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WEAK ACID-INDUCED RELEASE OF LIPOSOME-ENCAPSULATED CARBOXYFLUORESCEIN

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Leakage of the entrapped anionic fluorophore carboxyfluorescein was used as a measure of the permeability of liposomes to several different acids. Carboxyfluorescein leakage increased with increasing buffer concentration at a given pH and depended on its chemical nature: apolar weak acids such as acetic or pyruvic acids induced fast leakage at relatively high pH (4 to 5), while glycine, aspartic, citric and hydrochloric acids induced leakage only at lower pH. Fluorescence leakage measurements reflected the acidification of the liposomes' aqueous spaces, which was primarily caused by the diffusion of undissociated acid molecules across the lipid bilayer. A simple mathematical model in accord with this hypothesis and assuming that carboxyfluorescein leakage was directly related to the proportion of its neutral lactone form, described satisfactorily the carboxyfluorescein leakage kinetics and allowed rough estimation of permeability coefficients for carboxyfluorescein (neutral lactone form: $9 \cdot 10^{-9}$ cm $\cdot$ s^{-1}), acetic acid ($> 1 \cdot 10^{-7}$ cm $\cdot$ s^{-1}) and glycine (cation: $6 \cdot 10^{-9}$ cm $\cdot$ s^{-1}). These results are consistent with low effective proton permeability of liposomes ($< 5 \cdot 10^{-12}$ cm $\cdot$ s^{-1}) and with the permeability coefficient of HCl ($3 \cdot 10^{-3}$ cm $\cdot$ s^{-1}) reported by Nozaki and Tanford ((1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4324–4328). Diffusion of weak acid molecules across lipid membranes has implications for drug encapsulation and delivery, and may be of biological significance.

Introduction

Liposomes have been extensively studied as model systems for biological membranes. Their responses to changes of environment and in particular to pH changes if of major interest for studies of the regulation of pH gradients that are created between organelles and the cell cytoplasm. Our studies have demonstrated that monoclonal antibody-targeted liposomes are internalized by receptor-mediated endocytosis and transported to lysosomes or other low pH compartments from which their contents, the weak anions carboxyfluorescein and methotrexate were released into the cytoplasm [1,2]. The question we have posed in this paper is whether the leakage of these entrapped materials could be explained on the basis of the low lysosomal pH (approx. 4.6–4.8) [3] and, if so, by what mechanism the acidification could cause the release. Some authors have reported very low proton and hydroxide permeabilities for liposomes [4], and for planar phospholipid membranes [5]. In contrast, large proton permeability coefficients of the order of 10^{-4} cm $\cdot$ s^{-1} have also been reported [6,7].

Nozaki and Tanford [4] have suggested that in their experiments non-dissociated HCl or HNO_3

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide.

could be responsible for pH equilibration across phospholipid membranes, and Gutknecht and Walter [5] stated that proton transport occurred primarily by diffusion of molecular HCl across phospholipid/decane bilayers. This prompted us to study the effect of various acids, including weak acids, using measurements of carboxyfluorescein leakage to probe the acidification of the liposome aqueous compartment. Carboxyfluorescein is fluorescence quenched at high concentration [8], and its leakage from liposomes as a function of their destruction by detergent, temperature transition of their lipids [9], or by pH changes [10] has served as a useful marker in other experiments. Comparison of the ability of several acids to promote carboxyfluorescein leakage as a function of pH, temperature, and concentration are reported in this paper. Consequences for membrane biology, drug encapsulation and drug delivery are discussed.

Materials and Methods

Carboxyfluorescein. The commercial product from Eastman (Lot C4-F) was purified by crystallization from ethanol and Sephadex LH20 (Pharmacia) chromatography in water [11]. The K^+ salt was used routinely; the Na^+ salt was used in the experiment described in Table I. Titrations were performed at room temperature by addition of 1 M HCl or KOH solutions (Merck Titrisol) to a large volume of carboxyfluorescein (10 mM in water). pH was monitored with a Radiometer pH-meter equipped with a combined electrode. Carboxyfluorescein diluted (25 μM) into citrate or Hepes (100 mM) buffers adjusted to various pH between 3.0 and 8.0 was extracted with an equal volume of 1-butanol, 1-octanol, or a 1:1 (v/v) mixture of diethyl ether and heptane by vortexing and decantation. Duplicate aliquots of the organic phase were then back extracted into Hepes buffer (100 mM, pH 8.0). Carboxyfluorescein concentrations were determined spectrophotometrically at 492 nm (ϵ_M 492 = 72,000) [12].

Lipids. Dipalmitoylphosphatidylcholine (DPPC) was from Supelco and cholesterol from Fluka. All reagents were maintained as stock solutions (25 $mg \cdot ml^{-1}$) at −20°C in benzene/methanol (9:1, v/v; Merck).

Other reagents. HCl, KOH and sodium aspartate were from Merck, glycine and acetic acid from Carlo Erba, Hepes from Calbiochem, sodium pyruvate from Gibco, dimethyl sulfoxide (DMSO) from Prolabo, and valinomycin from Sigma.

Small unilamelar vesicles. 20 μmol DPPC and 10 μmol cholesterol, or in the experiment described in Fig. 4, 20 μmol DPPC alone, were mixed in a conical tube and organic solvents removed by evaporation under N_2 and overnight lyophilization. To the dried lipids was added 3 ml 80 mM or 40 mM carboxyfluorescein (or 40 mM Lucifer yellow CH, Sigma) in doubly distilled H_2O. The tube was heated to 50°C, vortex mixed into suspension, and sonicated under a stream of N_2 at 50°C with the microprobe of a CIT Alcatel/Pons sonicator for 20 min. The resulting clear liposome solution was centrifuged for 1 h at 100000 × g. Supernatant liposomes were passed over a 20 × 1.5 cm column of Sephadex G-50 (Pharmacia) equilibrated with 0.145 M NaCl/0.010 M Hepes (pH 7.4). Liposomes were sterilized by filtration through 0.45 μm Gelman filters, and kept at 4°C. All liposomes in this study were prepared less than one week before use, though we detected no deterioration of similarly prepared liposomes after many months.

Fluorescence measurements. Measurements of fluorescence were made on a Aminco SPF 500 fluorometer (carboxyfluorescein excitation 488 nm,

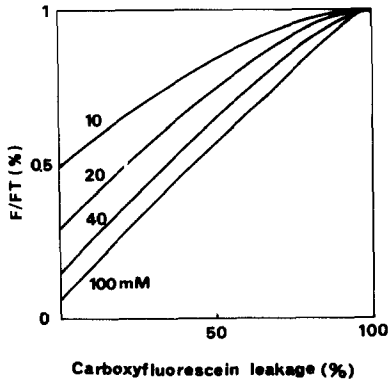

Fig. 1. The relationship between F/F_T and the percentage of carboxyfluorescein leakage from liposomes was drawn point by point (increment = 1% leakage) according to Eqn. 4. Several different starting concentrations of carboxyfluorescein were considered: 10 mM (Q = 52%), 20 mM (Q = 61%), 40 mM (Q = 85%), 100 mM (Q = 94%).

emission 520 nm; Lucifer yellow excitation 428 nm, emission 530 nm) using 20 nM carboxyfluorescein or 500 nM Lucifer yellow as fluorescence standards. Lysis of liposomes was via addition of Triton X-100 detergent (Fluka, final concentration: 0.5%).

Carboxyfluorescein leakage measurements. 10 μl of liposomes (stock preparation) were incubated at 4°C or 37°C with 100 μl of the specified buffer or acid for 1.5 or 2 h. 10-μl aliquots were then diluted into 1 ml Hepes buffer (200 mM, pH 7.4) at 4°C and the fluorescence was measured before and after addition of Triton, as described above. For kinetic experiments, 10 μl aliquots were diluted into 1 ml Hepes buffer at the specified time intervals during the 37°C incubation and fluorescence measured as above.

Results and Discussion

Dissociation constants and water-organic solvent partition of carboxyfluorescein

Titration of a solution of carboxyfluorescein (10 mM, potassium salt) with HCl gave a first transition (one equivalent) with a mid-point at pH 6.7. The addition of more HCl revealed the existence of two other acid functions which are barely resolved with pK around 5.0 and 4.3. The completely uncharged form of carboxyfluorescein is only sparingly soluble in water and some turbidity developed below pH 4.5. When the same solution was back titrated with KOH the turbidity disappeared only after the addition of two hydroxide ion equivalents (pH 5.0), and an hysteresis of pH was observed. The determination of approximate pK values was important for this study (see below), however, since carboxyfluorescein purified from commercially available material contains a mixture of two isomers [5,6] more precise measurements were not possible.

Dilute solutions of carboxyfluorescein in citrate or Hepes buffers at different pH were extracted with 1-butanol, 1-octanol and a 1:1 mixture of diethyl ether and heptane by vortexing and decantation. The amount of extracted carboxyfluorescein was measured after reextraction into Hepes buffer (pH 8) and expressed as the percentage of the total as a function of pH. Grimes et al. [13] have described an increased partition ratio between water and octanol at lower pH for carboxyfluorescein in the range 6.4–8.0. We studied a much broader range of pH and found that with butanol the extraction was complete below pH 6, with octanol below pH 5, and with diethyl ether/heptane only below pH 4.0. In pure heptane, only very small amounts of carboxyfluorescein were extracted at any pH between 3 and 8. Whatever the solvent and the pH, the extracted material was colorless with absorption maxima at 235 and 277 nm, which suggests that carboxyfluorescein is present in its uncharged lactone form and explains the complete loss of fluorescence of the organic phase [13]. The absorption maximum was shifted back to 500 nm upon addition of triethylamine. Thus, these results indicate that only completely uncharged carboxyfluorescein partitions significantly in non polar organic solvents.

Fluorescence self-quenching and measurements of carboxyfluorescein leakage

Concentrated solutions of carboxyfluorescein at neutral pH are fluorescence quenched. The property of fluorescence self-quenching has been used for monitoring liposome integrity and rates of carboxyfluorescein leakage [8]. Our analysis of the data published by Ralston et al. [11] showed that the fluorescence self-quenching (Q) variation with carboxyfluorescein concentration (C) can be represented satisfactorily by the equation.

$$\log(Q/(100-Q)) = a \log(C/A) \quad (1)$$

where a and A are two constants identified by least-squares fitting. A is the concentration for 50% fluorescence self-quenching (9.5 mM), and a is a dimensionless parameter equal to 1.3. Eqn. 1 was used to derive nomograms for the determination of percentage of carboxyfluorescein leakage. If no leakage had occurred at the time of a fluorescence measurement, the ratio of the fluorescence measured before (F) and after Triton addition (F_T) is simply

$$F/F_T = 1-(Q/100) \quad (2)$$

If leakage occurs, Q decreases in the liposomes (Q'), and unquenched carboxyfluorescein is released in the medium (fCF), the ratio F'/F_T is

then:

$$F'/F_T = (1-(Q/100))\cdot(1-(fCF/C))+(fCF/C) \quad (3)$$

which gives, according to Eqn. 1, with the percentage of leakage $L = 100\, fCF/C$:

$$100(F'/F_T)$$

$$= L+(100-L)/(1+Q(1-L/100)^n/(100-Q)) \quad (4)$$

Theoretical curves computed on a Hewlett-Packard HP 9825A according to Eqn. 4 for several different values of carboxyfluorescein concentration (C) are represented in Fig. 1. They were used as nomograms to calculate the percentage of carboxyfluorescein leakage from the experimentally derived parameter F/F_T. It has been verified that there is an agreement between these calculations and the experimental determination of encapsulated and free carboxyfluorescein after gel filtration (not shown).

Weak acid induced leakage of carboxyfluorescein

Sonicated liposomes containing 80 mM carboxyfluorescein (pH 7.4) were incubated for 2 h in the presence of acetate buffer (100 mM sodium acetate) adjusted to various pH with acetic acid, or with glycine buffer (100 mM glycine) adjusted with HCl, at either 4°C or 37°C. At the end of the incubation liposomes were diluted into cold Hepes buffer (200 mM, pH 7.4) and measured for fluorescence quenching. Percentages of carboxyfluorescein leakage were plotted against pH (Fig. 2). At 37°C carboxyfluorescein leakage was a function of pH and of the buffer. At 4°C carboxyfluorescein leakage was considerably reduced, but still depended on the pH and the buffer. The important effect of temperature on liposome permeability agrees with the 17 kcal/mol reported by Rossignol et al. [7] for the net proton transport across liposome membranes. Their experimental system is not different in essence from ours, even if their interpretation of the results is different. It should be pointed out that in both cases the lipid composition was such that no phase transition occurred in the temperature range 4°–37°C.

At pH 4 and 37°C acetate induced within 2 h a complete release of encapsulated carboxyfluorescein, while glycine had almost no effect. The effects of several other acids are reported in Fig. 3. Pyruvate induced an important leakage at

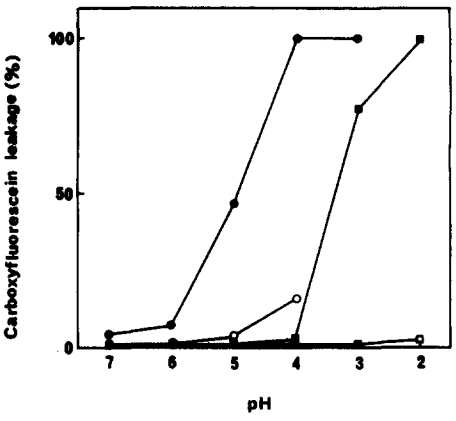

Fig. 2. The percentage of carboxyfluorescein leakage after 2-h incubation of DPPC/cholesterol small liposomes at either 4 or 37°C in the presence of acetate or glycine buffer was calculated according to Materials and Methods. Acetate buffer was prepared from 100 mM sodium acetate and adjusted to the different pH specified in the figure with acetic acid. Glycine buffer was 100 mM adjusted with HCl. Results are plotted against pH. ○, acetate 4°C; ●, acetate 37°C; □, glycine 4°C; ■, glycine 37°C.

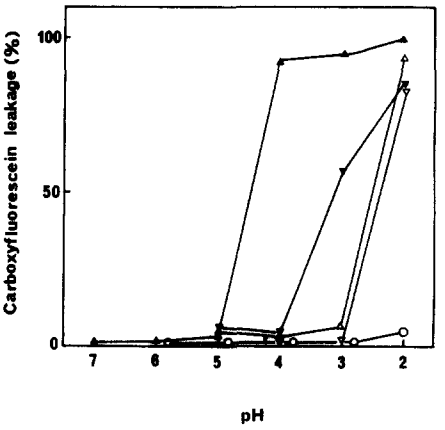

Fig. 3. Percentage of carboxyfluorescein leakage from DPPC/cholesterol small liposomes were measured and represented as for Fig. 2 but the incubation was only for 1.5 h and several other buffers were used. The incubation temperature was 37°C. Sodium pyruvate (▲), aspartate (▼), citrate (△) and chloride (▽) were 100 mM and adjusted to the specified pH with HCl. As a control, liposomes filled with Lucifer yellow CH were incubated in the presence of sodium acetate (○): no variation of fluorescence self-quenching was observed at the end of the incubation and no leakage could be detected by gel filtration.

pH 4, but more polar acids such as aspartate or citrate did not. Sodium chloride, adjusted to different pH with HCl did not induce significant leakage above pH 2. We have verified that the leakage of carboxyfluorescein depended on its neutralization and not on an acid-mediated disruption of the liposome bilayer as no release of encapsulated Lucifer yellow CH, a strongly acidic fluorescent probe [14], was detected under the same conditions of acid incubation (Fig. 3).

Fluxes of materials across membranes (J) are, as a first approximation, proportional to their molar activity (a) and therefore in dilute solutions, to their concentrations (C) and the permeability coefficient (P). The complete release of encapsulated carboxyfluorescein in 1.5 h requires an inward flux of acidic species at least sufficient for its neutralization. Thus a minimal order of magnitude for J can be computed (J_{min}) with an initial internal concentration of carboxyfluorescein equal to 80 mM and a mean liposome diameter of $6 \cdot 10^{-6}$ cm: $J_{min} = 3 \cdot 10^{-15}$ mol $\cdot$ cm$^{-2} \cdot$ s^{-1}. This would correspond, if only protons can diffuse, to a permeability coefficient (P_H) of the order of $3 \cdot 10^{-8}$ cm $\cdot$ s^{-1}, thus, a diffusion constant for protons much lower than that reported by Nichols et al. [6] and by Rossignol et al. [7] (10^{-4} cm $\cdot$ s^{-1}). In contrast, if P_H is taken from Nozaki and Tanford [4] as smaller than $5 \cdot 10^{-12}$ cm $\cdot$ s^{-1}, then even at pH 2 the diffusion of protons would be too slow and diffusion of molecular acids should be invoked to account for the experimental carboxyfluorescein leakage. At pH 2, in the presence of NaCl and HCl, the concentration of molecular HCl is about 10^{-9} M, assuming a pK of -6.1 for HCl [15]. Then using the permeability coefficient of $3 \cdot 10^{-3}$ cm $\cdot$ s^{-1} determined by Nozaki and Tanford [4], one finds $J = 3 \cdot 10^{-15}$ mol $\cdot$ cm$^{-2} \cdot$ s^{-1}. Given that the experimental conditions are not exactly the same (temperature, lipid composition and method of liposome preparation), this could be taken as a good agreement with their hypothesis that molecular HCl diffusion is responsible for pH gradient reversal under such conditions [4].

Liposomes made without cholesterol showed increased permeability compared to cholesterol-containing liposomes (Fig. 4). Nevertheless, for liposomes composed of DPPC alone, the effect of buffer composition on carboxyfluorescein leakage was qualitatively the same. Quantitative, carboxyfluorescein leakage was found to be slower than that reported by Szoka et al. [16] and by Weinstein et al. [12] and similar to that presented by Straubinger et al. [17], presumably as a result of the extensive purification of carboxyfluorescein which removes apolar contaminants probably responsible for increased membrane permeability.

Effect of counter-ion current on the movement of protons

Liposomes containing the sodium or potassium salts of carboxyfluorescein (40 mM) were incubated in glycine buffer in the presence or absence of DMSO or 1 or 10 μM valinomycin diluted in DMSO (Table I). In the presence of valinomycin, leakage of K^+-containing liposomes was increased about 2-fold in glycine buffer relative to liposomes containing the Na^+ salt. Thus, a diffusion barrier exists for protons in the absence of the movement of a counter-ion, as stated by Deamer and Nichols [18]. The glycine induced leakage of the K^+ salt of carboxyfluorescein in the presence of 10 μM valinomycin was still less than the acetate induced leakage without valinomycin

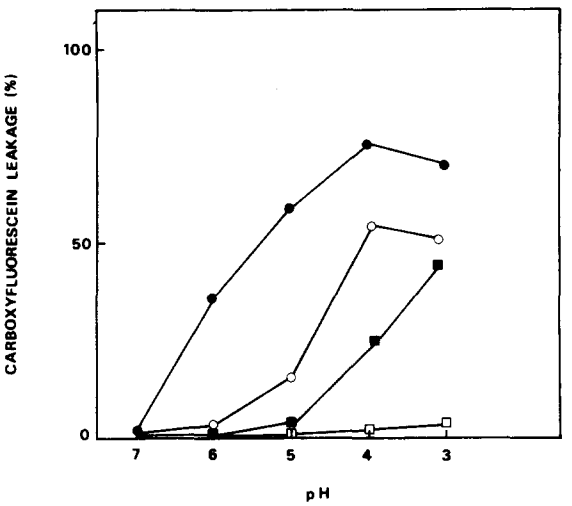

Fig. 4. The percentage of carboxyfluorescein leakage from liposomes made from DPPC only in the presence of acetate (●) or glycine (■) buffers was measured after 1.5 h of incubation at 25°C. For comparison the percentage of carboxyfluorescein leakage from DPPC/cholesterol liposomes was also measured under the same conditions (acetate (○); glycine (□)).

TABLE I

THE EFFECT OF VALINOMYCIN ON LEAKAGE OF CARBOXYFLUORESCEIN FROM LIPOSOMES IN VARIOUS BUFFERS

The effect of valinomycin (1 and 10 μM) was tested by adding to liposomes in 100 μl of buffer, 1 μl of a 100 μM or 1 mM solution of valinomycin in DMSO. DMSO alone was included as a control and had essentially no effect on carboxyfluorescein leakage. The liposomes contained 40 mM of either the sodium or the potassium salts of carboxyfluorescein. The incubation was 1.5 h at 25°C.

	Carboxyfluorescein leakage (%)							
	Liposomes containing sodium carboxyfluorescein				Liposomes containing potassium carboxyfluorescein			
	No addition	1% DMSO	Valinomycin		No addition	1% DMSO	Valinomycin	
			1 μM	10 μM			1 μM	10 μM
Hepes (pH 7.45)	0.3	0.0	0.0	0.9	0.8	0.4	7.1	12.0
Glycine/HCl (pH 5.0)	0.9	0.0	4.1	6.1	0.0	0.0	8.4	15.1
Glycine/HCl (pH 4.0)	1.9	2.2	6.9	8.4	3.1	3.0	14.2	25.2

at the same pH; however this difference might be due to the difference in concentration of the diffusing species (see below).

Effect of buffer concentration on carboxyfluorescein leakage kinetics

If non-ionized acids are responsible for carboxyfluorescein leakage, then the kinetics should depend on the buffer concentration at a given pH. The above mentioned pH dependence of carboxyfluorescein leakage would thus represent only the effect of pH on the proportion of undissociated acids in a buffer mixture. Illustrations of this are reported in Figs. 5 and 6: the same liposome preparation was incubated with acetate (pH 4) or HCl/glycine (pH 2) at different buffer concentrations and measured for fluorescence self-quenching at time intervals shown in the figures. A dramatic concentration dependence was found for both buffers, but the kinetics were very different: leakage beginning immediately in acetate and only after an important lag time in HCl/glycine. One possible explanation for such behavior is that uncharged acetic acid is able to diffuse very rapidly across membranes while cationic glycine is able to diffuse at a much slower rate. Since diffusion of a charged molecule would create a large electric charge in the liposomes that would be energetically unfavorable it is possible that cationic glycine diffuses together with a counter-ion. The permeability coefficient for chloride ions is approx. 10^{-10} cm $\cdot$ s^{-1} [19]. So with the high chloride concentrations present in these experiments chloride fluxes can be large (10^{-14} mol $\cdot$ cm^{-2} $\cdot$ s^{-1}) and not rate limiting. However, other explanations, such as the formation of an ion pair, cannot be excluded.

Simulation of carboxyfluorescein-leakage kinetics

A simple kinetic model was developed in order to test whether our assumptions on the mechanism of carboxyfluorescein leakage agree with the experimental data presented above. This model was based on the following hypotheses:

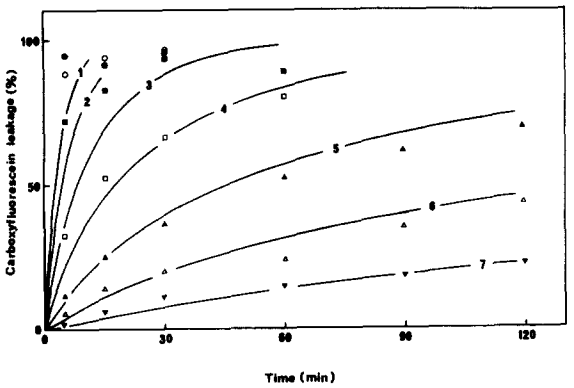

Fig. 5. The kinetics of carboxyfluorescein leakage at 37°C were monitored as described in Materials and Methods in the presence of various concentrations of acetate buffer (pH 4) diluted in 100 mM sodium chloride. The solid lines represent the computer simulations of the kinetics (see Results). The symbols for the experimental points are as follows: 1, ●, 100 mM; 2, ○ 50 mM; 3, ■, 25 mM; 4, □, 12.5 mM; 5, ▲, 6.3 mM; 6, △, 3.2 mM; 7, ▼ 1.5 mM. The undissociated acetic acid concentrations were calculated according to the mass action equation with pK = 4.8 for acetic acid.

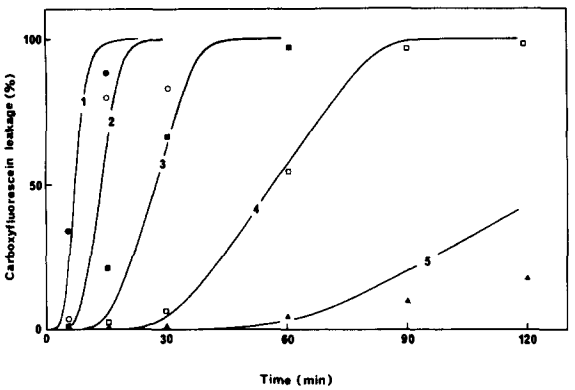

Fig. 6. The figure represents the same experiment as in Fig. 5, but in the presence of glycine buffer at pH 2. Note that the lag times apparent in the computer simulations (solid lines) were the result only of the low permeability coefficient assigned to glycine.

(1) Acids present in the medium surrounding liposomes diffuse with first order kinetics and the total volume of liposomes is sufficiently small that the external acid concentration remains constant. This would be true because of the small size of the liposomes (500–600 Å) and their large dilution in the buffer.

(2) Carboxyfluorescein is able to leak out of liposomes only in its completely uncharged form. This derives from the observation that at neutral pH the rate of carboxyfluorescein leakage was not measurable over days with sonicated, cholesterol containing liposomes and that only the neutral lactone was found to partition in apolar organic solvent.

(3) The acid-base reactions that take place in the liposomes as a consequence of acid entry and carboxyfluorescein neutralization are fast as compared to the diffusion of these molecular species. Then, pH and concentrations of the various species in the liposomes can be considered in equilibrium at any step of the kinetics, and can be calculated according to the classical mass action and electric neutrality equations. pK values were taken from our measurements for carboxyfluorescein and from the literature for acetic acid and glycine.

(4) Carboxyfluorescein and acid diffusion across liposome membranes was assumed electroneutral.

A computer program was based on a Runge-Kutta step by step integration of the system of two linear differential equations for diffusion of the acid species and of uncharged carboxyfluorescein (Eqns. 5 and 6 in Table II), associated with an iterative resolution of the mass action and electric neutrality equations (Eqns. 7 to 9 in Table II) at each step of the integration procedure. The integration step varied between 0.25 and 1 min, and the two permeability coefficients (P_A for the acid and P_{CF} for carboxyfluorescein), were adjusted manually to achieve the best graphical fitting simultaneously on all the kinetics. Thus, diffusion coefficients given in this paper should be consid-

TABLE II

EQUATIONS DESCRIBING THE MODEL USED FOR CARBOXYFLUORESCEIN LEAKAGE SIMULATION

Quantities in brackets stand for concentrations, subscripts indicate external (e) or liposome encapsulated (1) material. Permeability coefficients are as described in the text. Surface over volume ratio was taken as $6/D$ (D being the mean diameter of liposomes), assuming spherical shape. Superscripts indicate the ionization state of the molecule. A refers to total acid, HA to the non-dissociated form and A^- to the corresponding anion.

First order diffusion of uncharged carboxyfluorescein	$d[CF_1]/dt = -6(P_{CF}/D)[CF_1^0]$	(5)
First order diffusion of uncharged acid	$d[A_1]/dt = 6(P_{HA}/D)([HA_e]-[HA_1])$	(6)
Electroneutrality	$3[CF_1^{3-}]+2[CF_1^{2-}]+[CF_1^-]+[OH_1^-]+[A_1^-]=[Na_1^+]+[H_1^+]$	(7)
Carboxyfluorescein dissociation	$[CF_1]=[CF_1^{3-}]+[CF_1^{2-}]+[CF_1^-]+[CF_1^0]$ $\log([CF^0]/([H^+]\cdot[CF^-])) = pK_1(CF)$ $\log([CF^-]/([H^+]\cdot[CF^{2-}])) = pK_2(CF)$ $\log([CF^{2-}]/([H^+]\cdot[CF^{3-}])) = pK_3(CF)$	(8)
Acid dissociation	$\log([AH]/([H^+]\cdot[A^-])) = pK(A)$	(9)

ered as rough estimates, and only their order of magnitude and the possibility to correctly represent experimental data are meaningful. This program was run on a desk-top Hewlett-Packard HP 9825A computer connected to a digital plotter.

The results of such simulations are shown in Figs. 5 and 6 (solid lines). P_{CF} was taken as $9 \cdot 10^{-9}$ cm $\cdot$ s^{-1}. For acetate, P_A was set to $1 \cdot 10^{-7}$ cm $\cdot$ s^{-1}, but this is a lower limit since in this case the rate limiting step was carboxyfluorescein efflux (for planar membranes, Wolosin and Ginsburg [20] found $2.4 \cdot 10^{-4}$ cm $\cdot$ s^{-1}). For glycine, the best fitting was obtained with $P_A = 6.0 \cdot 10^{-9}$ cm $\cdot$ s^{-1}, but, as stated above, the chemical nature of the diffusing species is not certain. However, the assumption that the influx of acid is the rate limiting step allowed a satisfactory representation of experimental kinetics and in particular of the observed lag-time.

Conclusion

The purpose of the present experiments was to determine to what extent acids are able to cross liposomal membranes and induce the leakage of entrapped solutes. Experimental systems which have been designed for similar measurements have used large liposomes (obtained either by detergent removal or ether injection) containing highly buffered solutions (in general aspartate). These liposomes are more leaky (detergent removal), or more heterogeneous in size (ether injection) than sonicated liposomes. The reason for their use is the large amount of entrapped material necessary to induce significant pH changes in the surrounding unbuffered medium. The use of carboxyfluorescein in such systems offers several advantages. Carboxyfluorescein can be entrapped at high concentrations in liposomes prepared by any of these techniques. Its subsequent leakage can be monitored by simple fluorescence measurements with extreme sensitivity (less than 1 pmol carboxyfluorescein), by taking advantage of the fluorescence self-quenching [8]. We have simplified this technique by the introduction of nomograms that give the percentage of carboxyfluorescein leakage from the ratio of fluorescence measured before and after addition of Triton into the sample. Sonicated liposomes can be easily studied and the results do not rely upon the measurement of pH in unbuffered solutions near neutrality. Further advantages are that the experiment can be started by dilution of the liposome preparation in a buffer of known concentration, pH and temperature, and stopped at any time by further dilution in cold neutral buffer.

The experimental data presented in this paper clearly show that carboxyfluorescein leakage does not depend directly on the external pH. Instead, leakage depends on the rate at which acid molecules are able to enter into the liposomes and neutralize carboxyfluorescein. This was true whether or not liposomes contained cholesterol. These observations are strengthened by the fact that a simple mathematical model can account both qualitatively and quantitatively fort he present data and by the agreement with the data of Nozaki and Tanford [4] with respect to HCl diffusion. Gutknecht and Walter [5] reported much larger values for the HCl-permeability coefficient across planar phospholipid bilayers. Their experimental system was different and they used unbuffered solutions of concentrated HCl (100 to 300 mM) which were in contact with the lipid bilayer. This would be expected to modify considerably the phospholipid charges making their results difficult to compare with ours.

The main controversy in this domain arises from the very high permeability reported for protons by several authors, including Nichols et al. [6], Deamer and Nichols [18] and Rossignol et al. [7]. In the more recent papers, it seems that fast diffusion of protons was observed only when low pH gradients were studied [18]. In the present experiments the potassium ionophore valinomycin increased to some extent the rate of carboxyfluorescein leakage induced by HCl/glycine when liposomes contained the K^+ salt, indicating that the absence of counterion current slowed proton movement, as shown by Deamer and Nichols [18]. However, in the absence of added ionophore, the diffusion barrier is created well before any noticeable leakage of carboxyfluorescein had occurred. Thus operationally and as far as biological applications of liposomes are concerned, one should consider that proton permeability for lipid membranes is very low.

The computer simulations of our experimental

system show that the internal pH of the liposomes is, in the first step, lowered so that the proportion of neutral carboxyfluorescein increases and the leakage begins. This first step is almost instantaneous with acetate which diffuses rapidly, but is slower with glycine. Several straightforward implications of the model (Table II) were verified: the initial rate of carboxyfluorescein leakage depended on pH and concentrations of external acid and on their permeability coefficeints. The pK of the acid species determined the proportion of uncharged acid at a given pH and acid concentration. Depending on whether carboxyfluorescein (acetic acid experiments) or acid (glycine experiment) diffusion was rate limiting, carboxyfluorescein leakage started immediately or with a measurable lag-time. Multivalent acids, probably because of their low liposolubility, hardly induced carboxyfluorescein leakage. Very weak acids (with pK higher than that of carboxyfluorescein) were not studied. The model predicts that at pH 5 and constant total (anion and uncharged) acid concentration, fast diffusing acids of pK around that of the first negative ionization of carboxyfluorescein would be the most efficient in inducing carboxyfluorescein leakage.

Precise permeability coefficients were not derived from the kinetics of carboxyfluorescein leakage. This would have been, in any circumstance, impaired by the necessity to determine very accurately carboxyfluorescein diffusion coefficients and concentrations and also by the unavoidable heterogeneity of liposome preparations. Nevertheless, our results clearly demonstrate that the less polar acids such as acetate and pyruvate are very efficient at inducing carboxyfluorescein leakage while the polar ones were less effective, in perfect correlation with their solubility in apolar medium (see also Bakker and Van Dam [21]). With respect to liposomes as drug delivery systems and to the cell biology of the receptor mediated endocytosis of liposomes, this work shows that there is evidence that the acidic environment of lysosomes, which contain various organic acids as a consequence of proteolysis, could act by itself to induce carboxyfluorescein release from the liposomes. While methotrexate leakage was not directly measured here, its similarity in charge and molecular weight to carboxyfluorescein, and preliminary results from a methotrexate radioimmunoassay (our unpublished data) suggest that it behaves similarly. This would explain the inhibitory effect of lysosomotropic compounds in the intracellular delivery of methotrexate [1,22,23] and carboxyfluorescein [17,24], and suggest that the weak anion substances would be more easily delivered into cells than neutral or cationic ones.

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