Resonance Energy-Transfer and Fluorescence Intensity Studies of the Transport of Liposome-Encapsulated Molecules into Isolated Mouse Liver Nuclei

Tudor Arvinte, Philippe Wahl,* and Claude Nicolau
Centre de Biophysique Molêculaire du CNRS, 45071 Orlêans Cedex 2, France
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ABSTRACT: We present evidence that liposomes (composed of egg yolk L-α-phosphatidylcholine/phosphatidylethanolamine/cholesterol, in a molar ratio of 4:5:1) fuse with isolated mouse liver nuclei at low pH. Using the resonance energy-transfer assay, we determined the rate and extent of liposome and nuclear membrane lipid mixing. Fusion was substantial when the pH was below 5. The half-time of lipid mixing decreased by acidification of the solvent, reaching about 2 min at pH 4.5. In order to study the transport of the liposome–aqueous contents to the interior of the nuclei during the process, we developed fluorescence assays in which fluorescein isothiocyanate labeled dextrans of 150 kDa molecular mass (FITC-D150) were encapsulated in liposomes. These liposomes also included in their bilayers the fluorescent lipid *N*-tetramethylrhodamine-L-α-dipalmitoylphosphatidylethanolamine (*N*-Rh-DPPE). After incubation of these liposomes with mouse liver nuclei (pH 4.5, 37 °C, 30 min), we measured the fluorescence spectra of a suspension of washed nuclei and of nuclei treated by the detergent Triton X-100 (membrane-denuded nuclei). These Triton X-100 treated nuclei had no *N*-Rh-DPPE fluorescence while they showed a FITC-D150 fluorescence which amounted to 20% of that of the intact nuclei. In another assay, a laser beam was focused on single nuclei by a microscope epiexcitation device. The variation of the *N*-Rh-DPPE and FITC-D150 fluorescence with the nuclear radius was determined with the microphotometric attachment of the microscope. For the *N*-Rh-DPPE which was confined on the nuclear surface, the fluorescence intensity decreased strongly when the nucleus radius increased, as expected from the optical sectioning property of the microscope objective. This decrease was much weaker with the FITC-D150 fluorescence, showing that the dextran was partly distributed in the nuclear volume. By fitting these variations with formulas giving the light collection efficiency of the objective [Koppel, D. E., Axelrod, D., Schlessinger, J., Elson, E. L., & Webb, W. W. (1976) *Biophys. J. 16*, 1315–1329], we determined that 30% of the nucleus-associated dextrans were in the nucleus interior. To our knowledge, these are the first reported results showing that liposomes fuse with isolated mouse liver nuclei and that the liposome contents can be transferred into the nucleus interior. Our work suggests that liposome fusion with the nuclear membrane might be one of the steps in the process of transfer of liposome-encapsulated genes to eukaryote cells [Wong, T. K., Nicolau, C., & Hofschnieder, P. H. (1980) *Gene 10*, 87–94; Soriano, P., Dijkstra, J., Legrand, A., Spanjer, H., Londos-Gagliardi, D., Roerdink, F., Scherphof, G., & Nicolau, C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7128–7131].

It has been shown in a number of previous studies that low pH induces membrane fusion. The problem has been treated at different levels of complexity. Liposomes provide the simplest system. Sonicated unilamellar liposomes containing phosphatidylethanolamine and palmitoylcysteine fuse rapidly when the pH of the medium is lowered from 7 to 5 (Connor et al., 1984). Liposomes composed of oleic acid and phosphatidylethanolmaine aggregate, become destabilized, and fuse below pH 6.5 (Düzgüneş et al., 1985). More complex systems require, besides the low pH, the presence of proteins or fusogenic chemicals to induce or enhance the fusion of liposomes. For example, clathrin (Blumenthal et al., 1983; Hong et al., 1985), diphtheria toxin, tetanus toxin (Cabiaux et al., 1984, 1985), and polylysine (Uster & Deamer, 1985) were shown to induce fusion of phospholipid vesicles in acidic media.

The fusion between liposomes and viral membranes such as influenza virus (Stegmann et al. 1985), Semliki forest virus (White & Helenius, 1980), or fowl plague virus (White et al., 1982), between large unilamellar liposomes and membrane vesicles derived from *Bacillus subtilis* (Driessen et al., 1985), or between lipid-enveloped viruses and cells (Nicolau et al., 1978, 1979; White et al., 1980, 1981) is also induced by low pH.

In a recent study (Arvinte et al., 1986), we reported that liposome-bound lysozyme induces fusion between liposomes and erythrocyte ghosts. The maximum fusion rate occurred at pH 5.2, where the lysozyme enzymatic activity was optimum, suggesting that the enzyme active site is involved in the fusion process. Another interesting result of this study was the fact that at pH 4.4, liposomes without lysozyme also had very high fusogenic properties with erythrocyte ghosts.

In its ideal and physiologically relevant form, the fusion process of membranes requires mixing of membrane lipids and recombination of trapped volumes without leakage into the external medium. In the fusion of liposomes with biological systems, the mixing of membrane lipids can be studied relatively easily [i.e., using the resonance energy-transfer (RET)[1] method (Struck et al., 1981; Hoekstra, 1982)], while the recombination of trapped volumes is a more difficult problem to address, due to the diversity and complexity of the biological

[1] Abbreviations: Chol, cholesterol; DPPE, L-α-dipalmitoylphosphatidylethanolamine; EYL, egg yolk L-α-phosphatidylcholine; FRAP, fluorescence recovery after photobleaching; FITC-D150, fluorescein isothiocyanate labeled dextran of 150-kDa molecular mass; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Rh, tetramethylrhodamine; RET, resonance energy transfer; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TKM, 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, and 3.3 mM $CaCl_2$; kDa, kilodalton(s); *N*-Rh-DPPE, *N*-tetramethylrhodamine-L-α-dipalmitoylphosphatidylethanolamine; TLC, thin-layer chromatography.

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structures. A general modality for solving this problem is to encapsulate molecules in the liposomes and to prove that (i) after fusion these molecules are present in the biological system and (ii) the molecules entered only during fusion with the liposomes and not through other processes.

A method based on the fluorescence enhancement of a concentrated solution of carboxyfluorescein, initially confined in one compartment of the fusing systems, has been widely used in the past (Weinstein et al., 1977). This method cannot provide information in the case of the interaction of liposomes and nuclei, since the nuclei possess pores which permit free exchange of molecules with diameters smaller than 5 nm (Peters, 1983; Paine et al., 1975).

The results presented in this work show that at pH values lower than 5, liposomes (phosphatidylcholine/phosphatidylethanolamine/cholesterol in 4:5:1 molar ratio) fuse with mouse liver nuclei membranes. The mixing of membrane lipids during the fusion process is shown by using the resonance energy-transfer assay. A spectrofluorometric method and a microfluorometric method have been developed which indicate that part of the liposome contents passes the two nuclear membranes and is delivered into the mouse liver nuclei interior.

Materials and Methods

Chemicals. 7-Nitro-2,1,3-benzoxadiazol-4-yl (NBD) chloride and tetramethylrhodamine (Rh) isothiocyanate were purchased from Fluka. Fluorescein isothiocyanate labeled dextran (FITC-D150) (average molecular weight 150 000), and cholesterol (Chol) were from Sigma.

Lipids. Egg yolk L-α-phosphatidylcholine (EYL), grade 1, was purchased from Lipid Products. Phosphatidylethanolamine (PE) from egg yolk and L-α-dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma. All lipids showed single spots on TLC in the solvent mixture, $CHCl_3$/MeOH/H_2O, 65:24:4 (v/v). *N*-NBD-DPPE and *N*-Rh-DPPE were synthesized by the method of Monti et al. (1978) and Vanderwerf and Ullman (1980), respectively, in the laboratory of Dr. K. Hildenbrand in the Max-Planck-Institut für Strahlenchemie, Mülheim/Ruhr, West Germany, as described previously (Arvinte & Hildenbrand, 1984; Arvinte et al., 1986).

Vesicle Preparation. For the RET assay, the liposomes were prepared by sonication (Arvinte et al., 1986). Thus, stock solutions of egg yolk phosphatidylcholine, phosphatidylethanolamine, cholesterol, *N*-NBD-DPPE, and *N*-Rh-DPPE in a molar ratio of 4:5:1:0.05:0.1 (10 mg of lipids) were mixed. The solvent was evaporated, and 2 mL of 10 mM borate buffer, pH 8.5, containing 0.145 M NaCl was added to the lipid film. Liposomes were formed by sonication using a Bransonic 12 bath sonicator. The sonication was performed until the solution was clear (i.e., no change in turbidity was observed with further sonication).

Liposomes containing FITC-D150 were made from the same lipid mixture, without *N*-NBD-DPPE. The 2 mL of borate buffer that was added over the dried lipid film contained 40 mg of FITC-D150. After the sonication step, the unincorporated FITC-D150 was removed from the liposomes by filtration through a Sepharose 4B column (30 cm × 1 cm). The column was loaded with 1 mL of the liposome solution. The fraction volume was 1 mL.

Phospholipid concentration in the liposome suspensions was determined from the absorbance of the eluted fractions at the rhodamine absorbance wavelength (λ_{abs} = 545 nm). To use this method, the initial lipid concentration must be known, and a uniform distribution of *N*-Rh-DPPE in vesicles of different size had to be assumed. This procedure yielded results similar to those obtained by using the phosphate analysis method (Arvinte et al., 1986).

Liposome size distribution was determined from electron micrographs of negatively stained liposomes using a Siemens Elmiskop 102 electron microscope. Aliquots of liposome suspension in borate buffer were placed on collodion-covered grids, negatively stained with ammonium molybdate (pH 7.4), and examined. The diameters of individual liposomes were calculated from photographs printed at 4 times negative enlargements, multiplying the measured liposome diameter by 0.707, in the assumption that the measured surfaces represent disk surfaces resulting from collapsed spherical liposomes.

Preparation of Nuclei. Nuclei from mouse liver were isolated following the procedure of Blobel and Potter (1966), with some modifications. The liver was homogenized in an ice-cold buffer containing 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, 3.3 mM $CaCl_2$ (TKM buffer), and 250 mM sucrose, pH 7.2. After filtration through nylon gauze, the homogenate was concentrated by centrifugation at 600*g* for 10 min at 4 °C using a Hettich Rotanta/R centrifuge.

The volume of the pellet was measured, and then twice this volume of TKM buffer containing 2 M sucrose was added.

The suspension was layered over the TKM buffer containing 2 M sucrose and concentrated by centrifugation at 90000*g* for 30 min at 4 °C using a Beckman L8-70 ultracentrifuge (SW 50 rotor). The pellet of nuclei was stored at 4 °C in TKM buffer containing 25 mM sucrose and used for experiments within 8 h after preparation.

Nuclei concentrations in stock suspensions were determined by the counting method and by absorbance measurements at 260 nm (Aaronson & Blobel, 1974).

RET Measurements. Mixing of lipids from liposomes and nuclei was monitored by the resonance energy-transfer method (Hoekstra, 1982; Struck et al., 1981). The RET donor–acceptor pair, *N*-NBD-DPPE and *N*-Rh-DPPE, was incorporated into liposomes at 0.5 and 1 mol %, respectively, concentrations at which there was a strong transfer of energy from NBD to Rh. Fluorescence measurements were performed on a Fica 55 MK II spectrofluorometer in a thermostated sample holder at 37 °C. NBD was excited at 475 nm, and the lipid redistribution was followed by monitoring the changes in NBD emission intensity at 530 nm (Struck et al., 1981). An assay mixture in the cuvette contained 0.665 mL of sodium acetate buffer (0.02 N, 0.145 M NaCl), 0.011 mL of liposome suspension (in borate buffer, 0.5 mM final lipid concentration), and 0.025 mL suspension of nuclei (in TKM buffer containing 25 mM sucrose, 6.9×10^6 nuclei). To avoid artifacts due to settling of fluorescent materials, the cuvette medium was continuously mixed during the measurements. The pH values indicated in the text were obtained in separate experiments from mixtures of the three buffers.

Normalization of Emission Intensities. Steady-state NBD emission intensities of the incubation mixture of liposomes with nuclei were normalized to the NBD emission intensities of the samples after treatment with Triton X-100 (1% v/v final concentration). The difference between the intensity of the Triton X-100 treated sample (F_T) and the liposome fluorescence at zero time (before addition of the nuclei) (F_0) was considered to be 100. Thus, normalized equilibrium emission intensities were calculated as $[(F_{eq} - F_0)/(F_T - F_0)] \times 100$, where F_{eq} is the fluorescence intensity at equilibrium. This procedure has the advantage of a high reproducibility and offers fluorescence scale to compare differences in fusogenic properties. At the same time, it eliminates the problematical notion of "percent of fusion", which is difficult to define

precisely in our case due to the membrane complexity of the nuclei. Control experiments had shown that the steady-state emission intensities were independent of pH in the pH domain of our experiments.

Assay for Transfer of Liposome Contents to Nuclei by Triton X-100 Treatment and Spectrofluorometry. Liposome suspension (0.050 mL) containing FITC-D150 (2.5 mM final lipid concentration) was mixed with 1.4 mL of sodium acetate buffer and 0.050 mL of nuclei suspension (1.4×10^7 nuclei) and incubated for 30 min at 37 °C (the pH of the mixture was determined as previously described). After incubation, the sample was concentrated by centrifugation. A Savant Speed-Vac concentrator was used for 7 min at 1000*g*. After the first centrifugation, the pellet of nuclei, C_1p, was suspended in 1.5 mL of TKM buffer containing 25 mM sucrose and washed once more by centrifugation. The pellet after the second centrifugation (C_2p) was suspended in 0.5 mL of TKM buffer, and fluorescence spectra were taken. Triton X-100 was then added (1% v/v final concentration), mixed, and reacted for about 5 min under gentle shaking. The Triton X-100 soluble fraction of the mixture was removed by third and fourth centrifugations which led to the C_3Tp and C_4Tp pellets, respectively. The pellets after these centrifugations were resuspended in TKM buffer. The C_4Tp fraction will be termed envelope-denuded nuclei (Aaronson & Blobel, 1974). The fluorescence spectrum of these envelope-denuded nuclei was measured.

Microfluorometric Measurements of Fluorescent Dextrans Associated with Nuclei. The measurements were performed on the FRAP apparatus previously described (Wahl, 1985; Arvinte et al., 1986). A drop of the suspension of nuclei was spread between a coverslip and a microscope slide and was placed on the stage of a Zeiss universal microscope. While the sample was observed with the transmitted light of the microscope, the middle plane of the nucleus was focused and placed in the center of the optical field defined by the ocular reticle of a Zeiss microphotometer attachment (MPM01K). Fluorescence was excited by the attenuated laser beam of the FRAP apparatus (λ = 488 nm); the beam waist (width w = 1 μm) was focused on the nucleus center by the combination of intermediate optics, orientable mirrors, a dichroic filter (FT510) of a Zeiss IIIRS vertical illuminator, and the microscope objective (Zeiss 100/1.3 NA Neofluar Phase 3).

The nucleus fluorescence was collected by the microscope objective; passed through the dichroic filter, the Zeiss LP525 barrier filter, intermediate optics of the microscope and of the photometer, and the photometer measuring diaphragm (with a projected radius on the object plane s_0 = 3 μm); and fell on the photocathode of a 56TVP Radiotechnique PM cooled to −20 °C by a thermoelectric heat exchanger. Single photoelectron pulses from the PM were shaped by a 9302 ORTEC amplifier discriminator and counted by a 2071 Camberra 100-MHz counter. The counter was read through a GPIB bus, by a Minc 11/23 computer.

A measuring cycle comprised switching on and off the laser light shutter, changing the position of the photometer hinged mirror, starting the photocounter and stopping it after a given time, reading the counter, and measuring and subtracting the background count. These operations were performed by the computer driven by a program in BASIC.

Optical Sectioning Effect. Fluorescence intensity measured by a microfluorometer is influenced by the optical sectioning effect (Koppel et al., 1976; Schneider & Webb, 1981; Agard, 1984; Scholtz et al., 1985). That is, the collection efficiency of the system defined by the microscope objective and the photometer measuring diaphragm is maximum in the object focal plane and decreases when the distance of the emitting plane from the focal plane increases. Koppel et al. (1976) have calculated the collection efficiency in the case of epiexcitation by a laser beam. The relevant formula for our case has been given by Schneider and Webb (1981):

$$1/E(z) = 1 + z^2/2h^2 + w^2(z)/2s_0^2 \quad (1)$$

where E is the efficiency, z the distance of the fluorescent plane to the focal plane, $w(z)$ the laser beam radius, s_0 the radius of the measuring diaphragm projected on the object, and h the distance of half-efficiency of the collection optics defined by

$$h = s_0 \cot \alpha \quad (2)$$

where α is the aperture angle of the objective. The second term of the right-hand member of eq 1 can be considered as negligible, and one can write

$$E(z) = (1 + z^2/2h^2)^{-1} \quad (3)$$

These formulas did not take into account the spherical aberration of the objective and should be considered as approximate (Schneider & Webb, 1981; Sholtz et al., 1985).

Microfluorometry of Spherical Nuclei. When we carried out microfluorometric measurements of fluorescent dextrans associated with nuclei, the fluorescence emitters were included in a volume defined by the intersection of the axial laser beam with a centered nucleus which we shall assume to be spherical. The beam radius (w) was much smaller than the nucleus radius (R). It could be assumed to be constant throughout the nucleus, and the intersected volume could be considered with a good approximation to be a cylinder of base $S = \pi w^2$ and height $2R$.

We considered three different spatial distributions of the dye in the nucleus. In the first case, the fluorescence was assumed to be restricted to the nucleus surface with a uniform surface concentration σ. According to eq 3, the fluorescence intensity measured by the microfluorometer could be written

$$F_1 = 2kS\sigma(1 + R^2/2h^2)^{-1} \quad (4)$$

where k is a constant of proportionality.

In the second case, the fluorescent dye was assumed to be uniformly distributed in the nucleus volume with a concentration ρ. The fluorescence intensity was then

$$F_2 = 2kS\rho \int_0^R (1 + z^2/2h^2)^{-1}\, dz$$

which after integration can be written

$$F_2 = 2kS\rho h\sqrt{2} \tan^{-1}(R/h\sqrt{2}) \quad (5)$$

The third case was an intermediate one, when a part of the fluorescent dextran was uniformly distributed on the surface and the other part in the nucleus volume. One can then write

$$F = 2kS\sigma[(1 + R^2/2h^2)^{-1} + (\rho/\sigma)h\sqrt{2} \tan^{-1}(R/h\sqrt{2})] \quad (6)$$

Let us assume that we carried out microfluorometric measurements on a population of nuclei with different radii. If the dye was on the surface, eq 4 predicts that the fluorescence intensity should decrease when the radius increases. If the dye was distributed throughout the volume, eq 5 predicts that the fluorescence intensity should increase almost linearly

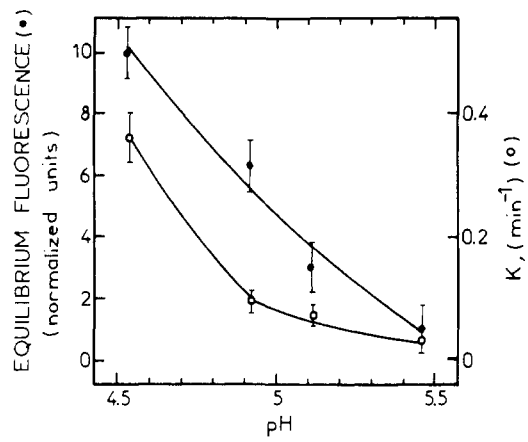

FIGURE 1: Assay of lipid mixing of liposomes and nuclear membranes at different pH values. Liposomes containing *N*-NBD-DPPE (0.5 mol %) and *N*-Rh-DPPE (1 mol %) were mixed with mouse liver nuclei as described under Materials and Methods. The increase in *N*-NBD-DPPE fluorescence intensity due to lipid redistribution was recorded continuously. The normalized equilibrium emission intensities were calculated as described under Materials and Methods. The fusion rate constant was defined as $k = \ln 2/t_{1/2}$, where $t_{1/2}$ was the time at which the intensity was equal to half the value reached at the end of the assay. Lines were drawn for clarity only and have no theoretical significance.

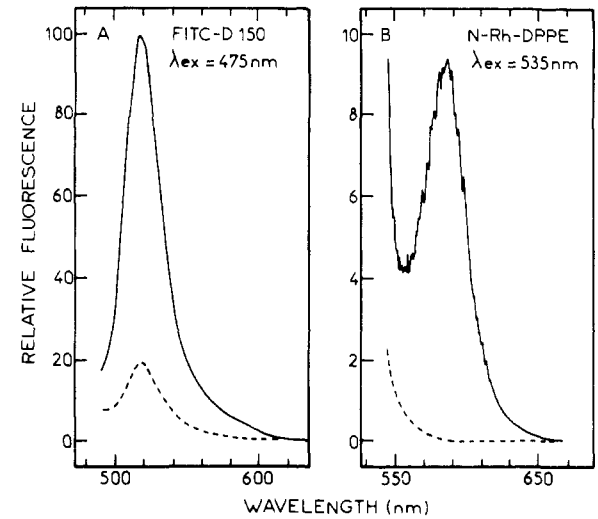

FIGURE 2: Fluorescence assay for the transfer of encapsulated FITC-D150 in liposomes to the nuclei interior. Liposomes encapsulating FITC-D150 and containing *N*-Rh-DPPE in their membranes were reacted at pH 4.53 with mouse liver nuclei, and the mixture was concentrated by centrifugation as described under Materials and Methods. (A) Spectra of FITC-D150 associated with the nuclei pellet (—) after incubation of the liposomes with nuclei and two centrifugations (C_2p nuclei). Triton X-100 treatment and washing followed by two centrifugations generated the envelope-denuded nuclei (C_4Tp, see Materials and Methods) which also contained FITC-D150 (---). (B) Spectra of *N*-Rh-DPPE associated with the C_2p nuclei pellet (—). After Triton X-100 treatment and centrifugation, the C_4Tp residual nuclei contained no measurable amount of *N*-Rh-DPPE (---).

with the nucleus radius. Finally, in case of a mixed distribution, the variation of the intensity with the nucleus radius will be determined by the ratio ρ/σ of the volume and surface dye concentrations as shown by eq 6.

RESULTS

Liposome Characterization. Electron micrographs of negatively stained liposomes showed that about 40% of the counted vesicles were unilamellar with diameters between 30 and 100 nm (maximum population of 50 nm). The multilamellar liposomes (60%) were between 50 and 150 nm in diameter with a mean diameter of 100 nm. Fluorescence and optical density assays permitted calculation of the molar ratio of lipids to FITC-D150 of 1700 ± 1000 to 1. This value is in good agreement with the value of 1800 to 1 which is obtained for the lipid to FITC-D150 molar ratio, knowing the initial FITC-D150 concentration in the sample and the experimentally determined size distribution of liposomes (considered unilamellar).

pH-Dependent Lipid Mixing during Incubation of Liposomes with Nuclei. Liposome and nuclei lipid mixing during the incubation was studied by using the RET method (Struck et al., 1981; Hoekstra, 1982). After mixing of liposomes containing 0.5 mol % *N*-NBD-DPPE and 1 mol % *N*-Rh-DPPE with mouse liver nuclei at different pH values, the *N*-NBD-DPPE fluorescence intensity was monitored (see Materials and Methods). Time-dependent fluorescence curves were recorded and analyzed to obtain the equilibrium emission intensities and the fusion rates (Figure 1). The amount of fusion and the fusion rates increased steeply as the pH decreased, a result similar to that obtained when the same types of liposomes were fused with erythrocyte ghosts (Arvinte et al., 1986).

Fluorescence Assay for Contents Mixing. To study concomitantly the fate of the liposome lipids and liposome contents during fusion, we developed the following fluorescence assay. Liposomes encapsulating FITC-D150 and containing *N*-Rh-DPPE in their membranes were incubated with the nuclei for 30 min at pH 4.53 and 37 °C. After being washed, the fluorescence spectra of the C_2p nuclei pellet were recorded (see Materials and Methods). As shown in Figure 2 (A and B, continuous line), the nuclei pellet contained FITC-D150 and *N*-Rh-DPPE. Control experiments have shown that under the conditions of centrifugation used, the liposomes were not pelleted. Therefore, a strong nuclei–liposome interaction occurred. The fact that we found FITC-D150 and *N*-Rh-DPPE in the C_2p pellet was not sufficient to prove a fusion of the liposomes with nuclei; that is, one can imagine that the liposomes adhered at the nuclei surface.

The C_2p pellet was then suspended in TKM buffer containing 25 mM sucrose, and Triton X-100 was added (1% v/v). The mixture was then concentrated by centrifugation. After resuspension and washing (see Materials and Methods), the fluorescence spectra of the C_4Tp envelope-denuded nuclei pellet showed only the presence of FITC-D150 fluorescence (see Figure 2A,B, dashed lines). This could be explained as follows: (a) The Triton X-100 solubilized the lipids (there was no residual *N*-Rh-DPPE fluorescence). According to a number of workers, at the Triton X-100 concentration used, the nuclear membranes are removed, and the residual nuclei which are obtained keep their internal structure (Aaronson & Blobel, 1974; Dwyer & Blobel, 1976; Kirschner et al., 1977; Smith & Wells, 1984). (b) About 20% of the initial nuclei-attached FITC-D150 remained associated with the envelope-denuded nuclei. To be sure that the FITC-D150 did not enter in the nuclei through processes other than membrane fusion, the following control experiments were done: (i) Nuclei were treated with Triton X-100 and washed, and the envelope-denuded nuclei were incubated with free FITC-D150 (30 min, pH 4.53, 37 °C). The concentration of free FITC-D150 was 1.5 times higher than the bulk concentration of FITC-D150 in the case of the experiments using liposomes, as determined by the fluorescence spectra. (ii) Intact nuclei were mixed with FITC-D150 [same concentration as (i)] and Triton X-100. The suspension was then incubated under the same conditions as in the case of experiments (i) (30 min, pH 4.53, 37 °C). After washing by repeated centrifugation, both residual nuclei

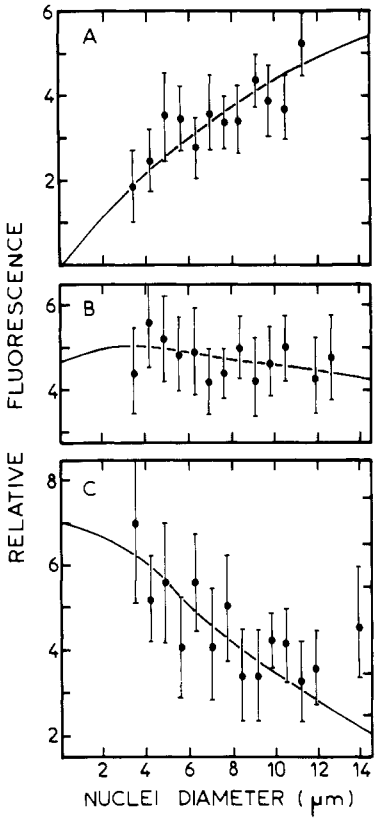

FIGURE 3: Microfluorometry of nuclei and envelope-denuded nuclei. The continuous line was the result of fits of eq 5, 6, and 4 to data of panels A, B, and C, respectively: the parameters were $h = 3.5$ μm and $\rho/\sigma = 0.12$ μm^{-1}. (A) FITC-D150 fluorescence intensity as a function of denuded nuclei diameter. After the liposome–nuclei incubation, the C_2p nuclei pellet was suspended in a buffer containing Triton X-100. The measurements were performed on the C_4Tp-denuded nuclei (see Materials and Methods). (B) FITC-D150 fluorescence intensity as a function of intact nuclei diameter (C_2p nuclei). (C) *N*-Rh-DPPE fluorescence intensity as a function of nuclei diameter. Liposomes containing only *N*-Rh-DPPE were incubated at pH 4.53 with nuclei. The *N*-Rh-DPPE fluorescence of the C_2p nuclei was measured. The average intensity of a group of 10–20 nuclei was determined. The error bars represent standard deviations. Fluorescence intensities were measured by a microfluorometric technique. Excitation was provided by a laser beam of radius 1 μm.

pellets from (i) and (ii) were not fluorescent. Therefore, fusion appeared to be necessary for the high molecular weight FITC-D150 to enter into the nuclei.

The same fluorescence assay for the contents mixing of liposomes and nuclei was also performed at pH 5.43. At this pH, the amounts of FITC-D150 associated with the nuclei and with the envelope-denuded nuclei structures (obtained after a Triton X-100 wash) were much less, 20% and 8%, respectively, of the values at pH 4.5. This result was in good agreement with the RET experiments where the amount of fusion at pH 5.4 was about 12% of the value at pH 4.5.

Dependence of the Nucleus Fluorescence upon Its Radius. (*a*) *Measurement with Intact Nuclei.* In order to obtain further information on the location of nuclei-associated FITC-D150 after incubation with liposomes, we have performed microfluorometric measurements on nuclei as a function of the nucleus diameter. The apparatus used has been described under Materials and Methods. The laser beam was focused on the nucleus center. Diameters of the nuclei were determined with an ocular micrometer. We measured 120 nuclei. The results are presented in Figure 3B and show that the fluorescence intensity decreased weakly when the nucleus radius increased. According to the consideration presented under Materials and Methods, such a behavior was intermediate between the uniform surface distribution and the uniform volume distribution. In a more quantitative evaluation, we performed microfluorometric measurements of nuclei stained on the surface with a fluorescent dye. These nuclei were prepared as follows: liposomes containing *N*-Rh-DPPE only were incubated at pH 4.53 with nuclei (30 min, 37 °C). After centrifugation, a strong *N*-Rh-DPPE fluorescence was found in the nuclei pellet. As shown in Figure 3C, the *N*-Rh-DPPE fluorescence intensity decreased with increasing nuclei radius, as expected from the "optical sectioning" effect (Koppel et al., 1976; Schneider & Webb, 1981; Scholtz et al., 1985). We fitted eq 4 with the experimental curve, by taking $h = 3.5$ μm. The resulting theoretical curve was also drawn in Figure 3C.

We then could fit the experimental points of Figure 3B with eq 6 for a value $\rho/\sigma = 0.12$ μm^{-1}. The resulting theoretical curve is also shown on the same figure. It followed that one could calculate that 33% of the fluorescent dextrans were localized in the nucleus interior for a nucleus with a 4-μm radius (the mean radius of the nucleus population).

(*b*) *Measurements with Triton X-100 Treated Nuclei.* The same measurements were performed on nuclei incubated with FITC-D150 liposomes and treated afterward with 1% Triton X-100. A total of 150 nuclei were measured, and the fluorescence intensity as function of the nucleus radius was plotted in Figure 3A. It can be seen on that figure that, contrary to the preceding measurements, the fluorescence intensities increased with the nucleus radius, showing that the fluorescent dextrans remained associated with the residual nuclei and were distributed in the nucleus interior. In the same figure, we drew a theoretical curve obtained by applying eq 5 with $h = 3.5$ μm and fitted to the experimental points.

We also determined the fraction of fluorescent dextran remaining on the nuclei after Triton X-100 treatment and multiple washings by using the microfluorometric method. This method avoided the errors due to the material loss which might falsify the spectrofluorometric experiments; the results of these measurements are given in Table I. It was found that the residual fluorescence resisted multiple washings and amounted to 29 ± 8% of the fluorescence intensity present in the initially intact nuclei. After performing the correction for optical sectioning (eq 5 and 6), it resulted that 20% of the fluorescent dextran present in the initial nuclei remained in the Triton X-100 treated nuclei. This value is in complete agreement with the spectrofluorometric measurements quoted previously and also with the analysis of curve 2B relative to

Table I: Effect of Multiple Washings on the FITC-D150 Fluorescence Intensity Measured on Nuclei and Envelope-Denuded Nuclei Pellets[a] after Fusion of Liposomes Containing FITC-D150 with Mouse Liver Nuclei

centrifugation	fluorescence of nuclei	fluorescence of residual nuclei[b]	% incorporated[c]
2	1.3 ± 0.2	0.3 ± 0.2	23 ± 6
4	0.9 ± 0.2	0.3 ± 0.1	33 ± 10
6	1.2 ± 0.2	0.4 ± 0.1	33 ± 10

[a] Liposomes containing FITC-D150 were incubated with mouse liver nuclei at pH 4.53 as described under Materials and Methods and then washed by centrifugation. The relative fluorescence intensities associated with the pellets were calculated from single nuclei fluorescence intensities measured by the microfluorometric method described in the text. Mean values ± SD of measurements on more than 15 different nuclei of the same size are presented here. [b] After the incubation of nuclei and liposomes (30 min, pH 4.53, 37 °C), 1% (v/v) Triton X-100 was added, and the resulting envelope-denuded nuclei were washed by multiple centrifugations. [c] The percent of incorporated FITC-D150 represented the value of the initial nuclear FITC-D150 fluorescence which remained in the envelope-denuded nuclei after centrifugation.

the intact nuclei.

Discussion

For an understanding of cell and organelle surface interactions, a knowledge of fusion properties of the lipid components of membranes is necessary. In this perspective, the study of the interaction of liposomes with biological membranes appears to be of great interest. Furthermore, by establishing the mechanism of liposome fusion with nuclear membranes, one may help to improve the yield of gene delivery to nuclei by liposomes (Nicolau & Senē, 1982; Nicolau et al., 1983; Soriano et al., 1983). In the present work, liposomes composed of a mixture of PC/PE/Chol (4:5:1 molar ratios) have been incubated with mouse liver nuclei. It has been found that both mixing of membrane lipids and transfer of liposome contents into the interior of nuclei occurred. It could be deduced that a fusion process had taken place between these two membranes.

In order to demonstrate membrane mixing, we used the RET assay where *N*-NBD-DPPE and *N*-Rh-DPPE were incorporated in liposomes. It has been shown in a number of studies that phospholipids having long acyl chains and labeled head groups are not exchanged between lipid vesicles either through the aqueous phase or in vesicle aggregates (Nichols & Pagano, 1981; Struck et al., 1981; Arvinte & Hildenbrand, 1984). No exchange was found with these labeled lipids in interactions between vesicles and cells (Struck & Pagano, 1980; Nichols & Pagano, 1981; Struck et al., 1981; Wojcieszyn et al., 1983). Using liposomes, it has also been shown that at low pH facilitated transfer of the fluorescent long-chain lipids is excluded (Driessen et al., 1985). In our experiments, the RET assay showed that the mixing of liposome lipids with the nuclear membrane was occurring in minutes. The results presented in Figure 1 show that the amount and the rate of lipid mixing increased by lowering the pH in a way similar to the pH fusion behavior observed in other systems, for example, liposomes with Sendai virus membranes (Haywood & Boyer, 1982), liposomes and membrane vesicles derived from *Bacillus subtilis* (Driessen et al., 1985), and liposomes and erythrocyte ghosts (Arvinte et al., 1986).

Mixing of lipids in liposome interactions is often an indication of fusion (Düzgüneş et al., 1985), which means that mixing of liposome aqueous contents must occur. However, the two phenomena do not always occur together. There are cases where lipid mixing is accompanied by leakage of liposome contents (Ellens et al., 1985). Thus, it was necessary to examine whether, under our experimental conditions, liposomes transferred their contents into the nucleus interior.

Previously reported fluorescence tests used for liposome aqueous content mixing involved the interaction of two liposome populations, one containing a fluorescent compound and the other containing a quencher or an enhancer (Wilshut & Papahadjopoulos, 1979; Smolanski et al., 1977; Ellens et al., 1984). These assays are clearly not appropriate for systems involving cells or organelles.

Experiments in which a self-quenching fluorescent component such as carboxyfluorescein is encapsulated in liposomes and its fluorescence enhanced by injection in cells (Weinstein et al., 1977) are not applicable to our case since these compounds can permeate freely into and out of the nucleus through the nuclear pores.

We were then led to develop a method in which a fluorescent dextran of high molecular weight, which does not permeate through the nuclear pores, was encapsulated in liposomes.

In addition, since the nucleus was surrounded by a double membrane, it was necessary to localize the fluorescent dextran in the nucleus after its incubation with the liposomes.

For that purpose, we studied the variation of the fluorescence associated with nuclei as a function of the nucleus radius. The fluorescence of a single nucleus excited by a focused laser beam was measured by a microfluorometer technique.

It was found that the fluorescence decreased slightly as the nucleus radius increased. In comparison, the fluorescence of nuclei previously incubated with *N*-Rh-DPPE-labeled liposomes decreased strongly with increasing nuclei radius. The *N*-Rh-DPPE was localized in the nuclear membrane with a uniform surface concentration. Emitters were then confined in two small planar disks situated on both sides of the focal plane and at a distance equal to the nucleus radius. By fitting the experimental data with a formula given by Schneider and Webb (1981) and based on a computation of Koppel et al. (1976), we determined the distance of half light collection efficiency of the microphotometer: $h = 3.5$ μm. This value was somewhat larger than the theoretical value given by eq 2. This discrepancy could be explained in part by the microscope objective aberration (Schneider & Webb, 1981; Scholtz et al., 1985). An additional effect could come from flattening of nuclei pressed between the coverslip and the microscope slide. Consequently, the nucleus width measured by the ocular micrometer was larger than the nucleus height which determined the distance of the fluorescent emitters from the focal plane.

The collection efficiency of the fluorescence of chromophores uniformly distributed in the nucleus interior was obtained by integrating eq 3 derived from the theory of Koppel et al. (1976). The nuclear radius dependence of the experimental dextran fluorescence was intermediate between the fluorescence dependence of a superficial and volumic distribution. For a nucleus of diameter 8 μm, we calculated that 30% of the dextran previously encapsulated in liposomes had been transferred into the nucleus interior.

In an additional study, the nuclei were treated by Triton X-100 after their incubation with dextran liposomes. This treatment removed the nuclear membrane and the liposomes which might be bound to the nucleus. It has been shown that the envelope-denuded nuclei thus obtained retained the pore complexes, the peripheral lamina, and the interior structure of intact nuclei (Aaronson & Blobel, 1974; Kirshner et al., 1977).

Spectrofluorometric measurements showed that 20% of the fluorescent dextrans intially associated with the intact nuclei remained in the envelope-denuded nuclei. Microfluorometric measurements confirmed this value and in addition showed that the fluorescence intensity was increasing proportionally to the nucleus radius. The quantitative analysis of these results showed that the dextrans were distributed uniformly in the interior of the envelope-denuded nuclei, suggesting that this dextran fraction was identical with the internal component of the dextran associated with the intact nuclei.

Nuclei are surrounded by a double lipidic membrane studded with nuclear pore complexes. The nuclear pores are permeable to small molecules but do not allow the passage of large molecules such as the FITC-D150 dextran (Paine et al., 1975; Peters, 1983). The most probable mechanism for the transfer of dextrans to the nucleus interior was therefore fusion between the liposomes encapsulating dextran and the nuclear membrane.

Since there was a double nuclear membrane, it can be assumed that the transfer involved two successive fusions and implied that liposomes had at least two lipidic bilayers. This latter condition was fulfilled since electron microscopic ob-

servations had shown that 60% of our liposomes were multilamellar.

The 70–80% of the nuclei-associated fluorescent dextrans which were localized on the nuclear surface could have been encapsulated in nonfused liposomes adhering to the outer membrane. Another fraction of these dextran molecules could be included in the perinuclear space (between the two membranes) either free or encapsulated in liposomes, depending on whether they originated from the fusion of unilamellar or multilamellar liposomes with the outer nucleus membrane.

We found that both lipid mixing and dextran transfer into the nucleus interior were favored at pH values below 5. Similar behavior has been reported for fusion of liposomes with biological membranes. This is the case for enveloped viruses such as influenza virus, Simliki forest virus, and fowl plague virus (Stegmann et al., 1985; White & Helenius, 1980; White et al., 1982) where the process is induced by fusogenic proteins. It has also been shown that liposomes composed of soybean lipids could be fused with the mitochondrial inner membrane (Schneider et al., 1980), chromatophores from *Rhodopseudomonas sphaeroides* (Costa et al., 1982; Snozzi & Crofts, 1984), and thylakoid membranes (Millner et al., 1983). More recently, liposomes composed of cardiolipids and dioleoylphosphatidylcholine were found to fuse with vesicles made from bacterial membranes (Düzgüneş et al., 1985). This fusion process was dependent on the bacterial membrane proteins.

These results suggest that nuclear membrane proteins could be involved in the fusion process of liposomes with the nuclear membrane. This is a difficult problem to address since treatments of the nuclei by a proteolytic enzyme have been shown to destroy the nuclei (Berenzney & Coffey, 1977).

To our knowledge, this work represents the first evidence of liposome fusion with nuclei.

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Blood Coagulation Induced by the Venom of *Bothrops atrox*. 1. Identification, Purification, and Properties of a Prothrombin Activator†

Hélène Hofmann‡ and Cassian Bon*
Unité des Venins, Unité associée Institut Pasteur/INSERM (285), Institut Pasteur, 75015 Paris, France
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ABSTRACT: In this paper, we show that the procoagulant action of *Bothrops atrox* venom is due in part to a protein component that activates prothrombin. The venom prothrombin activator was purified by ion-exchange chromatography and gel filtration. It was separated from a protease by affinity chromatography in a *p*-aminobenzamidine–CH-Sepharose column. It is a protein of about M_r 70 000, consisting of a single polypeptide chain. We have studied the kinetics of activation of prothrombin under different experimental conditions. The prothrombin activator from *B. atrox* venom is insensitive to reagents of serine and thiol proteases but is inactivated by ion chelators and by various divalent ions. These results suggest that it is a metalloenzyme. The prothrombin activator from *B. atrox* venom is inactive on the chromogenic substrates S-2337 and S-2238, and it is selective for prothrombin since it does not act on other blood coagulation factors such as fibrinogen and factor X. We have also studied the pattern of peptide cleavages produced in the human prothrombin molecule during the activation by the activator from *B. atrox* venom and compared it to that obtained with ecarin, a prothrombin activator from *Echis carinatus* venom. In the presence of thrombin inhibitors, e.g., hirudin, we found that the activators from *B. atrox* venom and ecarin act in a similar, or identical, manner by producing a thrombin intermediate, meizothrombin. In the absence of thrombin inhibitors, several peptides are generated, and α-thrombin is produced as a consequence of meizothrombin action.

Snake venoms, particularly those belonging to the families Crotalidae and Viperidae, induce blood clotting. In the case of *Bothrops* species, this activity has been attributed to a fibrinogen clotting enzyme, batroxobin (Stocker &, Egberg, 1973; Stocker & Barlow, 1976; Holleman & Weiss, 1976), which cleaves fibrinopeptide A from fibrinogen (Funk et al., 1971). An other enzyme purified from *Bothrops atrox* venom, thrombocytin, activates blood platelets but has a poor fibrinogen clotting activity (Niewiarowski et al., 1977, 1979; Kirby et al., 1979). In addition to their action on fibrinogen, the venoms of many *Bothrops* species can convert prothrombin into thrombin, either directly or indirectly (Eagle, 1937; Janzky, 1950; Breda et al., 1951; Michl, 1954; Nahas et al., 1964). Recently, we have identified and partially purified a factor X activator from *B. atrox* venom (Hofmann et al., 1983). This procoagulant component resembles that of Russell's viper (*Vipera russelli*) since it activates factor X in a calcium-dependent manner and is not sensitive to diisopropyl fluorophosphate (DFP)[1] (Hofmann et al., 1983).

We also demonstrated that the DFP-treated venom of *B. atrox* clots plasma in the absence of calcium (Hofmann et al., 1983). This indicates that in addition to batroxobin and thrombocytin, which are sensitive to DFP (Kirby et al., 1979), and in addition to factor X activator, which is calcium-dependent (Hofmann et al., 1983), the venom contains another component that acts on fibrinogen and/or prothrombin. The observation that *B. atrox* venom treated with DFP does not clot purified fibrinogen (Hofmann et al., 1983) favors the hypothesis that this venom contains a prothrombin activator.

In this study, we submitted the venom of *B. atrox* to ion-exchange chromatography and analyzed the effects of its fractions on purified fibrinogen, prothrombin, and factor X.

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‡Present address: Service de Biochimie, Hôpital Ambroise Paré, 92100 Boulogne, France.

[1] Abbreviations: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; S-2238, H-D-Phe-Pip-Arg-*p*-nitroanilide hydrochloride; S-2337, benzoyl-Ile-Glu-Pip-Gly-Arg-*p*-nitroanilide hydrochloride; TAME, *N*-tosylarginine methyl ester; TLCK, *N*-*p*-tosyllysine chloromethyl ketone; TPCK, *N*-*p*-tosylphenylalanine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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