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THE OPPOSING EFFECTS OF PRESSURE AND GENERAL ANAESTHETICS ON THE CATION PERMEABILITY OF LIPOSOMES OF VARYING LIPID COMPOSITION

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SUMMARY

The effect of pressure and general anaesthetics on lipid membranes of different composition was studied by noting the variation in the cation permeability produced in single bilayer liposomes. At concentrations producing a loss of righting reflex in the newt *Triturus cristatus carnifex* the logarithm of the liposome cation permeability increase was linearly proportional to the concentration of anaesthetic whereas the sugar permeability was generally unaffected.

Pressure compensated for the anaesthetic effect by decreasing the membrane cation permeability rather than by removing the anaesthetic from the membrane. The compressibility of the bilayer was estimated as $3 \cdot 10^{-5}$ atm^{-1}, similar to but lower than those of non-polar liquids. There was an excellent correlation between the narcotic concentrations required to increase cation permeability through 50% cholesterol liposome membranes and that required to block axonal conduction or protect erythrocytes against hypotonic lysis.

The correlation with the isonarcotic concentrations for general anaesthesia was best for the high cholesterol membranes, but in this case the more polar water soluble anaesthetics affected liposome cation permeability too much and non-polar anaesthetics were relatively inactive. This result suggested a site of action for general anaesthesia deeper in the bilayer than the surface region which is known to be rate limiting for liposome cation permeability.

Recent studies of the reversal of anaesthesia by pressure on newts and mice confirm the critical volume hypothesis which considers that anaesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a certain critical volume by the absorption of molecules of an inert substance[1,2]. Pressure thus appears to oppose anaesthesia by compressing this hydrophobic region. The original calculations supporting such an hypothesis used simple solvents such as benzene and olive oil to simulate the behaviour of the hydrophobic region. The success of such calculations, as with the Meyer–Overton anaesthetic–olive oil partition

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coefficient correlation, is strongly indicative of the general nature of the site of action of anaesthetics lying within the hydrophobic interior of a lipid structure, such as a bilayer, where the hydrocarbon chains are essentially in a liquid state. The earlier calculations were based on pure isotropic liquids because their properties are well known. While they provide some insight, only studies on more realistic simple systems will lead to a more detailed knowledge of the action of anaesthetics on structures within real biological membranes. For this reason a number of isolated bio-membranes have been studied but, from the physical point of view, these are overcomplex and often ill-defined in composition. An alternative approach to this problem was to extend earlier studies of anaesthetics and artificially prepared phospholipid membranes which exhibit some of the organisation of a bio-membrane and which may be prepared in a controlled manner[3,4]. The properties of such systems may, like the isotropic liquids, be studied with some precision and once obtained, may be evaluated for their biological significance[5].

One such simple membrane is the liposome or phospholipid microvesicle consisting of a single spherical bilayer shell, some 240–500 Å in diameter (depending on composition), surrounding a central aqueous compartment[6,7]. Anaesthetics were found to increase the cation permeability of these vesicles by causing an increase in the freedom of motion of the lipid molecules, particularly at the aqueous–lipid interface[8]. Such increased freedom of motion also implies an expansion of the membrane in a manner consistent with the critical volume hypothesis as suggested in preliminary reports[9,10]. Further evidence on the expansion of erythrocyte membranes by anaesthetics has been provided by Seeman *et al.*[11,12], whose results also suggest that membrane expansion is related to anaesthetic potency. Corroborative evidence from monolayer penetration studies[3,4,13], nuclear magnetic resonance[14] and spin label studies[10,15] shows that anaesthetics increase the randomness of the membrane structure, again implying an increase in volume. The relation between membrane expansion and anaesthetic action is thus of considerable interest. In this paper, therefore, the interaction between the effects of pressure and anaesthetics on the cation permeability of liposomes of various composition, are reported in more detail. Correlation of the behaviour of biological membranes with those of liposomes reveal several interesting trends.

MATERIALS

Phosphatidylcholine, supplied by Nigel Miller, was extracted from egg yolks and phosphatidic acid prepared from it by enzymic hydrolysis as described by Papahadjopoulos and Miller[16]. Some samples of phosphatidylcholine and phosphatidic acid were from Lipid Products, Redhill, England, all samples were tested chromatographically for purity as described by Papahadjopoulos and Miller[16] and found to be satisfactory. Phosphatidylserine was supplied by Applied Science Laboratories Inc., Pennsylvania, U.S.A. Cholesterol was Sigma's chromatographic grade. Valinomycin was obtained from Calbiochemicals. Isotopes came from the Radiochemical Center, Amersham, England or New England Nuclear, Boston, U.S.A. All other reagents were analytical grade. Chloroform and halothane were twice extracted with the 0.155 M KCl buffer immediately before use to remove the stabilizer.

METHODS

Preparation of liposomes

A stock phospholipid mixture containing 4% phosphatidic acid and 96% phosphatidylcholine was prepared and stored in chloroform solution under nitrogen. This is referred to subsequently as 4% phosphatidic acid–phosphatidylcholine. Approximately 40–80 μmoles 4% phosphatidic acid–phosphatidylcholine with or without cholesterol in chloroform solution were evaporated to dryness under reduced pressure in a 100-ml round-bottomed flask. 0.6 ml, 0.155 M ^{42}KCl (or ^{86}RbCl) buffered at 7.6 (20 °C) with 10 mM Tris–HCl was added to the lipids, under nitrogen. For the anaesthetic concentration–response measurements the aqueous mixture also contained 5 mM glucose with about 40 μCi [^{14}C] glucose. The thick, white suspension was transferred to a 1.3 cm diameter flat-bottomed glass vial and sonicated under nitrogen at 16 °C for about 1 h in a Kerry ultrasonic cleaning bath type KG 80/1 at 80 kHz or in a Mullard E 759 DB ultrasonic disintegrator at 10–25 kHz or in a Heat Systems, Model 5 × 5, 40 kHz. The lipid then appeared semi-transparent, and could be stored in the unopened vial at 4 °C until required (1–6 days). At the time of a permeability measurement, the liposomes typically contained about 0.025 μCi of the radioactive ion, and 0.25 μCi [^{14}C]glucose (if present).

Permeability measurements

The sonicated liposomes were separated from untrapped isotope by passage through a 30 cm column of Sephadex G-50 (coarse), anhydrous weight 3 g, which had previously been well washed and equilibrated in non-radioactive 0.155 M KCl–10 mM Tris–HCl buffer, pH 7.6 (20 °C). If the liposomes contained glucose, the buffered solution also had 5 mM glucose. In an experiment requiring valinomycin, 2–6 μmoles valinomycin per mole lipid were dried down in a large stoppered glass tube and the liposomes in 0.155 M KCl buffer solution were added. The control samples from the valinomycin mixture were diluted with 0.155 M KCl buffer, and the others with the appropriate quantity of anaesthetic dissolved in the buffer so that 1-ml samples contained 1–2 μmoles lipid which were then transferred to 8/32 Visking dialysis bags which had been soaked overnight in 0.155 M KCl buffer and repeatedly washed. Each bag was dropped into 10 ml 0.155 M KCl buffer (with or without anaesthetic) at 21 °C or 37 °C. Anaesthetic containing samples were done in triplicate, controls in quadruplicate. Liposomes not containing valinomycin were allowed a preliminary 30 min dialysis period in the control or anaesthetic KCl solution, they were then transferred to fresh 10 ml portions and incubated for about 3 h. Valinomycin-containing samples were incubated for 1.5–2 h after the addition of valinomycin. All samples were shaken mechanically in the water bath.

The times were noted when valinomycin was added, when the anaesthetic was added, when the pressure was applied to the tubes and when it was removed, and finally when the bags were taken out of solution. The bags were cut into fresh 9 ml portions of KCl to release the liposomes. The radioactivity escaping from the dialysis bags during the experiment and that released from the bags at the end was counted in 10 ml aqueous KCl in a scintillation counter by the Cerenkov radiation arising from the ^{42}K or ^{86}Rb. Decay corrections were required for the ^{42}K, but no quench corrections were necessary for the presence of lipid. [^{14}C]Glucose was counted

in Bray's solution after the ^{42}K had decayed, 10 days later. Again, no quench corrections were required as only count ratios were calculated.

Presssure system

Samples were exposed to hydrostatic pressure in cylindrical stainless steel pressure vessels of about 300 ml internal capacity. The dialysis bags were contained in "Vacutainer" tubes which were sealed with a rubber cap whilst still under the buffered salt solution. A hypodermic needle through the cap allowed expulsion of surplus fluid during the sealing process which was carried out so as to exclude air bubbles. Pressure was raised in the water filled pressure vessel either by gas pressure applied through a water filled pressure reservoir from a gas cylinder and pressure booster pump or (in later experiments) by a hydrostatic pump. Pressure was measured with Bourdon gauges to 1% accuracy.

Gaseous anaesthetics were also tested in these pressure vessels, the sample tubes were capped with punctured parafilm and were presaturated with gas for at least 2 h with vigorous stirring before the liposome containing bags were added. This procedure was essential to ensure equilibration of the gas.

Method of calculating permeabilities

A mathematical description of the method of calculating absolute permeabilities from the single compartment vesicles is given by Johnson and Bangham[6]. In these experiments it was found that the retardation of the alkali metal cations was negligible, provided that the dialysis bags were well soaked before hand, so the following simplified equation was used.

Let the permeability coefficient of the liposomes be P, their external surface area A_L and their internal volume V_c. If N is the count rate of the marker initially in the liposomes, and n the count rate of the material which has escaped from the liposomes at time t

$$\frac{dn}{dt} = \frac{PA_L}{V_c}(N - n) \tag{1}$$

from which

$$P = \frac{V_c}{A_L t} \ln \frac{1}{1 - n/N} \tag{2}$$

In the case of liposomes without valinomycin, n/N was very small so the logarithmic term could be expanded, hence

$$P = \frac{V_c n}{A_L t N} \tag{3}$$

Glucose permeabilities were treated like valinomycin-mediated potassium permeabilities.

The permeability of the control liposomes (P_0) was first calculated from Eqn 2 or 3. This permeability was then substituted back in Eqns 2 or 3 and the counts lost before the anaesthetic was added were calculated. Corrected values of N and n were resubstituted in the equation to allow the anaesthetic permeability (P_a) to be found and a further, similar correction, applied before the pressure permeability (P)

was obtained. Finally, the ratios P_a/P_0 and P/P_0 were calculated. The liposomes were normally under pressure for more than 1.5 h, the preliminary period was about 0.3 h.

Absolute values of P were derived as follows: A standard was made containing 0.01 ml of the isotopic solution used to prepare the liposomes diluted by 10 ml KCl buffer. V_c (ml) was calculated from

$$\frac{N \times 0.01}{\text{count rate of standard}}$$

The quantity of phospholipid phosphate present was measured by the method of McClare[17], and the area of liposomes was taken as twice that shown on the plot of area *versus* V_c per μmole lipid in ref. 6, since subsequent work[7] had shown that the vesicles are single rather than double-walled.

Measurement of partial molar volume of sodium pentobarbital

The density of an ethanol (C.S.C. Gold Shield) solution at 25 °C was found, using a calibrated density bottle with a covered stopper to prevent the evaporation of solvent. 0.5 g of sodium pentobarbital was placed in the dry bottle and was dissolved in the ethanol (25 ml) which was carefully added to fill the bottle. The volume of alcohol displaced by the sodium pentobarbital was calculated, and from this the partial molar volume was found to be 185 ml with an estimated accuracy of $\pm 2\%$.

RESULTS

Fig. 1 shows the effect of increasing concentrations of ether on the ^{42}K and ^{22}Na isotope exchange from liposomes with valinomycin (1 μmole per mole of lipid). It will be seen that the anaesthetic increases the permeability for both ions although

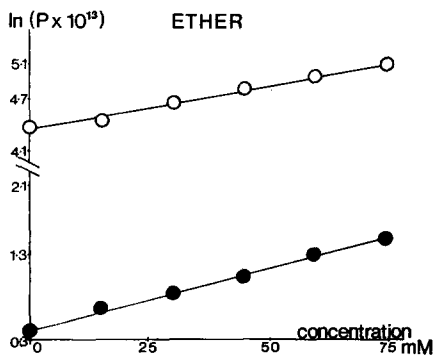

Fig. 1. The effect of increasing concentrations of diethyl ether on the alkali metal cation permeability of 4% phosphatidic acid–phosphatidylcholine liposomes at 37 °C in the presence of valinomycin, concentration 1 μmole/mole phospholipid. ○—○, K permeability; ●—●, Na permeability.

the absolute leak rate is 55 times faster for ^{42}K than ^{22}Na. Without valinomycin and at pH 7.3 (37 °C) the Na permeability is about 1.7 that of K. The linear relationship between $\ln(P_a/P_0)$ and the anaesthetic concentration was shown by all five liquid or solid anaesthetics listed in Table I. The linear relationship extended for at least

TABLE I

The increase in liposome cation permeability at 21 °C produced by the anaesthetic concentration causing a 50% loss of righting reflex in the newt, *T. cristatus carnifex* at 20 °C. The anaesthetics show a linear relation between anaesthetic dose and ln (permeability increase) ± (standard error of the gradient) so permeabilities at other anaesthetic doses may be readily calculated. The newt anaesthetic doses are published in Miller *et al.*[2].

Anaesthetics	*Newt anaesthetic concn (mM)*	*ln P/P₀ for K permeability with valinomycin 21 °C*				*ln P/P₀ with no valinomycin, 21 °C*	
		Cholesterol: —	*10%*	*50%*	*47.9%*	*4% phosphatidic acid 96% phosphatidylcholine*	
		Phosphatidic acid: 4%	*3.6%*	*2%*	*13.8%*		
		Phosphatidylcholine: 96%	*86.4%*	*48%*	*38.3%*	*Rb*	*Na*
Sodium pentobarbital	0.845	0.62 ± 0.06	0.58 ± 0.06	0.28 ± 0.02	0.23 ± 0.03	0.21 ± 0.04	0.23 ± 0.04
n-Butanol	17.0	0.35 ± 0.09	0.32 ± 0.02	0.243 ± 0.005	0.165 ± 0.006	0.35 ± 0.05	0.43 ± 0.05
Ether	25	0.25 ± 0.03	0.20 ± 0.03	0.21 ± 0.01	0.18 ± 0.01	0.15 ± 0.07	0.36 ± 0.05
Chloroform	0.90	0.12 ± 0.01	0.078 ± 0.009	0.091 ± 0.003	0.038 ± 0.003	—	—
Halothane	0.39	0.03 ± 0.005	0.03 ± 0.01	0.046 ± 0.003	0.013 ± 0.004	—	—

3-fold anaesthetic concentrations (6 times for halothane), higher anaesthetic concentrations were not tested (but see below), except for an 8-fold anaesthetic concentration of sodium pentobarbital on liposomes without valinomycin, for which the response was still linear. Replacing the 4% phosphatidic acid with 6% phosphatidylserine in the liposomes without cholesterol gave quantitatively similar permeability increases for ether with or without valinomycin, and for chloroform with valinomycin, 0.9 mM chloroform gave $\ln(P/P_0)=0.05$. Linear results could also be obtained with the gases if great care were taken over equilibration (Fig. 2). Because of transient temperature changes during compression the most reliable results were obtained at 37 °C where the contribution of such perturbations to the overall K^+ leak was negligible. We only studied N_2O in detail and found the relationship to be linear (Fig. 2) up to 23 times the anaesthetic pressure. Qualitative results for N_2 and SF_6

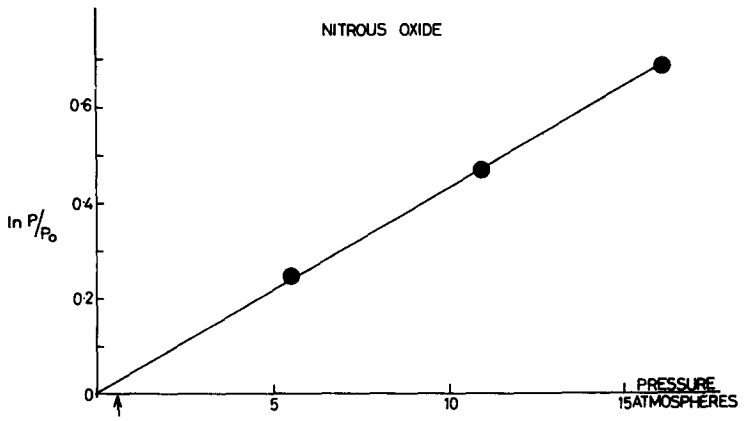

Fig. 2. The effect of increasing pressure of nitrous oxide on the valinomycin-mediated potassium permeability of 4% phosphatidic acid–phosphatidylcholine liposomes at 37 °C. The arrow shows the newt anaesthetic pressure at 30 °C, 0.75 atm, for which $\ln P/P_0=0.00220\pm0.00002$.

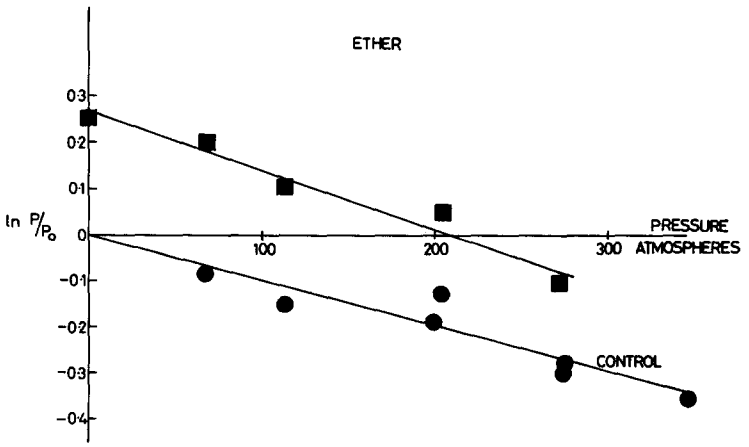

Fig. 3. The effect of the increasing pressure of the non anaesthetic gas helium on the potassium ion permeability of 4% phosphatidic acid–phosphatidylcholine liposomes at 37 °C. ●—●, liposomes without ether; ■—■, liposomes in the presence of 27.3 mM diethyl ether.

confirmed the low potency for these gases. Initial results demonstrating pressure reversal (using helium) with N_2 and SF_4 were not pursued because of the experimental uncertainties.

Effect of pressure and anaesthetic

Figs 3, 4A and 4B show the combined effect of anaesthetic and pressure on various liposome permeabilities with or without valinomycin. In the experiments represented in Figs 3 and 4A the pressure was applied with the non-anaesthetic gas, helium. In addition to the results depicted in Figs 3, 4A and 4B the effect of 136 atm hydrostatic pressure and anaesthetic on two preparations of 50% cholesterol, 2% phosphatidic acid, 48% phosphatidylcholine liposomes with valinomycin was

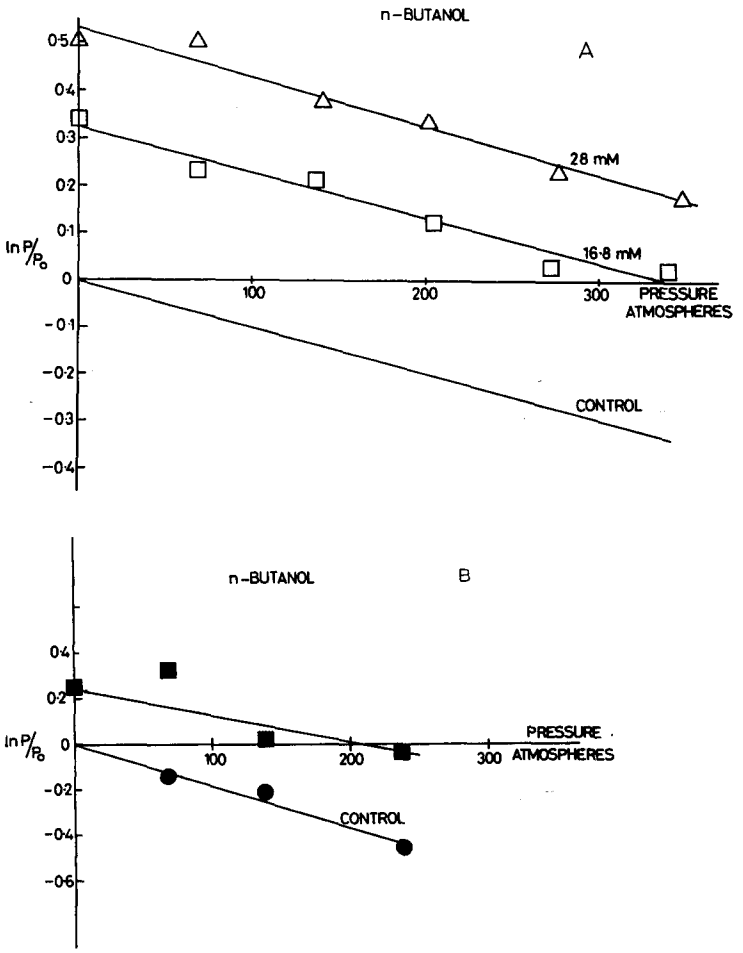

Fig. 4. (A) The effect of increasing helium pressure on the potassium permeability of 4% phosphatidic acid–phosphatidylcholine liposomes at 37 °C in the presence of *n*-butanol. △—△, 28 mM *n*-butanol; □—□, 16.8 mM *n*-butanol. The control line is the same as Fig. 3. (B) The effect of increasing hydrostatic pressure on the valinomycin-mediated potassium permeability of 50% cholesterol–2% phosphatidic acid–48% phosphatidylcholine liposomes at 21 °C. ●—●, liposomes without *n*-butanol; ■—■, liposomes with 17 mM *n*-butanol.

determined at 22 °C. The mean pressure-induced decreases in the value of ln P/P_0 for liposomes with anaesthetic were similar, namely 0.35 for 1.8 mM chloroform and 25 mM ether, 0.34 for 0.93 mM sodium pentobarbital and 0.29 for the control liposomes; the duplicate values agreed within ± 0.06.

Effects of general anaesthetics on the glucose permeability through liposomes

Glucose permeability was not increased by the presence of up to three times the anaesthetic concentration of chloroform and sodium pentobarbital, and up to six times the anaesthetic concentration of halothane for mixtures with or without cholesterol. Butanol, at twice the anaesthetic concentration produced an 8% increase, and ether produced an appreciable (20%) increase in glucose permeability at anaesthetic concentration.

DISCUSSION

Effect of anaesthetics on ion permeability of liposomes

It has been pointed out earlier that the Na^+ and K^+ permeabilities of liposomes are so low in comparison to natural membranes, at least in the absence of valinomycin, that the increase in permeability produced by the anaesthetics is in itself trivial, and significant only for what it reveals about the membrane, namely a disordering of bilayer structure[8], manifest also as an increase in membrane volume[3,4]. The increased cation exchange rate does not simply arise from an increase in the area across which the ion flux occurs, because no significant increase in glucose permeability is observed and the calculated increase in area is too small to account for the effect (see below). Similar arguments apply to the decrease in ion permeability observed under pressure. Thus the ability of the valinomycin–potassium ion complex to penetrate the bilayer may be regarded as depending upon the "fluidity" or "coherence" of the bilayer. Indeed below the thermal transition ("melting point") of the hydrocarbon chains the valinomycin mediated permeability is reduced to zero[18].

The effectiveness with which a given concentration of an anaesthetic increases the ion-permeability of liposomes of various compositions is examined in Table I. Raising the cholesterol content of 4% phosphatidic acid–phosphatidylcholine liposomes to 50%, for example, has a marked effect; the effectiveness of a given concentration of both the strongly polar molecules, sodium pentobarbital and *n*-butanol, is markedly reduced; that of the less polar compounds, ether and chloroform, is reduced somewhat whilst that of the relatively apolar halothane is increased. The effect of cholesterol on bilayer structure is not completely resolved at present, but at high concentration and depending upon the type of hydrocarbon chain, the main effects will be an increase in membrane area per phospholipid molecule, a reduction in the freedom of motion of the hydrocarbon chains in the interior, and a corresponding increase in thickness[19,20]. The decreasing effectiveness of those anaesthetics that are concentrated at or near the surface of the bilayer may be related to these changes, but might also result from a reduction of the bilayer aqueous partition coefficient of the anaesthetizing compound with increasing cholesterol content. Direct measurements of partition coefficients would clarify this point.

The effect of increasing phosphatidic acid was to decrease the effectiveness of all the anaesthetics, the less polar ones being most highly affected. We suggest that

the repulsion between phosphatidic acid head groups increases the head group area and consequently the hydrocarbon region disorder. The additional disordering effect of the anaesthetics is thus masked. The 6% phosphatidylserine–phosphatidylcholine liposomes were as sensitive to the anaesthetics as the phosphatidic acid–phosphatidylcholine liposomes.

Further confirmation that changes in permeability are associated with changes in bilayer volume is provided by estimation of these volume changes. The partition coefficient and partial molar volume data necessary to calculate the expansion of liposomes are not available, but in their absence we have used partition coefficient data obtained from measurements on erythrocytes[21] together with the volume occupied by the liquid anaesthetic at its boiling point, as the partial molar volume (except for sodium pentobarbital where the partial molar volume was measured in ethanol at 25 °C) (Table II). We note that these volumes are about 40–50 ml larger than those calculated by the Bondi method[22] and used in similar calculations on the erythrocyte membrane[11,12]. The discrepancy arises because Bondi's molecular volume, calculated from atomic van der Waal's radii, is the volume from which foreign electrons are excluded, and it is appreciably smaller than the volume from which other molecules are excluded under normal conditions of packing and of molecular motion.

TABLE II

The percentage volume expansion of 50% cholesterol–2% phosphatidic acid–48% phosphatidylcholine liposome membranes at 21 °C at doses of anaesthetic causing a 23% increase ($\ln P/P_0 = 0.21$) in valinomycin-mediated K^+ permeability. Membrane–buffer partition coefficients were those obtained for human erythrocytes by Seeman. The partial molar volume of sodium pentobarbital was measured experimentally in ethanol; those for butanol, ether and chloroform are molar volumes from Mullins' review[25]; that for halothane is its molar volume at 25 °C from data supplied by the manufacturer.

Anaesthetic	*Membrane/buffer partition coefficient*	*Partial molar volume (ml)*	*Concn giving 23% increase (mM)*	*% increase in liposome membrane volume*
Sodium pentobarbital	9.6	185	0.63	0.11
n-Butanol	1.5	98	14.7	0.22
Ether	1.18	105	25.0	0.31
Chloroform	18.7	81	2.08	0.32
Halothane	12.9	107	1.78	0.25
Mean				0.24

The results of calculations using such data are summarised in Table II where it may be seen that at equal increase in permeability the liposomes are indeed expanded very nearly equally by all anaesthetics. A concentration sufficient to increase permeability 23% causes a constant expansion of 0.24% within the errors of the estimate.

It seems probable that the net gain in membrane volume will not be reflected uniformly in the bilayer because of its anisotropic and co-operative structure. In particular an increase in head group area is generally associated with a decrease in

bilayer thickness because the hydrocarbon chains adopt a more randomised configuration. For example, it has recently been observed that the volume change at the crystalline–liquid crystalline phase transition for the dipalmitoyl phosphatidylcholine–water system is +1.4% whereas the thickness decreases 5 Å so that the head group area changes from 48 to 58 Å^2 (ref. 23). We might thus expect an increase in area of the liposome larger than 0.24% and accompanied by a decrease in thickness. *A priori* either or both of these changes could have an influence on permeability. It has been argued that the rate determining step for liposome cation permeability is close to the surface[8] and the change in surface area thus seems most significant in the present experiments, but in other cases, particularly with non polar permeants, reduction in the diffusion path across the belayer might play a part. The anomalously large increases in surface area produced by anaesthetics in erythrocytes[24] may be associated with the effects described above.

Pressure reversal of the anaesthetic effect

Pressure reversal of the raised cation permeability could result either from the anaesthetic being "squeezed out" of the membrane (because the partial molal volumes of most solutes are less in aqueous solution than in non-polar solvents)[25] or because of compression and re-ordering of the membrane. The latter appears to be the case for two reasons. First, the parallel decrease in permeability with pressure of liposomes in the presence and absence of anaesthetic (Figs 3, 4A and 4B) shows that a constant dose of anaesthetic causes the same increase in liposome permeability whatever the pressure. This strongly suggests that the amount of anaesthetic dissolved in the bilayer is the same at all pressures. This conclusion is supported by the variation of partition coefficient predicted thermodynamically from the difference in partial molar volumes in water and non-polar solvents. This variation is generally smaller than our errors. (The partition coefficient between water and oil would, for example, change only 5% for a 10 ml/mole difference in partial molar volumes). Secondly a completely self-consistent argument may be made on the basis of volume changes in the bilayer. Thus if one assumes equal permeabilities to occur at equal bilayer volumes, our estimate of the membrane volume gain at a 23% increase in permeability (Table

TABLE III

Estimation of the compressibility of 50% cholesterol–2% phosphatidic acid–48% phosphatidylcholine liposomes at 21 °C by use of data on the pressure reversal of the anaesthetic induced increase in cation permeability, together with the assumption that equal permeabilities occur at equal membrane volume.

Anesthetic	*% volume increase from Table II*	*Pressure (atm) to reduce leak rate 23%*	*Compressibility (10^{-5} atm^{-1})*
Sodium pentobarbital	0.11	85	1.3
n-Butanol	0.22	180	1.2
Ether	0.31	84	3.7
Chloroform	0.32	82	3.9
Mean			2.5

II) enables the compressibility of the bilayer to be estimated, and this estimate also acts as a consistency check. Table III shows the pressure required to reduce the valinomycin-mediated potassium exchange rate of anaesthetised 50% cholesterol, 2% phosphatidic acid, 48% phosphatidylcholine liposomes by 23% at 22 °C, which yields a mean compressibility of $2.5 \cdot 10^{-5}$ atm^{-1}. When compared to typical non-polar liquids this value of the compressibility is comparable but somewhat lower. Thus typical compressibilities are $6 \cdot 10^{-5}$ atm^{-1} for olive oil, $16 \cdot 10^{-5}$ atm^{-1} for hexane and $10 \cdot 10^{-5}$ atm^{-1} for palmitic acid[26]. It is possible that this relative incompressibility reflects the restricted, but fluid, state of the hydrocarbon chains particularly near the head group, suggested by recent spectroscopic studies[27-30], and it is interesting to note that the data for the more polar anaesthetics in Table III yield consistently lower compressibilities than the non-polar molecules, a trend which, if confirmed, would suggest that the compressibility of the bilayer increases towards the centre. However our estimate of β depends on values of the membrane partition coefficients and partial molar volumes of the anaesthetics which may not accurately represent those in the bilayers studied here. The above conclusions should, therefore, be regarded as tentative. Nonetheless the overall order of magnitude of the β obtained suggests our assumptions are at least self consistent.

Comparison of the effects of anaesthetics on liposomes and biological systems

The classical Meyer–Overton solubility hypothesis is based on olive oil as an analogue of the site of action of anaesthetics. It is consequently of some interest to examine the parallels between an apparently more realistic analogue (*i.e.*, the liposome) and real biological function.

The increase in cation permeability at an ED_{50} (dose giving 50% effect) concentration for loss of righting reflex in the newt (*Triturus cristatus carnifex*) is shown in Table I. These concentrations are virtually the same as those published by Miller *et al.*[2], and in good agreement with the anaesthetic concentrations of ether, chloroform and halothane obtained by Cherkin and Catchpool[31] for goldfish at 20 °C which were 29, 1.4 and 0.385 mM. (Chloroform appeared to produce toxic side effects in the newt, which may explain the higher concentration required for goldfish.)

Of all the liposome mixtures considered, the 50% cholesterol membranes gave the closest correlation with the newt anaesthetic concentrations, although the variation was by no means small even in this case. Nonetheless the result is encouraging in the light of known nervous tissue lipid compositions *e.g.*, myelin phospholipid: cholesterol molar ratio approximately 1:1 (ref. 32) and nerve trunk vesicles 1:0.45 (ref. 33) and tends to support the validity of the liposome as a model system.

Table I shows too, that for each of the liposomes, the strongly polar anaesthetics affect liposome cation permeability much more than the less polar anaesthetics, a result noted earlier for *n*-alcohols[3] when compared at equipotent newt doses. This suggests that the critical part of the membrane is closer to the aqueous interface for cation permeability than that for general anaesthesia[8,34]. This is consistent with kinetic and nuclear magnetic resonance studies which suggest that the rate determining step for valinomycin-mediated ion permeability is situated in the bilayer probably close to the most restricted hydrocarbon region[35].

The best correlations, however, are observed when the anaesthetics are com-

pared at nerve blocking concentrations. Thus in Table IV the correlation between equi-potent liposome anaesthetic concentrations for 50% cholesterol liposomes and those required to block axonal conduction in the cat is superior to that for general anaesthesia in Table I[36,37]. At these nerve block concentrations (at 37 °C) the liposome valinomycin-mediated ion permeability is raised 2.4-fold.

TABLE IV

Comparison between the doses of anaesthetic required to block axonal conduction in the cat with the dose required to increase 50% cholesterol–2% phosphatidic acid–48% phosphatidylcholine liposome permeability by 23% (ln $P/P_0=0.21$) at 37 °C. The cat anaesthetic doses are from Brink and Posternak[36] and Larabee and Posternak[37]. Temperature correction for valinomycin-mediated ion permeability in 4% phosphatidic acid–phosphatidylcholine liposomes from results in Johnson and Bangham[5] except for sodium pentobarbital which was measured at 37 °C.

Anaesthetic	*Cat axon concn (mM)*	*Liposome concn (mM)*	*Ratio of concns*
Sodium pentobarbital	3	0.63	4.8
n-Butanol	48	12.8	3.8
Ether	96	20.6	4.7
Chloroform	13.2	3.78	3.5

Table V compares liposome anaesthetic concentrations with concentrations required to block the axon potential in the frog sciatic nerve[38] and to give 50% protection against osmotic hemolysis of erythrocytes (whose lipids also contain approximately 50% cholesterol[39]). The correlation is close particularly between the liposome and the frog sciatic nerve, and it is tempting to invoke the primary role played by the lipid bilayer in all these cases. Armstrong and Binstock[40] showed that short-chain alcohol anaesthetics blocked the action potential sodium ion con-

TABLE V

Comparison between the concentrations of anaesthetic required to increase the cation permeability of 50% cholesterol–2% phosphatidic acid–40% phosphatidylcholine liposomes by 78% (ln $P/P_0=0.581$) at 21 °C, to block the axon potential in the frog sciatic nerve and to provide a 50% protection against the osmotic hemolysis of the human erythrocyte. The butanol concentration for the frog sciatic nerve was taken from Skou[38]. All other sciatic nerve, and erythrocyte protection concentrations from Seeman[39].

Anesthetic	*Liposome 78% increase concn (mM)*	*Frog sciatic concn (mM)*	*Erythrocyte concn (mM)*
Sodium pentobarbital	1.75	1.7	1.6
n-Butanol	41	68	42
Ether	69	50	90
Chloroform	5.8	5	9
Halothane	4.9	5	8

ductivity (g_{Na}) rather than the potassium ion conductivity (g_K). Hille[41] found that when g_{Na} was reduced by 100% by local anaesthetics, alcohols and detergents the g_K was only reduced by 5%. The excellent correlation between the amount of anaesthetic required to block the action potential and that required to approximately double the ion permeability across a lipid bilayer (Tables IV and V) suggests that the g_{Na} part of the axon potential is caused by a surface rearrangement which is prevented when the surface is expanded by anaesthetic, and that g_K is scarcely affected by a similar surface change. Spyropoulos[42] showed that very high pressure would virtually restore the original magnitude of the action potential of a squid axon anaesthetised by ethanol. Since our results show that none of the anaesthetics, and particularly *n*-butanol, are significantly squeezed out of the membrane by pressure, the effect of anaesthetics on g_{Na} is probably due to a physical expansion of the phospholipid membrane surface only, and not to interference by the alcohol itself. It is as well to remember, however, that because of its highly co-operative structure, perturbations on one part of the bilayer may be reflected to a greater or lesser extent in another part. Thus at sufficiently high concentrations even the non-polar general anaesthetics may cause nerve block. The correlations we have presented do indicate in a general way, however, that general and local anaesthetic activities appear to be located in different regions of the bilayer. Whether more complex, and possible second order, effects (such as those with membrane proteins) need to be invoked is a separate question. We may note, however, that the function of the polypeptide ionophore valinomycin is influenced by the effect of the agents studied in this work on the bilayer membrane.

Pressure reversal of anaesthetic effects in biological systems

Few systematic quantitative studies of the pressure reversal of anaesthesia have been reported. Data for the righting reflex of newts at 20 °C have been reported for several gaseous anaesthetics and these results have been shown to be consistent with the constant volume hypothesis[2]. Calculations were based on simple solvent analogues (for which solubility and partial molal volume data are available) of the site of action and indicated an average percentage expansion for loss of righting reflex at one atmosphere of 0.2% for the olive oil analogue or 0.5% for the benzene analogue and compressibilities of 3 and $6 \cdot 10^{-5}$ atm^{-1} respectively. This compares with 0.24% gain in membrane volume in the liposome (Table II) and an estimated compressibility of $2.5 \cdot 10^{-5}$ atm^{-1} (Table IV). Seeman and Roth[24] have estimated from measurements of the area increase caused by agents such as alcohols in erythrocyte membranes that general anaesthetics cause a 0.4% expansion in area at clinical doses. The consistency of these estimates is encouraging and tends to confirm the validity of the general approach outlined earlier.

Our results would also suggest that nerve block may be reversed by pressure and this has been demonstrated for ethanol in the squid axon and by nitrous oxide in frog sciatic nerve[43]. More generally any process which is dependent on the volume or degree of order in the bilayer should be sensitive to pressure. Thus the hyperexcitability observed in mammals (including man) at pressures above some 50 atm may be a result of membrane compression or "ordering".

This account of a compensation by pressure of anaesthetic effects in the bilayer is not necessarily exclusive. Any process in which anaesthetics induce an

increase in volume will be opposed by pressure. Thus anaesthetics reversibly reduce the luminescence of luminous bacteria, probably by partially denaturing luciferase (volume increase). This inhibition is reversed by pressure but only in some cases. Thus the effect of chloroform is strongly opposed, that of ether rather feebly so and that of the barbiturate, sodium barbital, not at all[44]. At the present time all protein systems studied show more specificity towards anaesthetics and pressure than is found in the whole animal. While a suitable protein may yet be found, it seems more reasonable to suppose that anaesthetics perturb the bilayer structure causing some protein disfunction. In the case of nerve block this perturbation probably prevents the specific sequence of events intimately related with impulse propagation[45]. Although such a generalized mechanism remains to be conclusively demonstrated for anaesthetics, the role of bilayer "fluidity" in protein function has been demonstrated in a number of other instances[46,47].

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REFERENCES

1 Lever, M. J., Miller, K. W., Paton, W. D. M. and Smith, E. B. (1971) *Nature* 231, 368–371
2 Miller, K. W., Paton, W. D. M., Smith R. A. and Smith E. B. (1973) *Mol. Pharmacol.*, in the press
3 Bangham, A. D., Standish, M. M. and Miller, N. (1965) *Nature* 208, 1295–1297
4 Bennett, P. B., Papahadjopoulos, D. and Bangham, A. D. (1967) *Life Sci.* 6, 2527–2533
5 Bangham, A. D. (1972) *Annu. Rev. Biochem.* 41, 753–776
6 Johnson, S. M. and Bangham, A. D. (1969) *Biochim. Biophys. Acta* 193, 82–91
7 Johnson, S. M., Bangham, A. D., Hill, M. W. and Korn, E. D. (1971) *Biochim. Biophys. Acta* 233, 820–826
8 Johnson, S. M. and Bangham, A. D. (1969) *Biochim. Biophys. Acta* 193, 92–104
9 Johnson, S. M. and Miller, K. W. (1970) *Nature* 228, 75–76
10 Trudell, J. R., Hubbell, W. L. and Cohen, E. N. (1972) *Fed. Proc.* 31, 549
11 Seeman, P., Kwant, W. O., Sauks, T. and Argent, W. (1969) *Biochim. Biophys. Acta* 183, 490–498
12 Roth, S. and Seeman, P. (1972) *Biochim. Biophys. Acta* 255, 207–219
13 Clements, J. A. and Wilson, K. M. (1962) *Proc. Natl. Acad. Sci. U.S.* 48, 1008–1014
14 Metcalfe, J. C., Seeman, P. and Burgen, A. S. V. (1968) *Mol. Pharmacol.* 4, 87–95
15 Hubbell, W. L., Metcalfe, J. C., Metcalfe, S. M. and McConnell, H. H. (1970) *Biochim. Biophys. Acta* 219, 415–427
16 Papahadjopoulos, D. and Miller, N. (1967) *Biochim. Biophys. Acta* 135, 624–638
17 McClare, C. W. F. (1971) *Anal. Biochem.* 39, 527–530
18 Krasne, S., Eisenman, G. and Szabo, G. (1971) *Science* 174, 412
19 Chapman, D. (1968) *Biological Membranes* pp. 125–199, Academic Press, New York
20 Rothman, J. E. and Engelman, D. M. (1972) *Nat. New Biol.* 237, 42
21 Seeman, P., Roth, S. and Schneider, H. (1971) *Biochim. Biophys. Acta* 225, 171–184
22 Bondi, A. (1964) *J. Phys. Chem.* 68–441
23 Träuble, H. and Haynes, D. H. (1971) *Chem. Phys. Lipids* 7, 324–335
24 Seeman, P. and Roth, S. (1972) *Biochim. Biophys. Acta* 255, 171–177
25 Mullins, L. J. (1954) *Chem. Rev.* 54, 289

26 National Research Council U.S. (1926) *International Critical Tables*, McGraw-Hill, New York
27 Hubbell, W. L. and McConnell, H. M. (1971) *J. Am. Chem. Soc.* 93, 314–326
28 Levine, Y. K., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1972) *Biochemistry* 11, 1416–1421
29 Horwitz, A. F., Horsley, W. J. and Klein, M. P. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 590–593
30 Chan, S. I., Seiter, C. H. A. and Feigenson, G. W. (1972) *Biochem. Biophys. Res. Commun.* 46, 1488–1492
31 Cherkin, A. and Catchpool, J. F. (1964) *Science* 144, 1460–1462
32 O'Brien, J. S. and Sampson, E. L. (1965) *J. Lipid Res.* 6, 537–544
33 Lagercranz, H. (1971) *Acta Physiol. Scand. Suppl.* 366
34 Papahadjopoulos, D., Nir, S. and Ohki, S. (1972) *Biochim. Biophys. Acta* 266, 561–583
35 Haydon, D. A. and Hladky, S. B. (1972) *Q. Rev. Biophys.* 5, 187–282
36 Brink, F. and Posternak, J. M. (1948) *J. Cell Comp. Physiol.* 32, 211–233
37 Larrabee, M. G. and Posternak, J. M. (1952) *J. Neurophysiol.* 15, 91–114
38 Skou, J. C. (1958) *Biochim. Biophys. Acta* 30, 625–629
39 Seeman, P. (1972) *Pharmacol. Rev.* 24, 583–655
40 Armstrong, C. M. and Binstock, L. (1964) *J. Gen. Physiol.* 48, 265–277
41 Hille, B. (1970) *Prog. Biophys. Mol. Biol.* 21, 1–32
42 Spyropoulos, C. S. (1957) *J. Gen. Physiol.* 40, 849–857
43 Roth, S. H., Smith, R. A. and Paton, W. D. M. (1972) *5th Symp. Underwater Physiology, Grand Bahama*
44 Johnson, F. M., Brown, D. E. S. and Marsland, D. A. (1942) *J. Cell Comp. Physiol.* 20, 269
45 Keynes, R. D. (1972) *Nature* 239, 29–32
46 Seelig, J. and Hasselbach, W. (1971) *Eur. J. Biochem.* 21, 17–21
47 Esfahani, M., Limbrick, A. R., Knutton, S., Oka, T. and Wakil, S. T. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 3180–3184