered that olive oil is a reasonably good solvent for the egg-lecithin membranes with 48% cholesterol. Then, for this case, the

Table 9. Least-squares estimates of parameters of Eq. (3)

System	log M [Eq. (3a)]			M [Eq. (3b)]		
	S_m	S_k	SD	S_m	S_k	SD
96% Egg-lecithin/ 4% PA (9)	−2.3	0.6	0.24	−0.012	0.6	0.23
81% Egg-lecithin/ 4% PA/ (8) 15% cholesterol	−1.3	0.6	0.22	−0.007	0.5	0.22
66% Egg-lecithin/ 4% PA/ (11) 30% cholesterol	−2.2	0.6	0.15	−0.014	0.6	0.15
48% Egg-lecithin/ 4% PA/ (20) 48% cholesterol	−2.6	0.8	0.19	−0.009	0.8	0.24
48% Egg-lecithin/ 4% PA/ (19) 48% cholesterol/ 0.48% nystatin	−2.4	0.55	0.18	−0.009	0.55	0.20
96% Dimyristoyl-lecithin/4% DCP gramicidin A (20)	−3.3	0.5	0.23	−0.012	0.5	0.30

The number in parentheses is the number of solutes used in the computation. SD is the standard deviation.

permeability can be expressed as,

$$P = K_{oil} D_m / l$$

where D_m is the diffusion coefficient in the membrane. Using this equation, relative diffusion values can be calculated and their dependence on the size of the solute investigated.

The logarithms of these calculated diffusion values P_{rel}/K_{oil} are plotted in Fig. 8*a* against the molar volume (Table 6) of the solutes. It can be observed that all members of the different homologous series fall closely in the same line. Figs. 8*b*, 8*c* and 8*d* illustrate the types of deviations that are found when olive oil/water partition coefficients are used to simulate the partition of the other membrane systems studied. Thus, in egg-lecithin liposomes without cholesterol (Fig. 8*b*), the P_{rel}/K_{oil} values for the hydrophilic solutes, malonamide (M), glycerol (G) and erythritol (E) are higher than for the members of the urea homologous series at equal molar volume. It can be seen that the same type of deviations occurs in the saturated liposomes

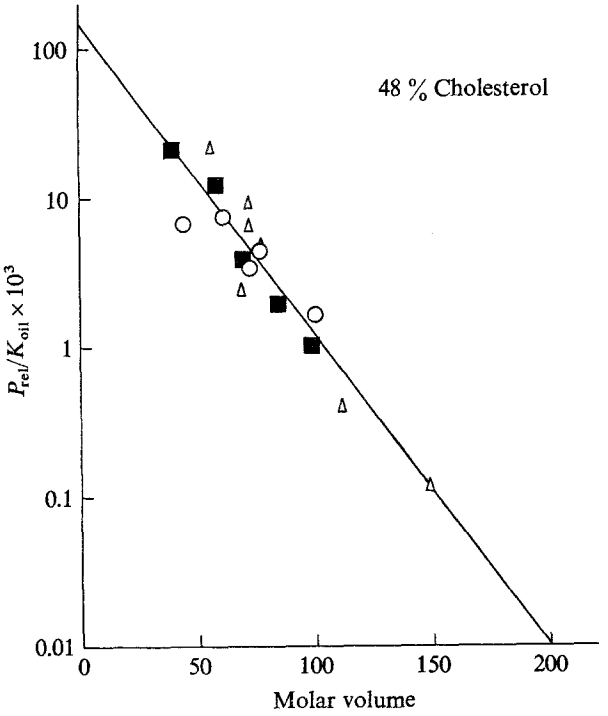

Fig. 8a

Fig. 8. Diffusion of nonelectrolytes across different liposome systems estimated as the ratio of the measured permeability values to their olive oil/water partition coefficients. Ordinate: P_{rel}/K_{oil} ($P_{urea}=1$). Abscissa: molar volume of the solutes. (*a*) Egg-lecithin/cholesterol/PA (48:48:4) liposomes. (*b*) Egg-lecithin/PA (96:4) liposomes. (*c*) Dimyristoyl-lecithin/DCP (96:4) liposomes containing gramicidin A. (*d*) Egg-lecithin/cholesterol/PA (48:48:4) liposomes containing nystatin. ○ alkyl-substituted urea, ■ aliphatic amides, △ other solutes. Malonamide (M), glycerol (G), erythritol (E), formamide (F)

containing gramicidin A (Fig. 8*c*). In this system, the P_{rel}/K_{oil} value for formamide (F) is also higher than expected from its molar volume. Finally, no apparent large deviations are observed in the egg-lecithin/48% cholesterol system in the presence of nystatin (Fig. 8*d*).

Discussion

The Effect of Cholesterol on the Permeability

The presence of cholesterol on egg-lecithin membranes produces a selective decrease in the permeability to nonelectrolytes. Thus, whereas the permeability to malonamide is reduced by 20 times on addition of 48%

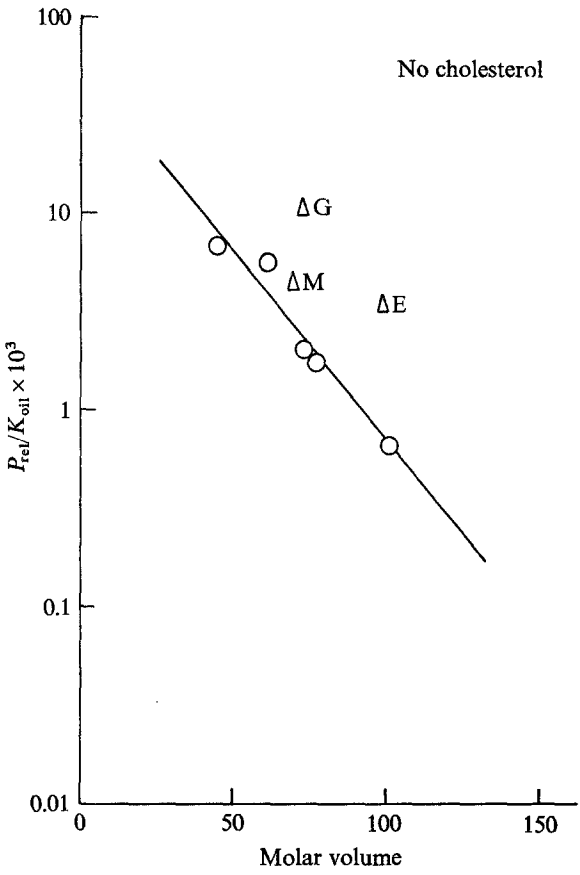

Fig. 8b

cholesterol, the permeability to 1,1-diethylurea is only reduced by 4.1 times (Table 1). The reduction in water permeability obtained by interpolation of the data in Fig. 7 is 5.9 times. This value should be compared with measurements of the water permeability of thin lipid films which have indicated that the rate of permeation is 5.6 times lower when the solution from which the thin lipid system is formed has an 8:1 cholesterol to egg-lecithin molar ratio (Finkelstein & Cass, 1967).

From the magnitude of such permeability changes, it can be considered that a variation in the membrane thickness exerts a very small contribution to the overall effect: the thickness of an egg-lecithin bilayer increases by a few angstroms when cholesterol is incorporated (Lecuyer & Dervichian, 1969).

The incorporation of cholesterol into egg-lecithin liposomes alters the permeability by affecting the dehydration step more than the two other terms

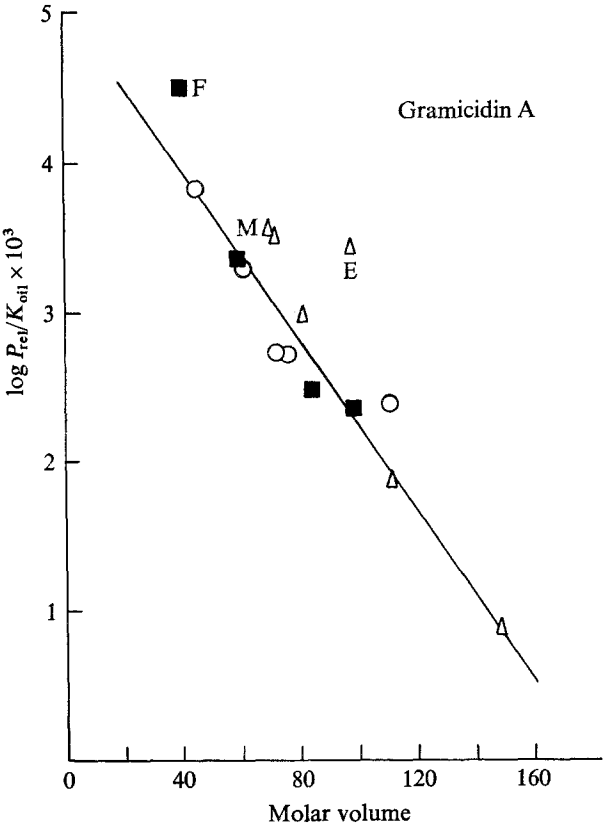

Fig. 8c

in Eq. (1). Thus, it has been shown that such changes in permeability are related to the capacity of the solutes to form hydrogen bonds in water and that the increase in the activation energy for permeation across cholesterol-containing membranes is nearly compensated for by an increase in entropy.

The precise portion of the bilayer where dehydration occurs cannot be specified because there is no experimental evidence about the role of hydrogen bonds in the free energy of adsorption at a lipid membrane/water interphase. However, it is interesting to note that the hydrocarbon part near the polar group is the portion of the bilayer that is most affected when cholesterol is incorporated (Hubbell & McConnell, 1971).

The Nature of the Partition Process of Nonelectrolytes in a Lipid Membrane/Water System

The rate of transfer of a solute from the aqueous phase to the hydrocarbon part of a lipid bilayer can be considered as dependent on the same

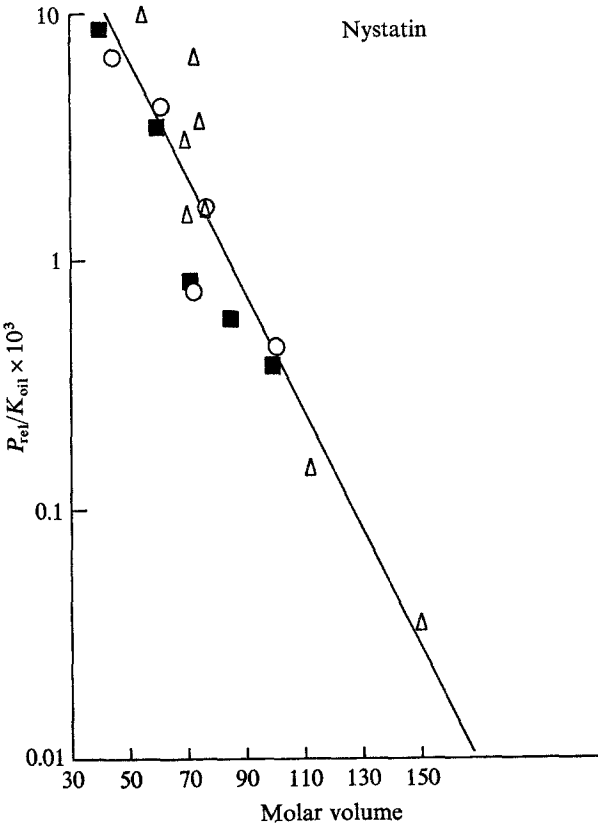

Fig. 8d

principles as those which determine the equilibrium of a solute between two immiscible solvents.

Since the work of Collander and Bärlund (1933), partition coefficients of solutes in olive oil have been used to simulate the partition properties of solutes in biological membranes. Olive oil is composed of a mixture of triglicerides containing oleic acid (83.4%) and palmitic acid (9.4%). The olive oil/water partition coefficients for most of the solutes whose permeability has been measured in the present work are tabulated in Table 6. The free energy of partition for those solutes can be calculated from the relation

$$K = \exp(-\Delta G_{par}/RT).$$

Although the partition coefficient K is dimensionless, its value depends on the method used to measure the concentration. For thermodynamic calculations it is convenient to express it in moles/mole, rather than in

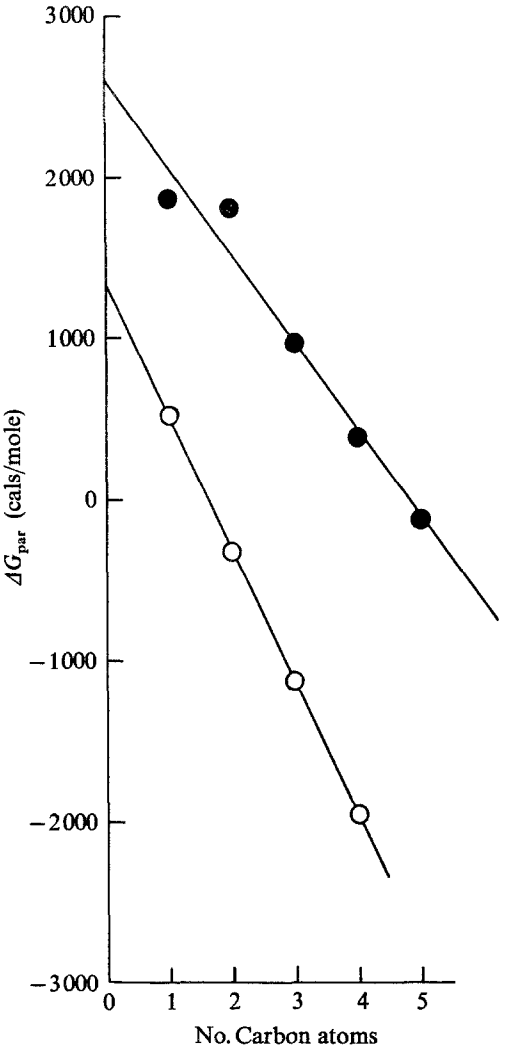

Fig. 9. Free energies for partition of aliphatic amides and alcohols plotted as a function of their number of carbon atoms. ○ alcohols, ● amides

moles/liter. This can be done by assuming that the molecular weight of olive oil is that corresponding to tri-olein with a density of 0.91. The calculated ΔG_{par}'s for partition in olive oil of the homologous series of alcohols and aliphatic amides are plotted against the number of carbon atoms in Fig. 9. It can be seen that there is a constant decrease in ΔG_{par} when a CH_2 group is introduced into a molecule. The ΔG_{par} per CH_2 group varies from 823.4 cal/mole for the alcohol series to 541.9 cal/mole for the amides. Extrapolation to zero number of carbon atoms gives the change in free

energy necessary to transfer the polar group from the aqueous phase to the solvent. These values are 1.34 kcal/mole for an OH group and 2.61 kcal/mole for an amide group.

Free energies of partition between water and a lipid solvent can be separated in two terms,

$$\Delta G_{\text{par}} = \Delta G_{\text{ads}} + \Delta G_{I/l} \tag{4}$$

where ΔG_{ads} is the free energy of adsorption of a solute at an oil/water interphase and $\Delta G_{I/l}$ is the free energy for the transfer of the hydrocarbon chains and the polar group from the interphase to the bulk lipid solvent.

The relative contribution of the changes in enthalpy and entropy to the free energies of partition in olive oil are not known. However, Haydon and Taylor (1960) have determined the effect of temperature on the free energy of adsorption per CH_2 group of alcohols at a hydrocarbon/water interphase. They have also measured the effect of temperature on the free energy of adsorption of rather polar molecules. In both cases, they have found that this effect is relatively small. These findings have suggested that for such an interphase the ΔG_{ads} term is mainly entropic, involving only the orientation of the polar groups and the hydrocarbon chains with no substantial dehydration involved.

An important consideration for evaluating the extent of the dehydration that a solute may suffer when partitioning into a lipid solvent is the amount of water in the solvent at equilibrium. In order to illustrate this point, a list of solvents, ordered by the magnitude of their dielectric constants and the amount of water they can contain is given in Table 10.

In a solvent with a very low dielectric constant, e.g. a saturated hydrocarbon such as decane, the interactions between solute and solvent molecules would be expected to be very small and the partition coefficient would be determined by the energy required to break the hydrogen bonds between solute and water molecules. The values for the enthalpies of partition of chloroform, 1.5 kcal/mole, ethylether, 3.5 kcal/mole, and butanol, 6.0 kcal/mole, obtained by Johnson and Bangham (1969) in a decane/water system agree with this prediction. Thus, they increase with the hydrogen-bonding ability of such compounds in water. On the other hand, in a solvent with a high dielectric constant and a relatively large amount of water, e.g. octanol, the polar part of a solute may interact with hydroxyl groups in the solvent and/or water molecules around it. For partition into such a solvent, there is no need for net breaking of H-bonds. This prediction is in line with the results of Klein, Moore and Smith (1971) who found that the ΔH's for

Table 10. Dielectric constants and water content at saturation of some solvents at 20 °C

Solvent	Dielectric const.[a]	Water conc.[b] (moles/liter × 10^3)
n-Heptane	–	3.3
n-Decane	1.99	–
Benzene	2.28	26.0
Olive oil	–	72.5
Oleic acid	2.46	–
Ethyl-ether	4.33	690.0
Chloroform	4.8	–
1-Octanol	10.2	2300.0
1-Butanol	17.8	9440.0
Methanol	32.63	–
Water	8037	–

[a] Taken from Handbook of Chemistry and Physics, The Chemical Rubber Co., 51st Edition, 1971.
[b] Leo, Hansch and Elkins (1971).

partition in octanol for rather polar molecules such as aminoacids are negligible or very small.

For partition into a solvent like olive oil, it would be expected that the contribution of the enthalpy term to the free energy of partition would be higher than in octanol and much nearer to that found in saturated hydrocarbons or liposomes (Cohen, 1975), according to the parameters in Table 10.

The partition coefficients of normal alcohols in dipalmitoyl-lecithin liposomes has been measured recently by Hill (1974) using the observed decrease in the transition temperature of this system induced by the alcohols as a measure of their concentration in the lipid membranes. This work has established that the free energy for partition of alcohols in liposomes is very near to the theoretical value for a process where only the entropy of mixing of the alcohols in the hydrocarbon phase is considered, suggesting that no breaking of hydrogen bonds is involved in such a process. This behavior was similarly found for partition of alcohols in octanol, ethylether or red cell membranes. However, for partition into dodecane the free energy of partition is about 3 kcal/mole higher than the theoretical value (M. W. Hill, *personal communication*).

The "partitioning step" for the process of permeation across liposomes involves adsorption of the solute at the water/membrane interphase and dehydration of the solute. Since the adsorption step does not appear to require any substantial breaking of hydrogen bonds, it can be simulated by

the partition data in octanol or liposomes. However, to account for the whole process it is necessary to use a solvent where a substantial dehydration occurs. This appears to be the rationale behind the observation that olive oil is a good solvent to simulate the permeation properties of a solute in liposomes and biological membranes.

The Nature of the Diffusion Process Across the Hydrocarbon Part of Lipid Bilayers

After the adsorption and dehydration steps have been completed, the movement of solutes across lipid bilayers proceeds by diffusion through the hydrocarbon chains. Information about the nature of this process can be obtained by analysis of the effect of the size of the solute on diffusion across different systems. Thus, the diffusion coefficient of a solute in a liquid medium is given by the Stokes-Einstein equation as,

$$D=\frac{\kappa T}{6\pi r\eta}$$

where κ is the Boltzman constant, r is the molecular radius of the solute and η is the viscosity of the medium. When the diffusing molecules are spherical, this equation predicts that,

$$DM^{1/3}=\text{constant}$$

M being the molecular weight of the solute. This prediction has been shown to be valid for diffusion of the solutes in water provided that they have an M value larger than 100 (Stein, 1967). For smaller solutes, Stein has found empirically that the mass coefficient that best fit the data is 1/2 rather than 1/3.

Relative diffusion values for diffusion through the hydrocarbon part of liposomes can be calculated by dividing the relative values of the permeabilities by the olive oil/water partition coefficients of the solutes as has been discussed before. The slopes of the correlations between the logarithms of these calculated diffusion values and their molecular weights are given in Table 11. In this Table are also listed the corresponding slopes for diffusion of some molecules in hydrophobic polymers and biological membranes such as *Chara* (Lieb & Stein, 1969). It can be observed that, for all liposome systems studied, the mass selectivity for diffusion is much greater than for diffusion in liquids and rather similar to diffusion across rubber or *Chara* membranes. This suggests that the rate of diffusion of solutes across the

Table 11. The relation between log D and log M

System	Temp. (°C)	Slope SD
(a) Liposomes		
48% Dipalmitoyl-lecithin/ 48% cholesterol/4% DPL−PA	30	3.0±0.6
48% Egg-lecithin/ 48% cholesterol/4% PA	10	3.6±0.4
96% Dimyristoyl-lecithin/ 4% DCP+gramicidin A	10	4.4±0.6 5.2±0.5[a]
96% Dipalmitoyl-lecithin/ 4% DPL−PA+gramicidin A	30	4.0±0.4 4.1±0.5[b]
(b) Hydrophobic polymers[c]		
Natural rubber, 1.7% sulfur	40	1.21
Natural rubber, 1.15% sulfur	40	1.43
Natural rubber, 11.3% sulfur	40	2.42
(c) Biological membranes		
Chara ceratophyla[d]	20–25	3.5±0.3

[a] Formamide, malonamide, erythritol, glycerol and trimethylcitrate were not included in the correlation (Fig. 8*c*).
[b] Formamide, glycerol and trimethylcitrate were not included.
[c] Stein and Nir (1971).
[d] Lieb and Stein (1969).

hydrocarbon part of liposomes and biological membranes is regulated by the amount of free volume or 'holes' between the hydrocarbon chains, in a way analogous to the diffusion process within polymers (Lieb & Stein, 1969; Cohen & Bangham, 1972).

The mass selectivity for the diffusion process across liposomes indicates the presence of a population of 'holes' of different sizes or molar volumes. These 'holes' may be formed by appropriate combinations of $2g1$ kinks, as suggested by Träuble (Träuble, 1971).

Comparisons of the mass selectivity for diffusion across egg-lecithin-cholesterol liposomes at 10 °C and of liposomes prepared from saturated lecithins at 30 °C (Table 11) reveal that a much lower temperature is needed by the unsaturated system to exhibit the same mass selectivity. This could be accounted for by the presence of a substantial proportion of double bonds of the *cis*-type in the egg-lecithin system which increases the free volume between the hydrocarbon chains.

It can also be observed in Table 11 that the diffusion process across liposomes containing gramicidin A below their transition temperature exhibit the highest mass selectivity. This region of high selectivity is thought to correspond to the lipid part of the membrane around the gramicidin pore (Cohen, 1975). It is interesting to see that this type of selectivity is also observed in red cell membranes (Lieb & Stein, 1969; Sha'afi *et al.*, 1971).

The Dimensions of the Aqueous Pores Created by Nystatin Molecules in Liposomes

The changes in selectivity obtained by adding nystatin to cholesterol-containing liposomes have shown clearly that this antibiotic increases only the permeability to hydrophilic solutes, e.g. solutes which have a partition coefficient in olive oil less than the K_{oil} value for water (Fig. 3). This indicates that nystatin molecules interact in the membranes, with cholesterol molecules, to form an aqueous pathway, leaving the rest of the membrane virtually unchanged.

The observation that the relative permeabilities in the presence of nystatin do not appear to depend on the amount of antibiotic used, suggests that this aqueous pathway is a route of permeation of fixed dimensions, in agreement with the results obtained in previous studies of the action of nystatin in black lipid membranes (Holz & Finkelstein, 1970).

The difference found between the water filtration coefficient and the diffusional coefficient of water when nystatin is present in thin lipid films (Holz & Finkelstein, 1970) has been used to estimate an "equivalent pore radius" of 4.6 Å (Solomon & Gary-Bobo, 1972) for the nystatin pore. The creation of a pore of such dimensions in liposomes would explain why solutes such as glucose are effectively excluded. (Glucose has a Stokes-Einstein radius $\simeq 4.0$ Å.) Holz and Finkelstein (1970) have reported an 8.3-fold difference between the permeabilities of glycerol and urea across the nystatin pore. However, in the present system, there were no significant differences between the nystatin-induced permeabilities to small hydrophilic solutes such as malonamide, glycerol, urea or thiourea, suggesting that a 4 to 4.6 Å pore is rather unselective with respect to the size of these solutes if judged by their cylindrical radius or molar volumes (Table 6). Certainly this is not the case in human red cell membranes where it has been found that urea is much more permeable than thiourea, glycerol or malonamide (Naccache & Sha'afi, 1973).

One of the consequences of the observed lack of discrimination for permeation across the nystatin aqueous pore would be that solutes cross

the pore largely "hydrated" rather than "unhydrated" and with similar activation energies to their diffusion in aqueous solutions. Unfortunately, no activation energies for permeation have been determined in this system.

The Dimensions of the Aqueous Pores in Human Red Cell Membranes

The observation that the magnitude of the activation energy for water permeation across human red cell membranes is comparable to that found for diffusion in water (Vieira *et al.*, 1970) has provided evidence for the presence in these membranes of an aquous pore. However, the calculated value for the dimensions of this pore (Solomon, 1968) appears to be too large to account for the observed selectivity between small hydrophilic solutes since the present results have shown that a nystatin pore of the same dimensions is rather unselective.

On the other hand, it is interesting to compare the permeabilities of the homologous series of ureas and amides across human red cell membranes as determined by Sha'afi *et al.* (1971) with the corresponding values for

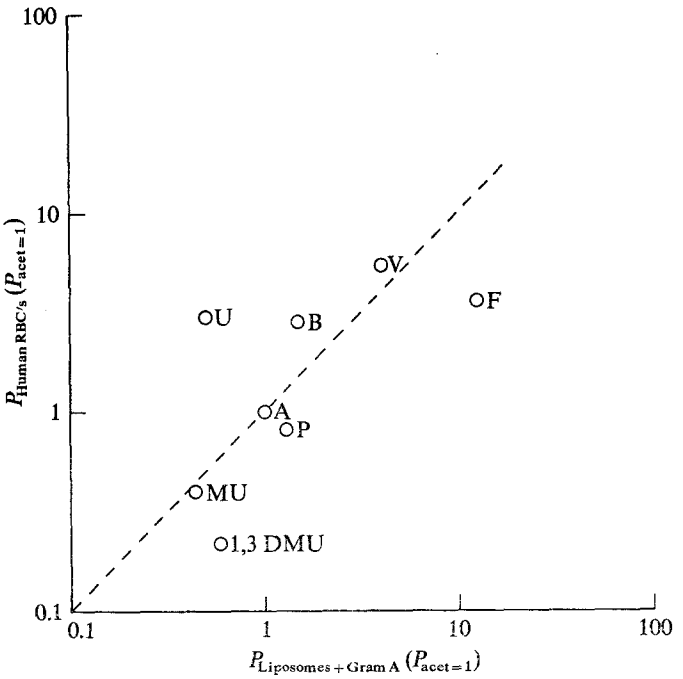

Fig. 10. Comparison between the relative permeabilities of alkyl-ureas and aliphatic amides across human red cell membranes and the corresponding values across dimyristoyl-lecithin liposomes containing gramicidin A. $P_{\text{acetamide}}=1$. The code for the different solutes is the same as in Fig. 1

those solutes across gramicidin-containing liposomes below their transition temperature where it is known that only urea and formamide (apart from water) are permeating across the gramicidin pore (Cohen, 1975) (Fig. 10). It can be observed in this Figure that the solutes that most strongly deviate from a one-to-one correlation are also urea and formamide, but they do so in opposite directions. Thus, whereas the permeability of formamide is higher in the liposome systems than in red cells, the permeability of urea is higher in red cells than in liposomes.

The increased permeability of urea across red cell membranes can be explained on the basis that this solute is permeating by a facilitated diffusion mechanism as its inhibition by phloretin has already indicated (Macey & Farmer, 1970). Since no pore for the permeation of formamide appears to be necessary, it is then concluded that only water is permeating substantially across aqueous pores in human red cell membranes. This conclusion has also been reached by Macey and Farmer (1970) from quite a different set of experimental evidence.

Conclusions

The present study of the permeability of nonelectrolytes across smectic mesophases of phospholipids (liposomes) has shown that their sequence of permeation follows an order corresponding to the partition coefficients of the solutes in a lipid solvent such as olive oil. This is precisely the sort of behavior that might be expected when considering the passage of molecules across continuous lipid thin films. However, it has also been observed that the introduction of a methyl group in formamide systematically decreases the rate of permeation whatever the lipid composition or the amount of cholesterol present. This indicates that the existence of a minimum or "sieve-like" effect in the permeability to a homologous series of nonelectrolytes is not necessarily an evidence of the presence of an aqueous pore, as has been suggested by other workers. This type of departure can be explained by a greater mass selectivity for the process of diffusion within the hydrocarbon part of lipid bilayers compared with diffusion in liquids.

The presence in liposomes of compounds that are known to form aqueous pores in lipid bilayers such as nystatin and gramicidin A, alter the order of permeation by inducing a selective increase in the permeability of small hydrophilic solutes. Such discrimination also leads to the appearance of minima for the permeabilities at the second or third members of the homologous series of ureas or aliphatic amides. These minima are more pronounced for the liposome systems containing gramicidin A than nystatin

in accordance with the reported values for the dimensions of such aqueous pores determined by independent methods.

Finally, the magnitude of the mass selectivity for diffusion within the hydrocarbon part of liposomes containing cholesterol is comparable to that found in biological membranes such as *Chara* cells. However, the increased selectivity for diffusion across red cell membranes is better simulated by the corresponding process across the lipid region around the gramicidin pore in liposomes that are below their transition temperature.

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