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A special lipid mixture for membrane fluidization

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The potency for membrane fluidization of mixtures containing neutral lipids (NL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from hen egg yolk was tested on human erythrocytes and lymphocytes. A specific mixture consisting of 70% NL, 20% PC and 10% PE was found to be a potent membrane fluidizer operating almost exclusively by extracting membrane cholesterol. Spectral results and electron micrographs indicate that aqueous dispersion of this mixture consists of chylomicron-like assemblies where the neutral lipids provide the hydrophobic core on the surface of which phospholipids are spread as a monolayer.

Introduction

Many cellular functions are inhibited when the lipid fluidity of the cell membrane is reduced, most commonly by increase in mole ratio of cholesterol to phospholipids [1,2]. In principle, such a reduction in cellular activity is at least partially reversible and can therefore be rectified by treatment with membrane fluidizers [1,2]. This approach bears an attractive possibility for an in vivo rehabilitation of physiological functions in aged [3,4] and drug addicted [5] subjects, where membrane rigidification is one of the most prominent symptoms [6–8]. For such cases membrane fluidization could be based on treatment with natural lipids like lecithin (i.e. phosphatidylcholine) [3] which eliminates any possible adverse effects.

Attempts in our laboratory to formulate an efficient lipid mixture for membrane fluidization, both in vitro and in vivo, have led to the discovery of a potent mixture consisting of egg-yolk neutral lipids, phosphatidylcholine and phosphatidylethanolamine. Preliminary results on the in vivo effects of this mixture in restoration of impaired brain structure and function [4,5], as well as lymphocyte responsiveness [4,9,10], were already reported elsewhere. The following paper presents an account on the biophysical nature of this mixture and its mode of action as a membrane fluidizer in vitro of human erythrocytes and lymphocytes.

Materials and Methods

All chemicals and solvents used were of the highest purity available. Sodium boro[^{3}H]hydride (20 Ci/mmol) and [^{3}H]cholesterol (40 Ci/mmol) were purchased from Amersham. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE), both from hen egg yolk and of > 99% purity, were purchased from Lipid Products (Nutfield, England).

Neutral lipids (NL) from egg yolk were isolated as follows: One hen egg yolk (approx. 20 ml) was first mixed with 180 ml isopropanol and after 1 h at room temperature was supplemented with 200 ml chloroform and mixed for another hour [11]. The precipitate was removed by filtration and the total lipid extract was recovered by evaporating the supernatant. The lipid was then dissolved in 50

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ml hot ethanol and mixed with 1 liter of 1 M KCl in water in a separation funnel. The upper layer of neutral lipids was separated, washed twice with distilled water and then dried by lyophilization. Thin-layer chromatography and chemical analysis indicated over 90% acylglycerols (mostly triacylglycerols), 2–3% cholesterol and 1–2% phospholipids.

^{3}H-labelled lipids. Partial hydrogenation of lipid double bonds was carried out with NaB^3H_4 on each lipid separately or in NL/PC/PE mixtures. 5 mg $NaBH_4$ + 100 mCi NaB^3H_4 in 1 ml ethanol containing 0.1 M NaOH was added to 100 mg lipid in 1 ml tetrahydrofuran. The catalyst mixture of 2 mg of chloroplatinic acid dissolved in 0.4 ml ethanol and mixed with 5 mg of active charcoal [12] was then added. The reaction was initiated by acidifying upon addition of 0.2 ml 1 : 9 concentrated HCl solution in ethanol and was allowed to proceed overnight at room temperature. The ^{3}H-labelled lipids were purified by preparative thin-layer chromatography with chloroform/methanol/water (65 : 25 : 4, v/v). The specific activity obtained ranged from 5 to 50 μCi per mg lipid which accounted for less than 5% saturation of the lipid double bonds.

Lipid treatment of blood cells. In all of the lipid treatments polyvinylpyrrolidone (PVP, M_r 40 000, Sigma) was used as the hydrophobic carrier [13,14]. The PVP medium consisted of 3.5% PVP, 1% bovine serum albumin and 0.5% glucose in phosphate-buffered saline (pH 7.4). The lipids were introduced into the PVP medium by 1 : 100 dilution of an ethanol solution, while in the control PVP medium 1% ethanol was included.

Human erythrocytes and lymphocytes were isolated from freshly drawn heparinized blood by the Ficoll-Hypaque sedimentation technique [15] and then washed twice with phosphate-buffered saline. $5 \cdot 10^7$ erythrocytes or $3 \cdot 10^6$ lymphocytes per ml PVP medium containing 0.5 mg/ml lipid were incubated at 37°C with gentle shaking in loosely capped glass vials. Non specific adherence of lipids to the cell surface, which is mostly governed by electrostatic interactions and which could introduce serious errors in fluidity measurements, was virtually completely eliminated by washing with high salt concentration (0.25 M CsBr). The treated cells were thus washed once with phosphate-buffered saline then with 0.25 M CsBr and once again with phosphate-buffered saline. The efficiency of the wash with 0.25 M CsBr was verified with lipid mixtures containing ^{3}H-NL, ^{3}H-PC and ^{3}H-PE. A single wash with 0.25 M CsBr was found to reduce the radioactive counts of the treated cells to background level.

For labelling of cells with [^{3}H]cholesterol incubation with PVP medium containing 10 μg/ml [^{3}H]cholesterol was carried out under the above conditions, and was allowed to proceed until an increase of about 1% in membrane cholesterol was reached.

Measurement of membrane fluidity. The standard technique of steady-state fluorescence depolarization with 1,6-diphenyl-1,3,5-hexatriene as a probe, was used [16,17]. Erythrocyte membranes [18] at a final dilution of 1 : 200 or intact lymphocytes ($2 \cdot 10^6$ per ml) in phosphate-buffered saline were labelled with diphenylhexatriene and the degree of fluorescence polarization, P, was determined as previously described [19]. The empirical linear scale of $2P/(0.46 - P)$ was used as an approximate presentation of the membrane lipid microviscosity [16,17]. Resolution of P which corresponds to the plasma membrane of human lymphocytes by the bixinoyl glucosamine quenching method [20] gave values which were less than 5% higher than those obtained with intact cells. Therefore, for our routine fluidity screening we used intact lymphocytes only.

Surface labelling of PE. The lipid impermeable reagent 2,4,6-trinitrobenzenesulfonic acid (TNBS) was used for measuring the availability of PE at the outer surface of the lipid assemblies [21,22]. Lipid mixtures (0.5 mg/ml) in phosphate-buffered saline (PBS) were sonicated for 5 min in the absence and in the presence of 0.1% Triton X-100. This detergent was applied for disintegration of the lipid assemblies to form permeable mixed micelles. Into 1 ml of each of the mixtures 10 μl of 100 mg/ml TNBS (colour free sodium salt from Research Organics Inc.) in water was added. In the other 1-ml samples, which served as reference, 10 μl of water was added. After 2 h incubation at room temperature the absorbance at 420 nm of the TNBS-labelled samples [20], in the absence (A_{PBS}) and in the presence of Triton X-100 (A_{Triton}), were measured versus their reference samples in tri-

plicate. The ratio A_{PBS}/A_{Triton} was taken as the fraction of PE available for TNBS labelling.

Lipid analysis. Total lipid extract from intact cells (10^8 per ml Hepes-buffered saline) was obtained by isopropanol-chloroform extraction [10]. Phospholipids and cholesterol were analysed with this extract by conventional colourimetric assays [23,24].

Results and Discussion

The principal determinant of the bulk lipid fluidity in cell membranes is the mole ratio of cholesterol to phospholipids, C/PL [1]. Membrane fluidization by reduction of C/PL can be mediated by external lipids through passive equilibrium-exchange of cholesterol [25] or by incorporation of external phospholipids [26]. For membrane fluidization in vivo the external lipid pool should exert its effect before being catabolized, namely within several blood cycles (i.e. minutes). The common fluidizing phospholipids, such as PC, do not comply with this prerequisite as their aqueous dispersions constitute stable lipid bilayers which are slow in extraction of membrane cholesterol [25] or in release of monomers for incorporation into cell plasma membranes [26].

To facilitate the fluidization activity of PC we have mixed it with PE at a mole ratio of 2 : 1 and with NL. PE is an abundant phospholipid which at high concentration forms hexagonal phase which is markedly different than the liquid crystalline bilayer [27]. At lower concentration it destabilizes lipid bilayers [27] and in aqueous mixtures of NL, PC and PE the lipids are likely to form chylomicron type assemblies where the acylglycerols serve as the hydrophobic carrier on the surface of which the phospholipids are randomly spread. This loosely integrated structure may facilitate the translocation of membrane cholesterol and may also serve as a source for monomeric phospholipids.

The membrane fluidization potency of the various NL/PC/PE mixtures was tested on human erythrocytes and lymphocytes (see Materials and Methods). The mixtures were all of 0.5 mg/ml and consisted of the phospholipids PC and PE (2 : 1) and increasing amounts of neutral lipids. In human erythrocytes incubation of over 12 h was required for an appreciable reduction in membrane microviscosity while with human lymphocytes a similar response was achieved after less than 2 h of incubation. Results obtained with these two cell types are shown in Figs. 1 and 2.

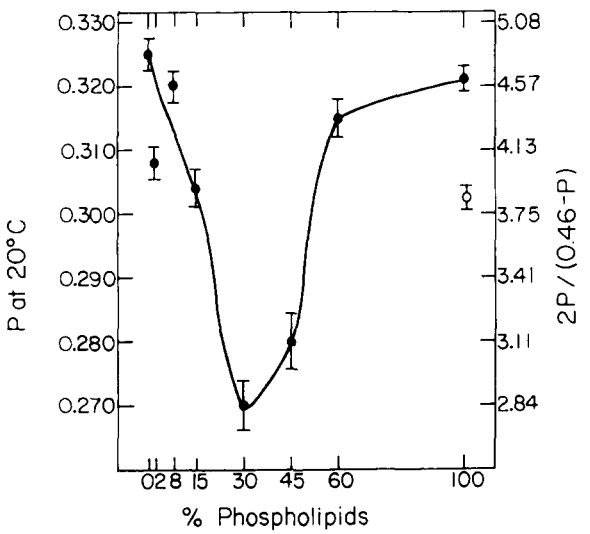

Fig. 1. Fluidization of human erythrocyte membranes ($5 \cdot 10^7$ cells/ml) with NL/PC/PE mixtures (0.5 mg/ml) after 18 h incubation at 35°C. Results are presented as the mean ± S.D. of the degree of diphenylhexatriene fluorescence polarization, *P*, obtained for membrane samples of ten humans (●). Results obtained with PC alone under the same conditions (○) are included for comparison.

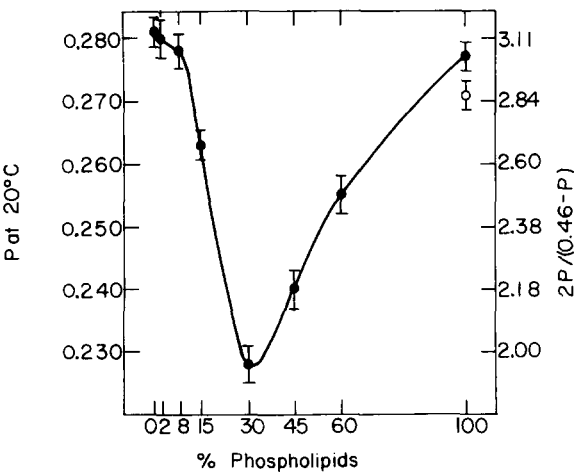

Fig. 2. Fluidization of human lymphocyte membranes ($3 \cdot 10^6$ cell/ml) with NL/PC/PE mixtures (●) or with PC (○) (0.5 mg/ml) after 3 h incubation at 37°C. Results were of six separate samples and are presented as in Fig. 1.

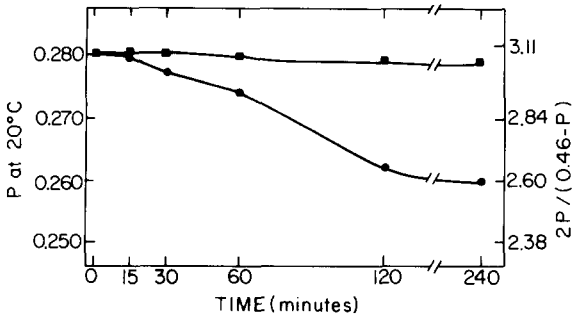

Fig. 3. Increase in membrane fluidity of human lymphocytes (one sample) incubated at room temperature with mixture 721 (0.5 mg/ml) for different periods of time (●). Results obtained with control medium (■) are also included.

The membrane fluidization profile in both cells is remarkably similar and indicates that the mixture of NL/PC/PE (7 : 2 : 1, 'mixture 721') is the most potent membrane fluidizer. In lymphocytes, mixture 721 mediates its effect within relatively short period of incubation even at room temperature (see Fig. 3) and is markedly more potent than PC alone (see Figs. 1 and 2). When similar experiments were carried out with cells which were surface labelled with ^{125}I no significant radioactivity was shed to the supernatant after the lipid treatment (data not shown) which excluded the possibility of shedding of membrane fragments.

The mechanism of membrane fluidization by NL/PC/PE mixtures was assessed by the ability to extract membrane cholesterol or to insert exogenous phospholipids into the cell membrane. Human peripheral blood lymphocytes with [^{3}H]cholesterol in their membrane were incubated with various NL/PC/PE mixtures under conditions similar to those in the fluidization experiments (see above). The radioactivity per cell population was measured after 0 and 3 h incubation time and the results are presented in Fig. 4. As shown, the profile of cholesterol extraction potency is similar to that observed in the membrane fluidization experiments (Fig. 2), indicating that mixture 721 is the most potent cholesterol extractor.

It should be noted that except for the fact that [^{3}H]cholesterol in the labelled cells could not be washed out with 0.25 M CsBr no direct information as for its mode of integration with the membrane lipids, was available. The finding that almost 50% of [^{3}H]cholesterol could be extracted with mixture 721 (see Fig. 4) indicates that during the 3 h of the experiment [^{3}H]cholesterol preferentially resided in the outer membrane leaflet, presumably due to a slow equilibration (flip-flop) with the inner leaflet.

The total cholesterol level in intact cells, expressed as C/PL, was found to be 0.65 ± 0.05

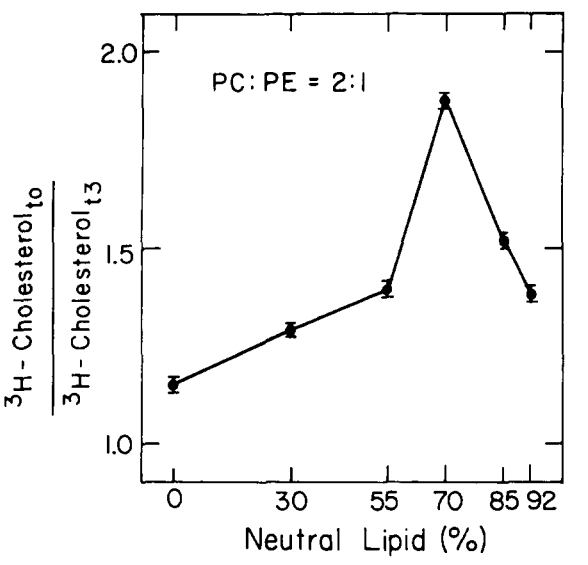

Fig. 4. Relative change in cholesterol content of human lymphocytes labelled with [^{3}H]cholesterol after treatment with NL/PC/PE mixtures (see legend to Fig. 2). The results are presented as the ratio of cpm at time 0 and after 3 h of incubation at 37°C.

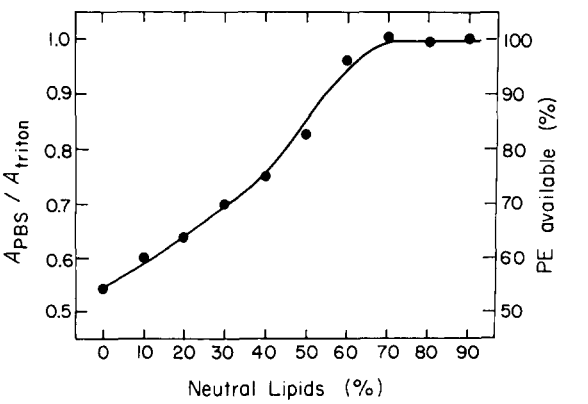

Fig. 5. Fraction of PE available for labelling with TNBS in mixtures of NL/PC/PE.

(mole ratio) in untreated lymphocytes and 0.55 ± 0.05 (mole ratio) in lymphocytes treated for 3 h with mixture 721, as above. This reduction in cholesterol content should be taken as the lower limit for the change in plasma membrane cholesterol, since internal membranes also contribute to this value. It nevertheless indicates again a significant reduction in membrane cholesterol after treatment with mixture 721.

When human lymphocytes or erythrocytes were incubated up to 8 h with mixture 721 containing either ^{3}H-NL, ^{3}H-PC or ^{3}H-PE, under conditions similar to those in the above experiments, no appreciable uptake of any of the radioactively labelled lipids was detected. These results indicate that lipid fluidization of erythrocyte or lymphocyte membranes by NL/PC/PE mixtures operates almost exclusively by extraction of membrane cholesterol. It should be noted, however, that preliminary experiments of fluidization of rat brain membranes (e.g. synaptic plasma membranes) with mixture 721 [4] indicated an appreciable incorporation of ^{3}H-PC as part of the fluidization mechanism.

The availability of the PE primary amine for labelling with the lipid impermeable reagent TNBS (see Materials and Methods) was measured in order to gain insight into the molecular arrangement of the various NL/PC/PE aqueous dispersions. Because of strong background staining these experiments were carried out in the absence of polyvinylpyrrolidone and bovine serum albumin. The results, shown in Fig. 5, indicate that in the absence of neutral lipids, close to 50% of the PE residues in the PC/PE (2 : 1) mixture is concealed from the outer aqueous domain. Addition of neutral lipids increases the exposure of PE and at 70% neutral lipids (i.e. in mixture 721), or above, all of the PE is exposed to the bulk aqueous medium. At these NL concentrations the phospholipids are presumably spread as a monolayer on the surface of the acylglycerol aggregates similarly to chylomicrons. Electron micrographs of mixture 721 in water comply with this suggestion and indicate globular structures, 0.1–1 μm in diameter, with electron dense material (i.e. phospholipids) on its surface (see Fig. 6). Based on these results a model for the lipid assemblies which constitute the aqueous dispersion of mixture 721, is presented in Fig. 7. No data as to the effect of polyvinylpyrrolidone

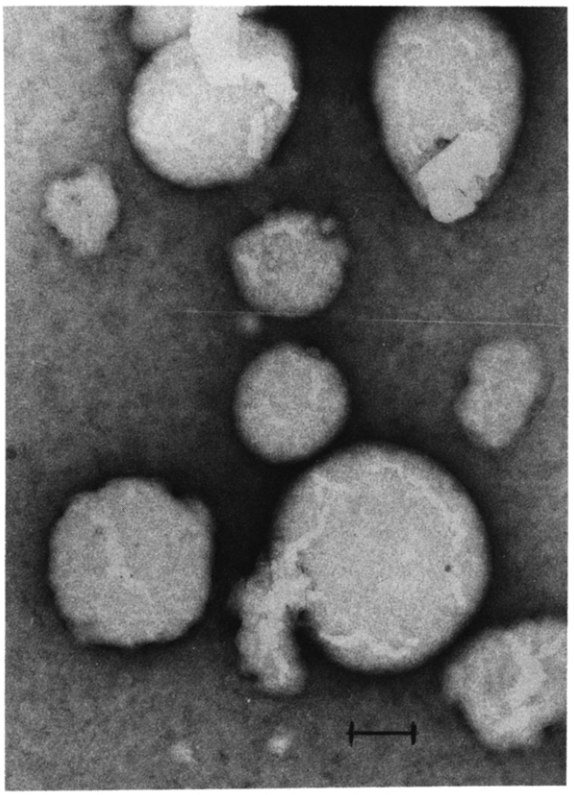

Fig. 6. Electron micrograph of mixture 721 in water after negative staining with uranyl acetate. The bar represents 0.1 μm (recorded by S. Himelhoch).

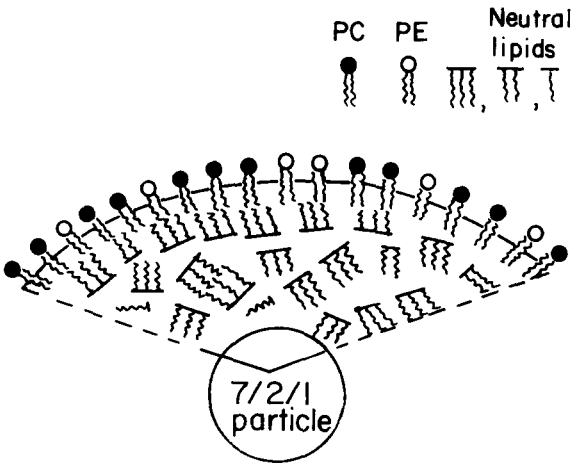

Fig. 7. A suggested model for the lipid assemblies of mixture 721 in water.

and bovine serum albumin on this structure is yet available.

Mixture 721 ('active lipid') was previously shown to be of practical use for membrane fluidization of brain tissues both in vitro and in vivo [4,5]. As demonstrated in this study, it also bears a great potential for fluidization of lymphocyte membranes and thus opens a route for modulation of immune responsiveness [4,9,10]. Detailed reports on the in vitro and in vivo rectification of impaired lymphocyte activity by mixture 721 in experimental animals and humans are now in preparation.

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