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THE EFFECT OF pH ON THE SIZE OF LIPOSOMES FORMED BY CHOLATE DIALYSIS

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The pH of the medium during the formation of liposomes by the cholate dialysis method affects their size. Liposomal size as measured by electron microscopy and volume equilibration is greatest if the dialysis step is carried out at pH 6 and decreases as the pH increases. The effect of liposome size on estimations of their incorporated choline carrier activity is illustrated.

Liposomes are being used increasingly as tools for the reconstitution and investigation of membrane function [1]. One of the most widely employed techniques to reconstitute solubilised membrane proteins into unilamellar liposomes is to form them from a solution of protein, lipid, and sodium cholate by removing the detergent by dialysis (cholate dialysis [2]). However, when these liposomes are used to measure reconstituted membrane transport functions, the sensitivity of the assay will vary, depending upon the size of the liposomes. The smaller the liposome, the more quickly will it reach equilibrium with its surroundings because the volume-to-surface-area ratio is proportional to the radius of the liposome. Therefore, measurements of uptake of a substance by unilamellar liposomes over a fixed time period (e.g., 30 s) will be a better approximation to initial rate of uptake in larger liposomes than in smaller liposomes. Since the measurement of specific transport phenomena usually depends upon the inhibition of initial rates of uptake by specific inhibitors, the larger the liposomes the greater the inhibition observed during the initial uptake period and, therefore, the more sensitive the assay.

While attempting to purify the molecule(s) involved in choline transport we observed that acid pH conditions during dialysis increased the size of the resulting unilamellar liposomes, and also increased the sensitivity of the assay for choline transport when membrane protein was incorporated into them.

[*methyl*-^{3}H]Choline chloride (60–85 Ci/mmol, ethanol solution) was obtained from Amersham International, U.K. The ethanol was removed by a stream of N_2 prior to use. Trypsin inhibitor (type II-S, from soybean), and phenylmethylsulphonyl fluoride were obtained from Sigma. Hemicholinium-3 was obtained from Aldrich Chemical Co. Asolectin (Soybean lipid extract) was obtained from Associated Concentrates, Woodside, New York, and acetone washed [2] before use. Triton X-100, cholic acid and sodium dodecyl sulphate were obtained from Sigma. Cholic acid was purified by ethanol precipitation [2]. The bath ultrasonicator was a 1.71 capacity 'Pulsatron 55' obtained from Kerry Ultrasonics, U.K. Millipore-filters (GSWP 02500) were obtained from Millipore (U.K.) Ltd. The neuronal membrane fraction was a bulk preparation of hypoosmotically ruptured crude synaptosomal fraction produced from ox brain cortex by the method of Ueda and Greengard [3], itself a modification of the procedure described by De Robertis et al. [4].

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Surface and non-intrinsic membrane proteins were removed by solubilisation with 0.05% Triton X-100, before the solubilisation of the remaining membrane components by the use of 0.5% Triton X-100 [5]. 10% v/v Triton X-100 was added (to a concentration of 0.05%) to the membrane preparation suspended in 100 mM NaCl 10 mM Tris-HCl (pH 7.4)/1 mM dithiothreitol/0.3 mM EDTA/0.1 mg/ml trypsin inhibitor/100 μM phenylmethylsulphonyl fluoride (protein concentration approx. 2 mg/ml). The suspension was stirred at 4°C for 30 min, and centrifuged at $100000 \times g$ (Beckman L265, SW27 rotor) for 1 h at 4°C. The solubilisation procedure was then repeated by resuspending the pellet in the same volume of the same buffer but this time with a Triton concentration of 0.5% (v/v). Centrifugation was again performed as above and the supernatant was retained. Asolectin was solubilised by Triton X-100 as described below, except that 2% (v/v) Triton X-100 was used instead of 2% (w/v) sodium cholate. Triton X-100 was removed from samples by stirring with Biobeads SM-2 (0.3 g beads/ml) for 2 h [6].

Cholate dialysis is designed to form unilamellar liposomes by the removal of detergent (during dialysis) from a mixture of lipid, sodium cholate, with or without solubilised protein [2,7]. All procedures were performed at 4°C. Asolectin was suspended in 100 mM NaCl/10 mM Tris-HCl (pH 7.4)/0.3 mM EDTA/2% (w/v) sodium cholate to a concentration of approx. 25 mg/ml. The suspension was sonicated in a sealed tube under N_2 to clarity using a bath sonicator (containing ice and water) for 30 min. This suspension was centrifuged at $100000 \times g$ (Beckman L265, SW27 rotor) for 1 h to remove any insoluble material. The supernatant was diluted in the above buffer to give a lipid concentration of 5–15 mg/ml. This solution was then dialysed for 16–20 h against 300–1000 vol. 100 mM NaCl/10 mM Tris-HCl (pH 7.2)/0.1 mM $CaCl_2$/0.1 mM $MgCl_2$/10 μM phenylmethylsulphonyl fluoride/0–5 μM choline chloride. If a pH of less than 7.2 was required during dialysis, the 10 mM Tris-HCl buffer was replaced by 20 mM sodium dihydrogen orthophosphate/disodium hydrogen orthophosphate buffer at the required pH. The choline chloride concentration used in the dialysis fluid was 5 μM. Liposomes were concentrated by centrifugation before the uptake measurements [7]: 15–20-ml batches of liposome suspension were spun at $40000 \times g$ (20000 rpm, Sorvall RC2B, SS34 rotor) for 30 min at 4°C, and the pellets from each batch resuspended in 0.4–2 ml of supernatant (to a maximum lipid concentration such that 20 μl liposomes would not block the Millipore filters during filtration and that the process of filtration and washing (see below) could be done in less that 20 s, this being decided empirically for each batch of liposomes).

Choline transport was measured by observing the uptake of [^{3}H]choline chloride with or without the presence of the specific inhibitor of choline transport Hemicholinium 3 [8] or saturating concentrations of non-radioactive choline chloride (10 mM). The liposomes were separated from the surrounding medium by millipore filtration, followed by washing [9]. The method used has been described previously [15]. Proteins in solution were measured by a modification of the method of Lowry et al. [10], with the addition of 0.5% w/v sodium dodecyl sulphate to overcome the interference of Triton X-100, and help micellise large quantities of lipid [11]. Proteins trapped on millipore filters were measured by staining the filters with Amido Schwarz 10B [12]. Phospholipids were measured by lipid extraction [13], followed by perchloric acid digestion and measurement of inorganic phosphate [14].

The average diameter of unilamellar liposomes was measured either by electron microscopy or by incubating the liposomes for 2–3 h with [^{3}H]choline (after which time the liposomes have approximately reached equilibrium with their surroundings [15]). The diameter of these liposomes can then be estimated roughly using the following assumptions:

(1) That the [^{3}H]choline uptake by the liposomes after 2–3 h is approaching equilibrium, is mostly present in the internal aqueous compartment of the liposomes, and that the internal aqueous [^{3}H]choline concentration is the same as the concentration in the incubation medium at equilibrium (i.e., it is assumed there is no large membrane potential).

(2) That the surface area of a fully hydrated lecithin headgroup in a phospholipid bilayer is 70 Å^2 [16] that this figure is approximately the same for other phospholipid headgroups, and that the

contribution to the surface area of the lipid bilayer by proteins or non-phosphorus containing lipids is small.

(3) That the mean molecular weight of phospholipids is 900.

(4) That the liposome bilayer has an approximately equal number of phospholipid molecules on the outside and inside surfaces.

(5) That the liposomes are spherical and uniform in size.

Fig. 1 shows electron micrographs of liposomes made from asolectin at pH 6.0 and 8.0. Estimates of liposomal diameter were made by measuring all clearly defined ring structures in rectangles of equal area but visual inspection reveals that those formed at pH 6.0 are larger than those formed at pH 8.0. It is also seen that the ring structures are negatively stained for the pH 6.0 liposomes, but darkly stained for the pH 8.0 liposomes. Negative staining of liposomes has been found to vary in its effect depending upon the conditions on the electron microscope grid during the staining procedure [19]. It has been postulated that in some cases the stain may form a meniscus around the liposomes [19], which could well account for the appearance of the pH 8.0 liposomes in Fig. 1.

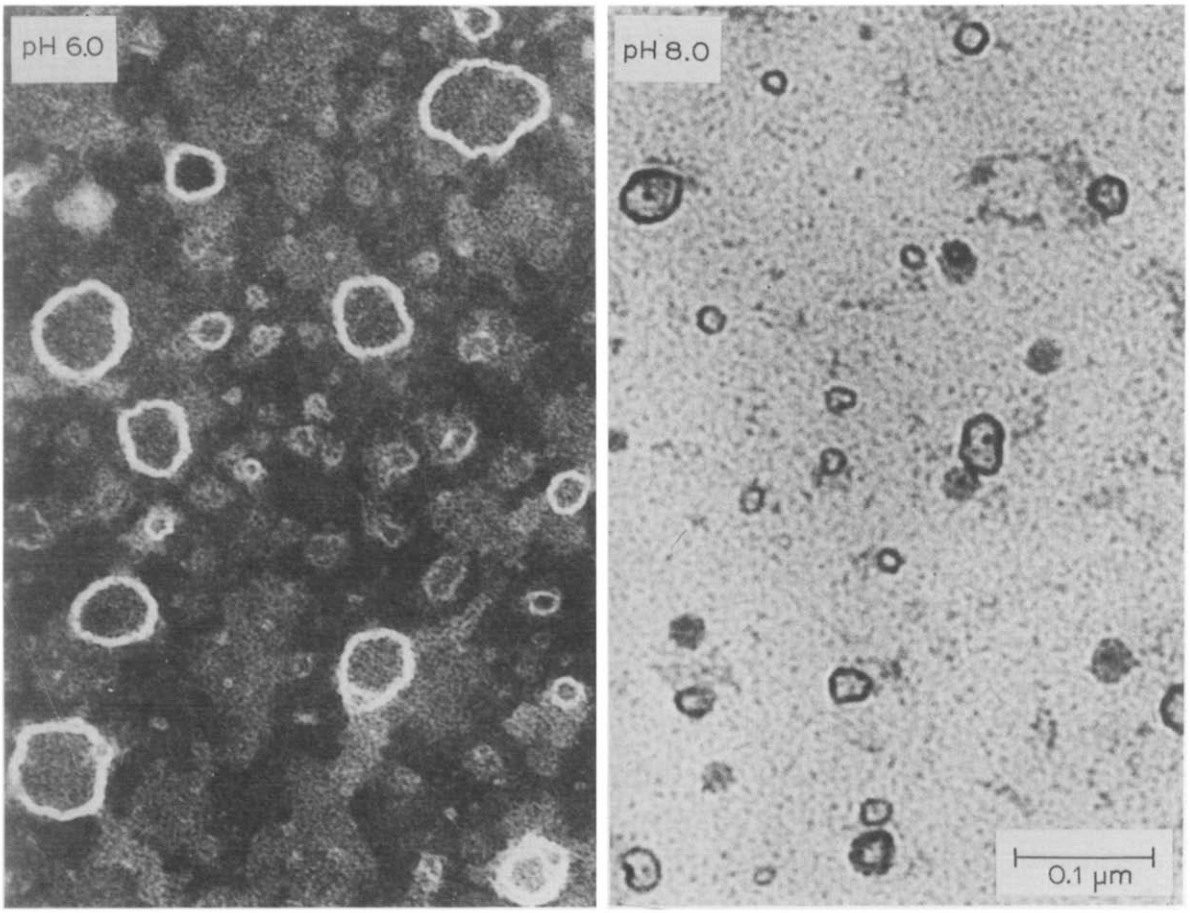

Fig. 1. Liposomes were made and concentrated by centrifugation as in Table I. They were then prepared for electron microscopy by negative staining [18]. To 0.1 ml liposomal suspension was added 0.1 ml 10% (w/v) formaldehyde in dialysis buffer (made to pH 7.0 with 0.33 M NaOH). After 5 min a small drop was placed on a collodion coated specimen grid (held by forceps), and most of this material was removed by placing a piece of filter paper lightly against the grid, thus leaving a thin film. A drop of 2% (w/v) phosphotungstic acid (neutralised to pH 7.4 with 2 M NaOH) was applied, and then all but a thin film removed as above. This was allowed to dry, and then the grids were viewed under an electron microscope. The electron micrographs shown are both at magnification 180000, and show typical fields from the liposomes made at pH 6 and at pH 8.

TABLE I

EFFECT OF pH ON THE FORMATION OF LIPID LIPOSOMES BY CHOLATE DIALYSIS

Liposomes were made using acetone-washed asolectin (10 mg phospholipid/ml) by cholate dialysis as described in the text, except that immediately prior to dialysis the cholate solubilised asolectin was divided into three equal batches, and each batch dialysed against dialysis medium at the pH shown above. Liposomes were concentrated by centrifugation prior to both uptake measurements and electron microscopy. During the uptake of [^{3}H]choline (5 μM) the pH for each batch of liposomes was the same as the pH of the dialysis medium in which that batch had been dialysed, thus avoiding the deliberate creation of a pH gradient across the liposomal membrane. Estimation of liposomal size from electron micrographs (electron microscopy as in Fig. 1) was performed by measuring diameters of all clearly defined ring structures within a rectangle of equal area from a micrograph of each batch of liposomes. Figures show mean ± S.E. (n).

	Liposomes dialysed at:		
	pH 6.0	pH 7.0	pH 8.0
3 h [^{3}H]choline uptake pmol/mg phospholipid	9.0 ± 0.5 (4)	5.4 ± 0.7 (4)	2.9 ± 1.0 (4)
Diameter of liposomes (nm) using assumptions in text	48 ± 3 (4)	29 ± 4 (4)	16 ± 6 (4)
Diameter of liposomes (nm) estimated from electron micrographs	47 ± 4 (13)	35 ± 4 (15)	24 ± 2 (16)
% $\frac{[^3\text{H}]\text{choline uptake in 40 s}}{[^3\text{H}]\text{choline uptake in 3 h}}$	12 ± 5 (4)	21 ± 4 (4)	39 ± 10 (4)

Table I shows the results of an experiment where liposomes were made from asolectin by cholate dialysis at different pH values. Estimations of liposomal diameter (using the above assumptions) are larger at the lower the pH of dialysis in the pH range 6.0–8.0. Also, estimations of liposomal diameter from electron micrographs show the same trend, and are similar (for each pH condition) to the estimates made using the above assumptions.

Also shown in Table I are figures for [^{3}H]choline uptake into liposomes within 40 s as a ratio of [^{3}H]choline uptake in 3 h. These figures are greater the smaller the estimated liposomal size, which is to be expected, since smaller liposomes should reach equilibrium with their environment more quickly than larger liposomes provided the permeability of their membranes is the same.

As we have reported previously [15] when liposomes are made containing protein derived from solubilised neuronal synaptic plasma membranes

TABLE II

THE EFFECT OF pH ON SIZE OF PROTEOLIPOSOMES, AND ON THE ASSAY FOR CHOLINE TRANSPORT

Triton X-100-solubilised neuronal membrane fraction and Triton-solubilised asolectin were mixed (0.45 mg protein/ml, 10 mg phospholipid/ml) and then used to form liposomes by removing the Triton with Biobeads SM2, adding sodium cholate to 2%, and dialysing against medium at pH 6.8. One batch was made to pH 6.6 immediately prior to dialysis, and the other to pH 7.4 (buffered by adding 0.5 M sodium phosphate buffer to 20 mM at the required pH). S.E. values are from four filtrations, and [^{3}H]choline concentration during uptake was 5 μM. HC-3, hemicholinium-3.

		pH immediately prior to dialysis:	
		6.6	7.4
2 h liposomal [^{3}H]choline uptake (pmol/mg phospholipid)		15 ± 1.2	3.4 ± 0.2
Estimated liposomal diameter (nm)–assumption as in text		80 ± 6	18 ± 1
35 s liposomal	No inhibitors	14 ± 1	8 ± 2
[^{3}H]choline uptake	100 μM HC-3	3 ± 1	2 ± 2
(pmol/mg protein)	10 mM choline	2 ± 3	3 ± 4
Mean HC-3 inhibitable [^{3}H]choline uptake (35 s) (pmol/mg protein)		11	6

(by cholate dialysis), these liposomes demonstrate uptake of [^{3}H]choline which is saturable, inhibited by hemicholinium-3 (a specific choline transport inhibitor) and activated from the *trans* position by preloading with non-radioactive choline. One problem we have encountered in attempts at purification of the choline transport system (using liposomes as an assay for choline transport), is the variability of liposomal size, and the insensitivity of the assay. However, as shown in Table II, if the pH of the lipid, protein, and detergent mixture is made to pH 6.6 immediately prior to dialysis (compared with pH 7.4), then the resulting liposomes are larger (80 ± 6 nm vs. 18 ± 1 nm), and also demonstrate greater hemicholinium-sensitive [^{3}H]choline uptake in 35 s (11 pmol/mg protein vs. 6 pmol/mg protein). Thus the larger the liposomes (made from the same material), the greater the sensitivity of the assay for the choline transport system. A lower limit is placed on the pH of the cholate dialysis procedure, however, since the pK of the cholic acid is pH 5.5 [17] and at a pH of below 6.0, non-micellised cholate will precipitate.

Although our interest has been in the reconstitution of the choline transport system [20], the results we describe on the effects of pH on liposomal size and the resultant increased sensitivity of a transport assay may well apply to the reconstitution of other membrane transport systems.

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