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CHLORIDE DIFFUSION FROM LIPOSOMES

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SUMMARY

1. Cl^- efflux from egg phosphatidylcholine liposomes is faster than that of K^+, both against exchangeable anions such as Cl^- itself, and against impermeable external anions such as SO_4^{2-}.

2. At 27 °C, Cl^- is released from such liposomes at a rate of 30–40 % per h against Cl^-, Br^- and SCN^-, 20 % per h against I^-, NO_3^- and acetate, and 10 % per h or less against SO_4^{2-}.

3. Valinomycin increases both Cl^- and K^+ efflux rates against external Na_2SO_4, but both valinomycin and uncoupler are required to produce a movement of Cl^- and K^+ equal in rate to the Cl^-–Cl^- exchange.

4. Liposomes composed of dimyristoyllecithin and dipalmitoyllecithin are more permeable to both K^+ and Cl^-, above their transition temperatures, than are egg lecithin liposomes. Valinomycin promotes both Cl^- and K^+ efflux from dipalmitoyllecithin liposomes, but uncoupler has almost no additional effect.

5. It is concluded, in agreement with McGivan (Ph. D. dissertation, University of Bristol (1968)) and Singer (Can. J. Physiol. Pharmacol. (1973) 51, 523–531), that the overall process of Cl^- movement is by a compulsory anion (e.g. Cl^-–OH^-) exchange. But it is suggested that this may be a result of the formation of HCl by reaction of Cl^- with membrane water, followed by the diffusion of that HCl molecule across the membrane and the release of Cl^- on the other side, thus creating a localised pH gradient in the membrane.

INTRODUCTION

Cl^- diffuses more readily from phospholipid vesicles than do most cationic species [1, 2]. This phenomenon raises two kinds of question: (a) why are the membranes more permeable to this anion than to cations of comparable size? and (b) how are electrical equilibrium and pH neutrality maintained? Neither question has been given a satisfactory answer.

Abbreviations: MOPS, morpholinopropane sulphonate; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone.

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Bangham et al. [3] showed that the exchange diffusion rates for Cl^- and I^- in multilamellar liposomes of 90 % phosphatidylcholine and 10 % dicetylphosphate were much higher than the rates for cations or the anions F^-, SO_4^{2-}, HPO_4^{2-}, and NO_3^-. In such systems $^{36}Cl^-$ exchanged completely within a few minutes. The same workers [4] showed that $^{36}Cl^-$ within multilamellar liposomes could exchange rapidly with a number of external anions, the order of effectiveness decreasing according to the sequence: $I^- > Cl^- \cong$ formate $> NO_2^- \cong Br^- >$ acetate $> F^- > HCO_3^- > NO_3^- > H_2PO_4^-$. The 50 % exchange time for Cl^- was about 1 min, compared to 100 h for K^+ at 22 °C, a differential of nearly 10^4.

Using phosphatidic acid to form negatively charged liposomes, Papahadjopoulos and Watkins [2] found rather lower ratios of Cl^-–K^+ diffusion rates, between 20 and 100 for sonicated (single bilayer) vesicles and only between 2 and 4 for multilamellar (hand dispersed) liposomes. McGivan and co-worker [5, 6] reported large variations in anion permeability of vesicles containing different negatively charged groups; dicetylphosphate-containing vesicles were much more anion permeable than cardiolipin-containing vesicles. In all cases, however, anion permeability exceeded that of small cations. On the other hand, the electrical flux created by Cl^- movement across black membranes is very much less than such isotopically measured exchange rates [7, 8]. Bangham [1] has therefore suggested that in the case of chloride HCl, rather than Cl^-, is the mobile form; this proposal was recently supported by Hauser et al. [9]. McGivan [5], on the other hand, by analogy with mitochondrial substrate anions, preferred to postulate an electroneutral 'antiport'. And Singer [10] has advocated a similar process of obligatory Cl^-–counter-anion exchange.

We have carried out experiments to compare the movements of labelled Cl^- and K^+ from both sonicated (single vesicle) and unsonicated (multilamellar) liposomes, using uncouplers and ionophores in an attempt to distinguish possible mechanisms. Our results with various types of phospholipids and different counter-ions tend to support in part the concepts of McGivan [5] and Singer [10] and to place some further restrictions on possible mechanisms. A preliminary report of some of these results has appeared [11].

MATERIALS

All phospholipids used in these experiments were analysed by two-dimensional thin-layer chromatography and shown to be >99 % pure. The preparation of egg yolk phosphatidylcholine and phosphatidic acid has been described earlier [12]. Dipalmitoylphosphatidylcholine, dimyristoylphosphatidylcholine, gramicidin and morpholinopropane sulphonic acid (MOPS) buffer were obtained from Sigma, London, Ltd. Only batches of synthetic saturated phospholipids having greater than 98 % palmitate or myristate by gas–liquid chromatography analysis were accepted. The saturated phospholipids were converted to the corresponding phosphatidic acids by the method of Kornberg and McConnell [13], using the modification of Klein [14]. All lipids, at known concentrations in chloroform, were stored under N_2 at −20 °C until required. Valinomycin was obtained from Calbiochem., and *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone (FCCP) from Dr P. G. Heytler, DuPont Chemical Co., Delaware, N.J.

All isotopes were supplied by The Radiochemical Centre, Amersham, England.

$^{42}K_2SO_4$ was prepared by titrating irradiated $^{42}K_2CO_3$ with 0.5 M H_2SO_4. 8/32 inch dialysis tubing (Visking) cut to approx. 8 inch lengths, was boiled for 5 min in 1 mM EDTA to remove heavy metal cations and a compound containing sulphur. Sephadex G-50 coarse (Pharmacia, Uppsala, Sweden) was similarly treated by boiling in water to remove an alcohol (presumably used in its drying process) and then well rinsed prior to column packing. All other chemicals were A. R. grade. Water was twice distilled, the second time from $KMnO_4$.

METHODS

Liposome preparation: 300 μmoles 4 % phosphatidic acid–96 % phosphatidylcholine were evaporated to dryness, under reduced pressure, in a 50-ml round bottomed flask. 4 ml of a salt solution containing 30 μCi ^{42}K, 10 μCi ^{36}Cl or $^{35}SO_4$ (and/or 20 μCi [^{14}C]glucose) were added and gentle agitation used to bring the liposomes into suspension. The latter were then allowed to equilibrate overnight (multilamellar liposomes). Single-vesicle liposomes were prepared by sonicating for 1 h under N_2 in a Kerrys 80/1 ultrasonic cleaning bath. All dimyristoyl and dipalmitoyl systems were swollen and sonicated above their transition temperatures (24.5 and 41 °C, respectively). These transition temperatures were checked by J. Hoyland with a Perkin–Elmer DSC-1B calorimeter*.

Liposomes containing sequestered isotopes were isolated at one column volume on Sephadex which had been previously equilibrated with an equi-osmolar salt solution (usually 67 mM Na_2SO_4, 5 mM MOPS, pH 7.0). Aliquots of 1 or 2 ml of this eluent were then pipetted into dialysis bags, which were well rinsed and then put into test tubes containing 10 ml "unlabelled" dialysing medium at time zero. 1-ml samples were taken at (usually) 30, 90 and 210 min for counting in a Packard Tri-Carb scintillation counter. ^{42}K was measured by Cerenkov radiation. 10 days later, after the ^{42}K had decayed, the ^{36}Cl and ^{14}C were counted in 10-ml Brays (Dioxan based) scintillator. Quenching corrections were necessary for I_2 by adding to each vial a small amount of $Na_2S_2O_4$. Lipid phosphorus was determined by the method of Fiske and SubbaRow [15].

RESULTS

Egg lecithin liposomes

Table I compares the present and previous [2] results for diffusion of Cl^- and K^+ out of liposomes. The Cl^- exchange rate for 4 % phosphatidic acid–96 % egg phosphatidylcholine single vesicles (line 2) is about 40 times that of K^+. When Na_2SO_4, instead of KCl, is used as the external salt, the rate of appearance of external Cl^- label decreases by at least 70 %, while the rate of K^+ leak remains the same. The Cl^- is therefore still moving out more than 10 times as fast as K^+. Fig. 1 compares the effects of several external anions on rates of Cl^- and K^+ release. Movement of Cl^- against KCNS (top) is as fast as the self-diffusion rate, and is little affected by the addition of either FCCP or valinomycin (which markedly accelerates the K^+

* The transition temperature of dipalmitoylphosphatidylcholine was almost unaffected by the presence of 4 % phosphatidic acid (less than 1 °C shift).

TABLE I

RATES OF DIFFUSION OF Cl^- FROM LIPOSOMES

27 °C, 4 % phosphatidic acid-containing vesicles (except where indicated). Percent diffusion given for first hour against 100 mM KCl or 67 mM K_2SO_4 or Na_2SO_4 buffered with 5 mM MOPS. Figures in brackets (lines 1 and 2) taken from ref. 2 (22 °C, pure egg lecithin). v_{Cl^-}/v_{K^+} gives ratio rates of Cl^- and K^+ diffusion.

	Lipid	Liposomes	Diffusion into KCl counter-ion system (self-diffusion)		Diffusion into K_2SO_4 counter-ion system	
			Percentage Cl^-/h	Ratio v_{Cl^-}/v_{K^+}	Percentage Cl^-/h	Ratio v_{Cl^-}/v_{K^+}
(1)	Egg-lecithin	multilamellar	(6)	(3)	3.5	2.2
(2)		single vesicle	30–40(18)	30(45)	7–13	10–12
(3)	Dimyristoyl-lecithin	multilamellar	65	1	65	1
(4)		single vesicle	(no trapping obtained)			
(5)	Dipalmitoyl-lecithin	multilamellar*	55**		55**	
			1–2**	1	1–2**	1
(6)		single vesicle*	22	3.2	17	2.3

* Experiments carried out at 43 °C (above transition region for dipalmitoylphosphatidylcholine).

** Markedly biphasic response: first figure gives extrapolated first-hour rate, second figure gives slow steady rate after first hour (see text).

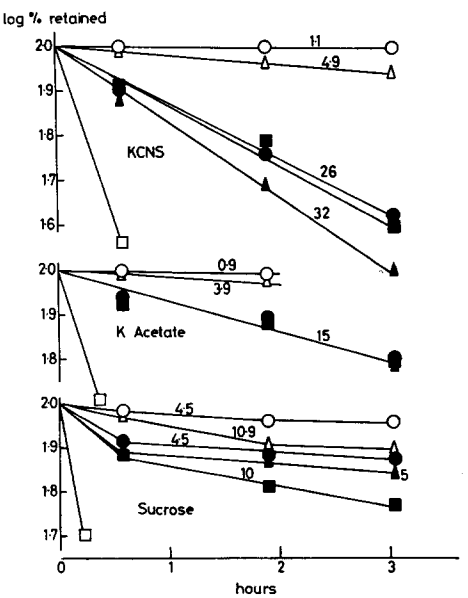

Fig. 1. Cl^- and K^+ efflux from sonicated liposomes. Effect of different external counter-ions. Log % ion retained plotted against time (h). External medium contained 5 mM K_2SO_4, 5 mM MOPS buffer, pH 7, plus 100 mM KCNS (upper curves), 100 mM potassium acetate (centre curves) or 200 mM sucrose (lower curves) at 24 °C. Open symbols, K^+ efflux. Closed symbols, Cl^- efflux. ○, ●: no additions; △, ▲: plus 1.13 μg/ml FCCP (calculated as diluted into 11 ml); □, ■: plus 0.450 μg/ml valinomycin (idem). Liposomes (4 % phosphatidic acid–96 % egg phosphatidylcholine) containing approx. 100 mM trapped KCl prepared as described in Methods. Figures on slopes indicate rates in percentage per h.

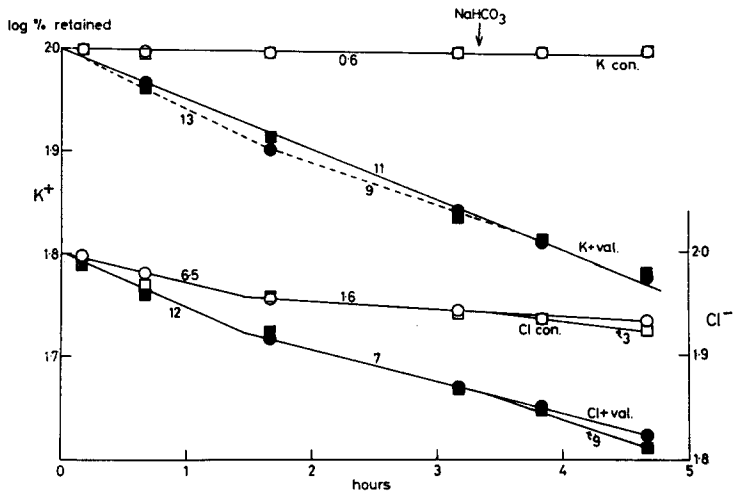

Fig. 2. Cl^- and K^+ efflux from sonicated liposomes against Na_2SO_4 as counter-ion system. Log % ion retained plotted against time. External medium contained 67 mM Na_2SO_4, 5 mM (Na) MOPS buffer at 26.5 °C. Upper curves (right hand ordinate), K^+. Lower curves (left hand ordinate), Cl^-. ○, □: controls; ●, ■: plus 9 ng/ml valinomycin. 10 mM $NaHCO_3$ added to two systems (□, ■) at 3 h 20 min as indicated. Other conditions as in Fig. 1 and Methods. 4 % egg phosphatidic acid–96 % egg phosphatidylcholine liposomes.

exchange). Potassium acetate is a poorer counter-ion system (Fig. 1, centre), while in sucrose (plus 5 mM MOPS) there is very little difference between Cl^- and K^+ efflux rates after the first 30 min. In this last case valinomycin seems to have some effect on Cl^- efflux. Fig. 2 illustrates the result of a more prolonged experiment with Na_2SO_4 as the external medium. Although Cl^- movement is now much slower in this system, especially after the first hour, it is still faster than that of K^+ (approx. 10 times as fast during the first hour, and three times as fast thereafter). Valinomycin (with no external K^+ present) definitely stimulates movement of both K^+ and Cl^-.

Against sulphate as the counter-ion system, therefore, Cl^- tends to follow K^+ electrophoretically when movement of the latter is induced with valinomycin. But the efflux of Cl^- in the absence of valinomycin must reflect either a movement as Cl^- plus H^+ (i.e. HCl movement) or the exchange of Cl^- for some external anion. The only possibilities are OH^-, HCO_3^-, and SO_4^{2-}. HCO_3^- is a possible exchangeable anion available to all such systems open to the air. But experiments carried out under N_2 gas showed no significant differences from those in air. Furthermore the addition of HCO_3^- (Fig. 2, arrow at 3 h 20 min) had a minimal effect on the movement of Cl^-, both in the presence and in the absence of valinomycin.

Although the doubly charged SO_4^{2-} normally moves only very slowly through the phospholipid bilayer, the possibility remained that Cl^- could induce the movement of an electrically equivalent amount of SO_4^{2-} electrogenically or by a compulsory exchange mechanism. Fig. 3, however, shows that this does not occur. Labelled $^{42}K_2$-$^{35}SO_4$ (together with traces of labelled glucose to check gross changes in permeability) was loaded inside liposomes instead of KCl. Whether allowed to leak against SO_4^{2-} (upper traces) or Cl^- (lower traces) as counter-anion system, SO_4^{2-} came out much more slowly than K^+, and was not significantly affected by the addition of either

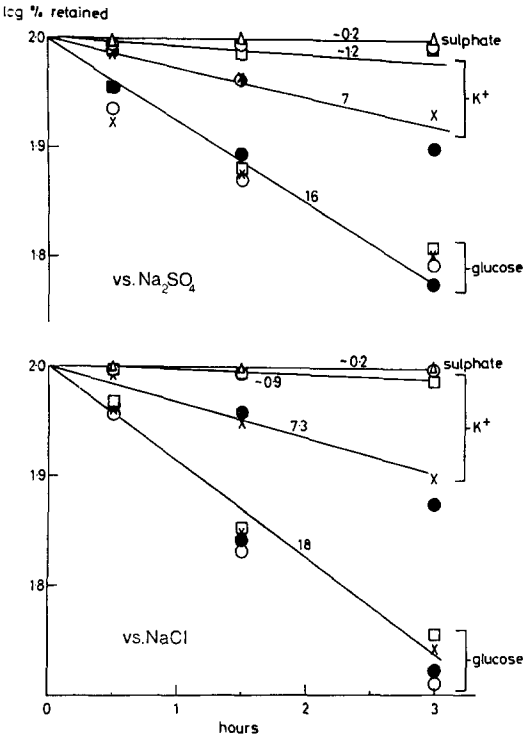

Fig. 3. SO_4^{2-}, K^+ and glucose efflux from sonicated egg phosphatidylcholine liposomes. Effect of permeant and impermeant counter-ions. External medium contained 67 mM Na_2SO_4, 5 mM MOPS (upper traces) or 100 mM NaCl, 5 mM MOPS (lower traces) at pH 7.0, 26 °C. Liposomes containing $^{42}K_2$ $^{35}SO_4$ and [^{14}C]glucose were prepared as described in Methods (4% phosphatidic acid–96% egg phosphatidylcholine). △, efflux (all conditions); K^+ and glucose efflux for ○, control; □, plus 230 ng/ml FCCP; ×, plus 9 ng/ml valinomycin; ●, plus both FCCP and valinomycin.

TABLE II

EXCHANGEABILITY OF Cl^- AGAINST DIFFERENT COUNTER-IONS

pH 7.0, 27 °C, 5 mM MOPS, 100 mM KCl.

	Counter ion	Diffusion of Cl^- (%/h)*	v_{Cl^-}/v_{K^+}
(1) Halides	Cl^-	36	40
	Br^-	43	—
	I^-	(20)**	—
(2) Strong acid anions	SCN^-	40	24
	NO_3^-	22	—
	SO_4^{2-}	13	12
(3) Weak acid anion or inert solute	acetate	15	17
	sucrose	18	4

* First hour.

** Measured after decolourisation with $Na_2S_2O_4$. Experimental conditions as in Fig. 1 and in Methods.

valinomycin or uncoupler. Cl^- therefore cannot induce the movement of SO_4^{2-}.

Table II compares the effectiveness of various diffusible counter-ions in facilitating Cl^- movement. Apart from Cl^- itself and SCN^-, only Br^- seems to be freely exchangeable. I^-, NO_3^- and acetate are less effective, although they are all more active than SO_4^{2-}. Sucrose solutions, which permit a rapid movement during the first half hour, tend to impede efflux in the subsequent steady state (Fig. 1, lower).

Although valinomycin or FCCP separately have little (Fig. 2) or no (Fig. 1) effect on Cl^- movement, when added together they increase the Cl^- flux against SO_4^{2-} to a level similar to that found in the Cl^-–Cl^- exchange system (Fig. 4). We have found that (a) valinomycin brings the ratio of Cl^- to K^+ flux down to about unity and (b) FCCP promotes both Cl^- and K^+ efflux against non-exchangeable counter-ions. Table III summarizes these results for single vesicles of egg phosphatidylcholine, as well as for dipalmitoyllecithin (discussed below). In analogous experiments carried out with hand-shaken (multilamellar) liposomes, qualitatively similar synergistic effects of valinomycin and FCCP were seen, although the rates were slower and the time courses even less 'first order' in character than those obtained with sonicated vesicles. The rate of Cl^- efflux rose from 3 to 8 %/h while that of K^+ increased from 1 to 11 %/h; v_{Cl^-}/v_{K^+} therefore decreased from about 3.0 to 0.7 in presence of valinomycin and FCCP.

Although such a combined effect of valinomycin plus uncoupler would normally support the concept of HCl movement, this possibility is rendered less likely by the failure of acetate (which can presumably cross the membrane as acetic acid) to act as an effective counter-ion (Fig. 1). Moreover, valinomycin alone does permit an increased efflux of Cl^- (Fig. 2) although not at the rate achieved in the presence of FCCP (Fig. 4). Hauser et al. [9] have stated that Cl^- exchange is faster at more acid

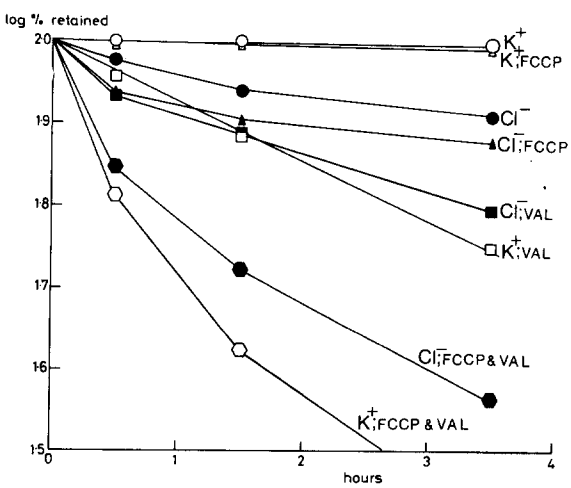

Fig. 4. Cl^- and K^+ efflux from sonicated egg phosphatidylcholine liposomes into an impermeant counter-ion system. Effect of valinomycin and FCCP. External medium contained 67 mM Na_2SO_4, 5 mM (Na) MOPS buffer, pH 7.0 at 27 °C. Liposomes containing $^{42}K^{36}Cl$ and MOPS were prepared as described in Methods (4 % phosphatidic acid–96 % egg lecithin). Open symbols, K^+ efflux. Closed symbols, Cl^- efflux. ○, ●: no additions (or with 0.1 % ethanol); △, ▲: plus 230 ng/ml FCCP; □, ■: plus 45 ng/ml valinomycin; ◇, ◆: plus both FCCP and valinomycin.

TABLE III

EFFECT OF FCCP AND VALINOMYCIN ON Cl^- AND K^+ EFFLUX FROM LIPOSOMES

Other conditions as in Figs 4 and 7.

Lipid	Additions	% efflux per h		Ratio (v_{Cl^-}/v_{K^+})
		Cl^-	K^+	
(1) Egg phosphatidylcholine (27 °C) (sonicated)	None	10.0	0.6	17
	Ethanol	10.5	0.6	17
	FCCP	16.5	0.9	18
	Valinomycin	19.0	15.0	1.3
	FCCP+valinomycin	40.0	48.0	0.8
(2) Dipalmitoyllecithin (43 °C) (sonicated)	None (±ethanol)	17.0	7.0	2.4
	FCCP	19.0	10.0	1.9
	Valinomycin	34.0	41.0	0.8
	FCCP+valinomycin	45.0	55.0	0.8

pH values. Bur their results do not show the 10-fold decrease with each pH unit that the simple HCl hypothesis would require. In fact, over the midrange of pH (where there is less possibility of gross changes in the liposomes), H^+ have only a small effect. Our results at pH values between 6 and 8 tend to confirm this. Initial diffusion rates were always between 30 and 50 %/h and showed no distinct trend with H^+ concentration. Although the Cl^- self-diffusion rate is thus relatively independent of pH, it is dependent on the external Cl^- concentration (Table IV). When isotonic KCl is replaced by 5 mM KCl the rate declines to that obtaining in 5 mM MOPS buffer alone, except in the case of liposomes prepared in low KCl media (line 3 in Table IV). The latter appear to be rather swollen (column 2, Table IV).

Liposomes (65 μmoles phospholipid) were prepared in 100 mM KCl and the suspension was pipetted into 5 ml unbuffered 200 mM glucose solution (KCl-free).

TABLE IV

DIFFUSION RATES AND TRAPPING OF Cl^- AT THREE Cl^- CONCENTRATIONS

Cl^- (in trap medium)	Apparent trapped volume (μl/μmole lipid)	Cl^- self-diffusion (%/h) vs					
		(1) isotonic KCl***		(2) 5 mM KCl		(3) MOPS alone	
		Initial*	Final**	Initial	Final	Initial	Final
154 mM	0.72	39	15	20	5	16	2
54 mM	1.07	34	8	17	6	15	1.5
14 mM	2.69	31	3	28	1.5	30	1.5

* First hour.

** Second 2 h.

*** i.e. 154 vs 154 mM, 54 vs 54 mM and 14 vs 14 mM NaCl plus 5 mM MOPS (pH 7). Other conditions as in Methods. Columns equilibrated with 10, 26, and 100 mM Na_2SO_4 (plus 5 mM MOPS). 100 μmoles phospholipid added/column.

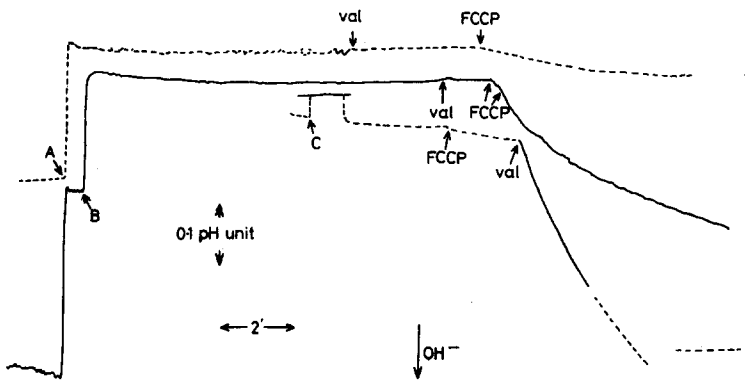

Fig. 5. H^+ and OH^- movements from hand-shaken (multilamellar) egg lecithin liposomes containing various trapped ions (acetate and phosphate). Effect of FCCP and valinomycin. Liposome suspension (65 μmoles lecithin) pipetted into 225 mM (unbuffered) glucose solution degassed with N_2. Initial pH approx. 7. 30 °C. Liposomes prepared in (A) 100 mM potassium acetate (pH 7.4); (B) 87.5 mM potassium acetate, 10 mM phosphate (pH 7.0) and (C) 80 mM potassium and sodium phosphate (pH 6.8). Final volume approx. 5 ml. 10 μl valinomycin (0.1 mg · ml^{-1}) and 20 μl FCCP (0.25 mg · ml^{-1}) solutions added as indicated.

We expected to see an acidification of the external medium. If HCl diffusion can occur (or, indeed, Cl^-–OH^- exchange), the Cl^- gradient should set up a pH gradient (with an external pH shift of about 1.5 units). These experiments were uniformly unsuccessful (see also McGivan [5]); but as Fig. 5 shows, even potassium acetate-containing vesicles (which must contain finite quantities of permeable acetic acid) fail to generate the expected external acidity, when added to unbuffered glucose solution. The pH remains steady until FCCP and valinomycin are added. An external alkalinisation then occurs, the extent of which depends on the internal buffer capacity of the vesicles. This alkalinisation reflects the exchange of internal K^+ for external H^+.

Saturated lecithin liposomes

When similar experiments involving Cl^- permeability were carried out with liposomes containing saturated phospholipids, considerable differences emerged. Firstly, dimyristoyllecithin systems (whose side chains are fluid at 27 °C, our working temperature with egg lecithin liposomes) failed to trap any KCl when sonicated as described in Methods. According to Johnson (Johnson, S., personal communication) a large proportion of this lipid after sonication sediments in the ultracentrifuge as if it had adopted a micellar rather than a microvesicular configuration. Multilamellar (hand-shaken) dimyristoyllecithin liposomes were therefore studied, and found to be very leaky above the transition temperature of 24 °C (Table I). K^+ and Cl^- leave together, whether exchangeable KCl or non-exchangeable Na_2SO_4 solutions are used as external media. Valinomycin and FCCP both induced a slight stimulation of the (already rapid) rates of Cl^- and K^+ exit. Even below the transition temperature (at 21 °C), about 40 % of the trapped ions could diffuse out rapidly. Haest et al. [16] showed that dimyristoyllecithin liposomes prepared above 32 °C became K^+ permeable at lower temperatures when subjected to a cooling 'shock'. Conversely, even above the transition region, in the absence of thermal shock the measured K^+ perme-

ability was low. With dipalmitoyllecithin (Table I) sonication did produce closed vesicles containing trapped KCl. Below the transition temperature of 41 °C, these vesicles are almost impermeable (<1 %/h) to both K^+ and Cl^-. Above the transition temperature, Cl^- permeability is less than that of egg lecithin vesicles at 27 °C, while the K^+ permeability is much higher (Table I). Substituting SO_4^{2-} for Cl^- as the external anion has little effect on this rate, but the addition of valinomycin (Table II) increases both K^+ and Cl^- rates to about 40 %/h. Addition of FCCP in this system has little effect, perhaps because dipalmitoyllecithin liposomes are already H^+ permeable.

Multilamellar dipalmitoyllecithin vesicles are even more heterogeneous in behaviour than are hand-shaken egg lecithin vesicles. Papahadjopoulos et al. [17] reported dramatic changes in the permeability of such systems at the transition temperature. Fig. 6 illustrates such permeability changes for the present type of experiment. Both K^+ and Cl^- release (measured as diffusion over 45 min) increase markedly at 41 °C. The permeability of K^+ is increased by gramicidin both below and above this temperature, while that of Cl^- is unaffected. Both Cl^- and K^+ show an apparent negative temperature coefficient [16] above 41 °C which is abolished for K^+ in the presence of gramicidin.

An examination of the time course of ion release suggests that this negative coefficient is not a true kinetic effect but the result of a decrease in the size of a leaky compartment as temperature is increased. Figs 7 and 8 compare the patterns of K^+ and Cl^- release, both above and below the transition temperature, for both sonicated (single vesicle) and hand-shaken (multilamellar) dipalmitoyllecithin liposomes. Fig.

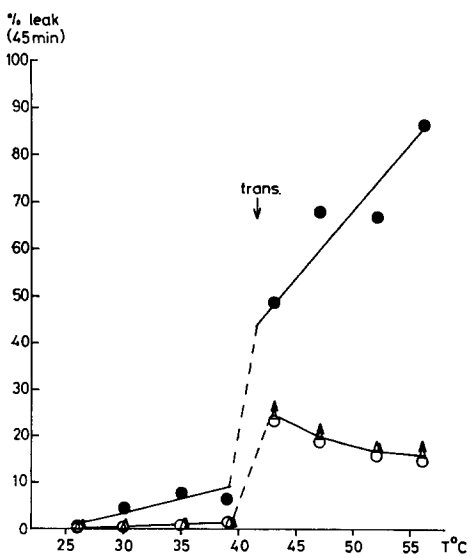

Fig. 6. Effect of temperature on apparent efflux rate of K^+ and Cl^- from (sonicated) dipalmitoylphosphatidylcholine liposomes (transition temperature 41.5 °C). Percent leak in 45 min plotted against temperature. Circles, K^+ leaks. Triangles, Cl^- leaks. ○, K^+ control; △, Cl^- control; ●, K^+ (plus 90 ng/ml gramicidin); ▲, Cl^- (plus 90 ng/ml gramicidin). External medium contained 67 mM Na_2SO_4, 5 mM (Na) MOPS, pH 7.0, buffer. Liposomes loaded with $^{42}K^{36}Cl$ as described in Methods (4 % dipalmitoylphosphatidic acid–96 % dipalmitoyllecithin).

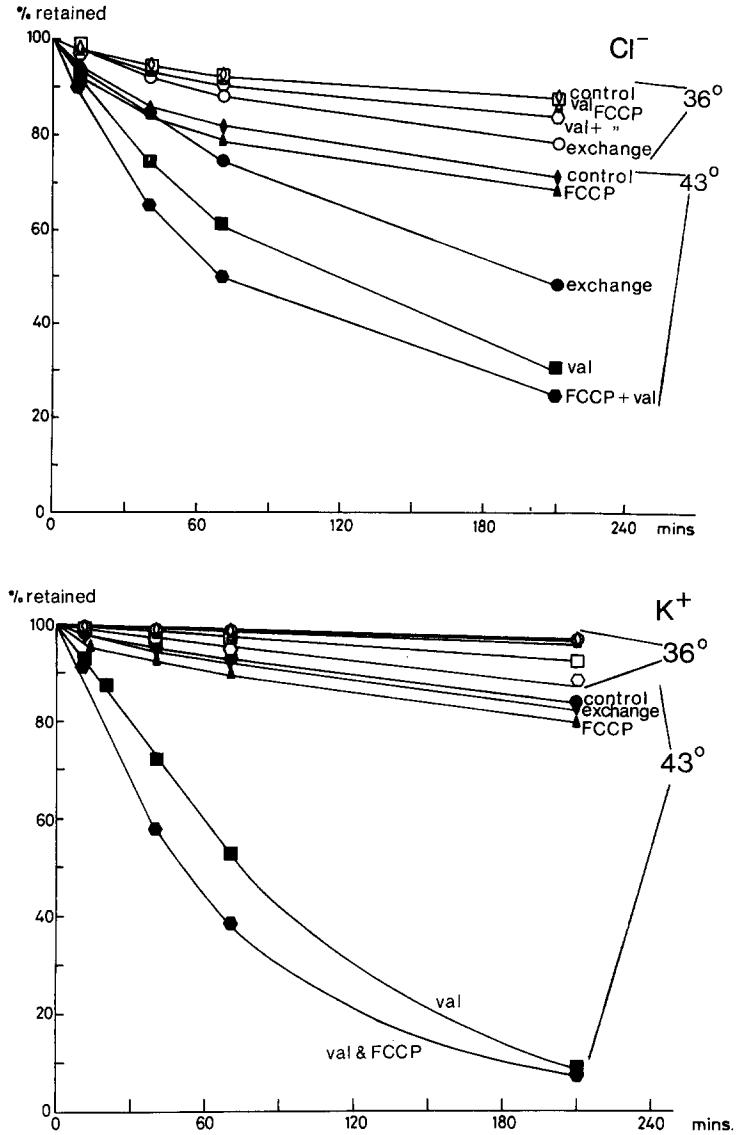

Fig. 7. (A) Cl^- efflux from sonicated dipalmitoyllecithin liposomes above and below the transition temperature. Percent retained plotted against time (min). External medium 5 mM MOPS buffer plus 100 mM KCl (○, ●) or 67 mM Na_2SO_4 (other symbols). Open symbols, 36 °C (below transition). Closed symbols, 43 °C (above transition). ○, ●: Cl^-–Cl^- exchange rate (no addition) (exchange); (a) ◇, ◆: Cl^- diffusion against sulphate (control); △, ▲: (a) plus 230 ng/ml FCCP; □, ■: (a) plus 45 ng/ml valinomycin; ⬡, ⬢: (a) plus both FCCP and valinomycin. Liposomes prepared and loaded with $^{42}K^{36}Cl$ as described in Methods (4 % dipalmitoylphosphatidic acid–96 % dipalmitoyllecithin). (B) K^+ efflux from sonicated dipalmitoyllecithin liposomes above and below the transition temperature. Same experiment as in A. Percent retained plotted against time (min). Symbols as in A. Open, above transition. Closed, below transition. ○, ●: K^+–K^+ exchange rate (exchange); (b) ◇, ◆: K^+ diffusion against Na^+ (control); △, ▲: (b) plus FCCP; □, ■: (b) plus valinomycin; ⬡, ⬢: (b) plus both FCCP and valinomycin. Other conditions as in A.

7A (note linear rather than logarithmic vertical axis) shows the diffusion of Cl^- from the single vesicles and Fig. 7B the corresponding efflux of K^+ in each case. As with egg lecithin liposomes (cf. Fig. 4), valinomycin promotes both K^+ and Cl^- movement. Figs 8A and 8B show the movement of Cl^- and K^+ from the multilamellar system.

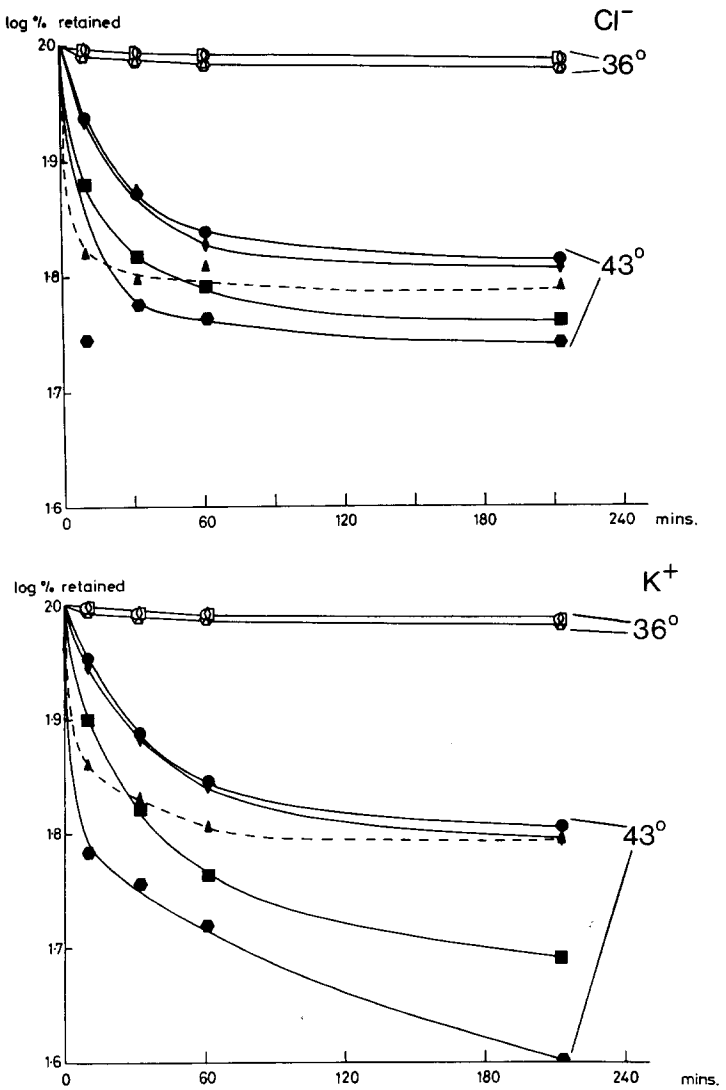

Fig. 8. (A) Cl^- efflux from hand-shaken (multilamellar) dipalmitoyl liposomes above and below the transition temperature. Log % retained plotted against time (min). Other conditions as in Fig. 7A. Open symbols, 36 °C. Closed symbols, 43 °C. ○, ●: Cl^-–Cl^- exchange, ◇, ◆: Cl^- efflux vs SO_4^{2-}; △, ▲: effect of FCCP; □, ■: effect of valinomycin; ⬡, ◆: effect of FCCP plus valinomycin. (B) K^+ efflux from hand-shaken (multilamellar) dipalmitoyl liposomes above and below the transition temperature. Same experiment as in A. Other conditions as in Fig. 7B. Open symbols, 36 °C. Closed symbols, 43 °C. ○, ●: K^+–K^+ exchange; ◇, ◆: K^+ efflux vs Na^+; △, ▲: effect of FCCP; □, ■: effect of valinomycin; ⬡, ◆: effect of FCCP plus valinomycin.

A marked heterogeneity is apparent (cf. Table I). The initial rate of release of both ions exceeds that in the sonicated system, but the final rate is much smaller. At 43 °C, 40 % of the internal salt leaves during the 'fast' phase, while 60 % remains more securely trapped. FCCP appears to increase the fast leak rate, while valinomycin accelerates the subsequent slow rate. As the temperature rises (between 43 and 56 °C) the rate of the fast phase increases but its extent diminishes, from 40 % at 43 °C to about 20 % at 56 °C. Addition of gramicidin renders all the K^+ rapidly permeable, removing the slow phase. Hence the apparent negative temperature coefficient (Fig. 6) is due to a decline in the size of the leaky compartment; and the positive coefficient for K^+ in the presence of gramicidin occurs because gramicidin abolishes that heterogeneity.

DISCUSSION

How is KCl held inside liposomes? Presumably it is retained partly in solution and partly in association with charged groups on the internal layer of lipid. Johnson [18] has suggested, on the basis of the similarity in apparent trap volumes for labelled K^+ and sugars, that the internal phosphatidic acid may not be completely ionized. With both K_2SO_4 and KCl, however, we have found apparent trap volumes to be rather greater for K^+ than for the anion. Thus under the conditions of Fig. 3 (70 mM K_2SO_4) we obtained 70 mmoles K^+ trapped/mole lipid and 27 mmoles SO_4^{2-}/mole, equivalent to trap volumes of 0.47 and 0.40 l/mole, respectively. However, the labelled glucose retained indicated a trap volume of 0.5 l/mole, even greater than that for the cation. In an experiment similar to that given in Fig. 2 we obtained 56 mmoles K^+ and 33 mmoles Cl^- trapped/mole lipid from a 100 mM KCl solution. These values are close to those for a Donnan equilibrium involving a single ionisation of the internal phosphatidic acid (20 mmoles internal negative charge/mole for 4 % phosphatidic acid) and internal concentrations of 130 mM K^+ and 77 mM Cl^- (0.43 l water/mole lipid). The apparent trap volumes for non-electrolytes seem to be a little higher than the corresponding volumes for electrolytes; there is thus no great excess of K^+ over glucose [18]. At higher ratios of phosphatidic acid to phosphatidylcholine the Donnan relationship does seem to break down (Nicholls and Miller, unpublished). At phosphatidic acid concentrations greater than 15 %, there may be suppression of internal ionization [18], or a tendency for phosphatidic acid to accumulate preferentially on the outside of the liposome. But with the present liposomes, satisfactory results are obtained if it is assumed that 30–50 % charge lies inside the liposome, bearing a single negative charge at pH 7 (the pK of phosphatidic acid in liposomes is between 8 and 9). In such systems, then, about 35 % of the internal K^+ will be non-diffusible in the absence of another cation (such as Na^+) which can enter to balance the internal fixed charges.

Various answers have been suggested to the two questions posed at the beginning of this paper. Dawson and Selwyn [19] and Haydon and Myers [20] discuss the possibility that phosphatidylcholine bilayers tend to have a positively charged lipid interior with a negative head-group region as a consequence of the surface dipole ordering of the phosphorylcholine radicals. Such an arrangement could facilitate the movement of anions and impede that of cations. An explanation of this type would not of course be needed if Cl^- movement were by means of HCl molecules, as suggested by Bangham [1] and by Hauser et al. [9]. Kornberg et al. [21] also favour HCl as

the mobile species, on the basis of experiments with induced pH gradients monitored by tempotartrate distribution. Although the effective pK of HCl in 100 mM KCl at pH 7 is not known precisely, it can be estimated as being close to 0. The resulting concentration of 10 nM HCl in the aqueous phase would demand a permeability of 10 $\mu m \cdot s^{-1}$ for HCl to account for the observed movement of Cl^-. Such a permeability is feasible, although high and close to that for water. But the absence of adequate pH effects [9] and the poor activity of acetate as a counter-ion (Fig. 1) tend to reduce the attractiveness of the HCl hypothesis. That Cl^- can move electrophoretically is suggested by the Cl^- response to valinomycin addition in systems devoid of external K^+ (Fig. 2). On the other hand, Cl^- movement alone (apart from requiring a high electrical conductivity for Cl^-, which is not observed [7, 8]) cannot account for the synergistic action of FCCP and valinomycin (Table III; Fig. 4A). This combined requirement for uncoupler and ionophore may also apply to the saturated lecithins. The anticipated FCCP dependence is diminished (Table III; Fig. 7) by a high intrinsic proton permeability.

McGivan [5] and Singer [10] have proposed an electroneutral diffusion process for Cl^- that does not involve HCl. McGivan [5] prefers a Cl^-–OH^- antiport system, perhaps to stress the analogy with the mitochondrion. The red cell Cl^- carrier also appears to be electroneutral [22]. And Selwyn and his co-workers [23] were able to show that movement of KCl into mitochondria (monitored by light-scattering changes consequent on swelling) could be accelerated by a combination of three carriers (valinomycin for K^+, triethyltin for Cl^-, and FCCP for H^+), indicating a compulsory antiport process in the presence of the anion carrier triethyltin. It now seems possible that most anion movement across phospholipid membranes proceeds by an exchange mechanism, and the systems found in mitochondria may thus represent only a special case of a more general phenomenon. No mechanistic interpretation of such exchanges is offered by theories that simply give Cl^- alone an intrinsically greater permeability [19, 20]. There are other problems. Two of these were first clearly formulated by McGivan [5]: (i) the failure of a Cl^- gradient (or indeed an acetate gradient, Fig. 5 above) to induce an appreciable pH gradient remains inexplicable on the simple exchange theory; and (ii) the failure of valinomycin alone (cf. Fig. 4) to induce a rate of Cl^- movement equivalent to the Cl^-–Cl^- exchange rate requires some special rule that makes antiport (Cl^-–OH^-) more favourable than symport ($Cl^- + K^+$).

We should like to propose our adaptation of an idea put forward for mitochondrial anion movement by Fonyo [24]. This is that Cl^- approaches the membrane as an anion [5, 10, 23], but crosses it as undissociated HCl [1, 2, 9]. The H^+ needed is taken from the membrane water (there is about 10 mM water in the phospholipid) on one side, thus leaving OH^-, and returned to it on the other side, creating H_3O^+. The consequences of the Cl^- transfer will then depend upon the separation of these OH^- and H_3O^+. If they are completely separated, there will be no membrane potential, but a local pH gradient in the membrane; if they are less than completely separated, there will be a combination of membrane potential and local pH gradient. The uncoupler requirement would then be to collapse the local H^+–OH^- separation and not a bulk H^+–OH^- separation.

McGivan's two 'paradoxes' can then be rationalised as follows: (i) the low rate of Cl^-–OH^- exchange compared with Cl^-–Cl^- exchange is due to the reluctance

of H^+ and OH^- to leave their membrane milieu, especially against the pH gradient, whereas the exchange reaction automatically collapses the gradients within the membrane. (ii) the poor rates of K^+–Cl^- symport are due to the fact that although electroneutrality is preserved in the bulk medium, such symport leaves an uncompensated local pH gradient in the membrane.

Unfortunately at the present time there is no direct evidence for the existence in the membrane of any such gradients. Only when such evidence is obtained, or a satisfactory alternative hypothesis elaborated, will it be possible to claim an understanding of the mechanism of membrane penetration by Cl^- and other anions.

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