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# IN VITRO INTERACTION OF ZAJDELA ASCITES HEPATOMA CELLS WITH LIPID VESICLES

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## Summary

We studied the in vitro interaction between Zajdela ascites hepatoma cells and small unilamellar vesicles, consisting of $^{14}C$-labeled phosphatidylcholine, cholesterol, and phosphatidylserine (molar ratio 5 : 4 : 1), containing high intravesicular concentrations of carboxyfluorescein or fluorescein isothiocyanate tagged dextran.

The entrapped markers were found to be associated with the cells to a lesser degree than the vesicle membrane marker. This discrepancy, which is slightly less pronounced for fluorescein isothiocyanate tagged dextran than for carboxyfluorescein, increases with incubation time and decreases with increasing vesicle lipid concentration in the incubation mixture. Vesicle-plasma membrane exchange of the vesicle lipid marker could not entirely explain the observed discrepancy. It is tentatively concluded that the gap mainly arises from a selective loss of entrapped dyes from vesicles actually interacting with the cell surface. Both spectrofluorimetric and fluorescence microscopic observations, as well as the relative insensitivity of vesicle uptake towards the presence of metabolic inhibitors, exclude a major contribution of endocytosis as a vesicle uptake route. We therefore conclude that vesicles are primarily internalized by a vesicle-cell fusion-like process. The observed discrepancy in uptake between entrapped materials and vesicle lipid is discussed in terms of a two-site vesicle-cell surface interaction model.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; RPMI 1640, Roswell Park Memorial Institute tissue culture medium, type 1640; FITC, fluorescein isothiocyanate.

## Materials

Phosphatidylcholine and phosphatidylserine were isolated from total egg yolk lipids and from bovine brain extract (Sigma), respectively, by preparative thin layer chromatography. Cholesterol (CH-S grade), cholesteryl oleate, sodium azide, 2-deoxyglucose, and Hepes were purchased from Sigma. RPMI 1640 tissue culture medium was from Flow Labs. Inc. All lipids used gave one spot upon thin layer chromatography.

[1$\alpha$,2$\alpha$(n)-$^3$H]Cholesterol (43 mCi/$\mu$mol), cholesteryl[1-$^{14}$C]oleate (15.4 Ci/mol), and [6,6'(n)-$^3$H]sucrose (1 Ci/mmol) were from The Radiochemical Centre (Amersham, U.K.). [*Me*-$^{14}$C]phosphatidylcholine was prepared from egg yolk phosphatidylcholine according to Stoffel et al. [1]. Its specific activity varied between 2 and 3 Ci/mol. carboxyfluorescein was bought from Eastman Kodak Co. (Rochester, NY), and purified as described by Blumenthal et al. [2]. 100 mM stock solutions of carboxyfluorescein in $H_2O$, adjusted to pH 7.4 with 1 M NaOH, were kept in the dark until use. FITC-dextran was obtained from Pharmacia. This compound had an average molecular weight of 3000, and carried 1 FITC residue per 100 glucose units. For entrapment in vesicles 100 mM FITC-dextran solutions in 0.135 M NaCl/0.010 M Hepes, brought to pH 7.4 with 1 M NaOH (this buffer will be referred to as NaCl/Hepes buffer), where used.

## Methods

*Cells.* Zajdela ascites hepatoma cells (in the following referred to as Zajdela cells) were a generous gift from Dr. F. Zajdela, I.N.S.E.R.M., Paris. The cells, derived from a dimethylaminoazobenzene-induced hepatoma [3], were grown in the peritoneal cavity of adult female Wistar rats, and passed weekly to fresh animals. Cells were harvested by aspiration of ascitic fluid 5 days after implantation, in yields of approx. $3 \cdot 10^8$ cells per animal, and subsequently freed of contaminating non-tumor cells by repeated centrifugations and washings in NaCl/Hepes buffer. Cell counts were performed in a Bürker hemocytometer. Throughout the experiments more than 95% of the cells excluded Trypan Blue, indicating preserved viability.

*Liposomes.* Chloroform solutions of phosphatidylcholine, cholesterol, and phosphatidylserine were mixed in a molar ratio of 5 : 4 : 1, and taken to dryness, under reduced pressure, in a rotary evaporator. The dried lipid film was vortex-dispersed either in NaCl/Hepes buffer for preparation of empty vesicles or in 100 mM aqueous solutions of carboxyfluorescein or FITC-dextran for preparation of loaded vesicles. Generally the final total lipid concentration in the dispersions was 8.75 mM. The lipid dispersions were exhaustively sonicated at room temperature under a $N_2$ atmosphere, either during 1 h intermittently (50% of the duty cycle) with a Branson B-15 sonifier equipped with a titanium microprobe, or during at least 2 h in a Bransonic 220 bath sonicator. Both methods, as judged by Sepharose 4B chromatography, gave similar vesicle populations with respect to average size, with more than 95% of the lipid present as small unilamellar vesicles. Fluorophore containing vesicles were then freed from non-entrapped dye by chromatography on Sephadex G-100 (for

carboxyfluorescein), or Sepharose 6B (for FITC-dextran). Columns (25 × 1 cm) were equilibrated and eluted with NaCl/Hepes buffer.

*Incubations.* Unless stated otherwise (see legends of Figs. and Tables), $5 \cdot 10^7$—$2.5 \cdot 10^8$ Zajdela cells were incubated for 1 h at 37°C in a shaking water bath with 5—25 μmol of vesicle lipid, in a final volume of 5—25 ml, in open Erlenmeyer flasks. The incubation medium consisted of a 1 : 5 (v/v) mixture of NaCl/Hepes buffer and RPMI 1640 medium,the latter supplemented with 25 mM Hepes and 10 mM $NaHCO_3$. The medium was brought to pH 7.4 with 1 M NaOH. No serum was added. At suitable times, duplicate 1-ml samples (containing $10^7$ cells and 1 μmol of vesicle lipid) were withdrawn from the incubation mixture, transferred to polystyrene tubes, centrifuged (600 × *g*, for 30 s) and washed twice with NaCl/Hepes buffer at room temperature. The samples were then taken to fresh polystyrene tubes, centrifuged and washed again, and finally dispersed in 3 ml NaCl/Hepes buffer. For determination of cell-associated radioactivity 1 ml aliquots of the washed cell suspensions were pipetted into glass vials, mixed with 5 ml of xylene-based scintillation mixture [4] and counted in a Nuclear Chicago MKII liquid scintillation counter. The remainder of the 3 ml cell suspensions was used for fluorescence measurements with a Perkin Elmer MPF43 fluorescence spectrophotometer. Excitation and emission were at 490 and 520 nm, respectively. The instrument was calibrated with a $10^{-4}$ M solution of quinine sulfate in 0.05 M $H_2SO_4$. Cell-associated fluorescence was read in a 1 cm light path quartz fluorescence cuvette against series of freshly prepared carboxyfluorescein or FITC-dextran standards in NaCl/Hepes buffer. Details on the processing of fluorescence measurements are described in the Appendix. Fluorescence microscopy was performed with a Leitz Orthoplan fluorescence microscope, equipped with a filter combination for fluorescein.

## Introduction

In the past few years a vast number of investigations on the interaction between liposomes * and cells has been published (for reviews see Ref. 5, 6). Since many investigators have indicated a possible role for liposomes as carriers of antitumor drugs in the chemotherapeutic treatment of cancer, we were interested in the details of the interaction between liposomes and the cells of an experimental animal tumor model system. For this study the highly anaplastic Zajdela ascites hepatoma [7] was chosen. Our first aim was to find an answer to some crucial questions: are Zajdela ascites hepatoma cells capable of taking up liposomes? Does this uptake **, if occurring, consist of mere adsorption of intact vesicles to the cell surface, or are vesicles internalized within the cells? Furthermore: if internalization can be demonstrated, what is the mechanism of uptake? In 1977, developing an idea of W.A. Hagins, Weinstein et al. [8]

* In this report the terms 'liposomes' and 'vesicles' will be used interchangeably.

** The use of the term 'uptake' does not imply internalization of vesicles by cells, but is used as short for 'association with cells'. Whenever internalization is meant, this will be explicitly stated. If, in the text, terms like 'total cell-associated carboxyfluorescein' are followed by a symbol, e.g. ($II_i$), the reader is referred to the Appendix for its meaning. The term 'adsorbed carboxyfluorescein' is to be read as short for: carboxyfluorescein, whose self-quenching is relieved upon addition of detergent.

introduced an elegant and sensitive method to quantitate vesicle-cell interaction. This method (for details see Refs. 2, 8, 9) utilizes high, self-quenching concentrations of a water-soluble fluorophore, carboxyfluorescein, trapped in the inner aqueous compartment(s) of lipid vesicles. The primary interaction between cell and vesicle will involve the attachment of the vesicle membrane surrounding the vesicle inner aqueous compartment(s) to the cell. If this is to be followed by vesicle-cell fusion, the subsequent transfer of the inner aqueous compartment(s) of the vesicle into the cytoplasm will result in a vast dilution of the entrapped concentrated dye into the cellular cytoplasm, causing complete relief of self-quenching and giving rise to an immediate fluorescent signal from the interior of the cell. If, on the other hand, the vesicles are predominantly endocytosed, the development of a fluorescent signal will depend mainly on the rate of leakage of the concentrated fluorophore from endocytotic vacuoles and/or secondary lysosomes into the cytoplasm. Addition of a detergent (e.g. Triton X-100) to cells and vesicles will instantaneously set free any entrapped, self-quenched dye and thus allow determination of the fraction of carboxyfluorescein, which is still contained in intact vesicles, either adsorbed to the plasma membrane or present in endocytotic vacuoles and/or secondary lysosomes.

In this report small unilamellar vesicles consisting of phosphatidylcholine, cholesterol, and phosphatidylserine in a molar ratio of 5 : 4 : 1 were used for several reasons. The choice of the lipid composition was based on the fact, that phosphatidylcholine/phosphatidylserine (molar ratio 9 : 1) vesicles have been suggested successful in transferring entrapped materials to a number of different cells, most likely via a vesicle-cell fusion mechanism [10]. Incorporation of at least 40 mol% cholesterol into the vesicle membrane was required (see Results section) to keep carboxyfluorescein leakage rates at acceptable levels during 1 h incubations. We preferred the use of small unilamellar vesicles to the use of multilamellar vesicles because, in case of vesicle-cell fusion, all vesicle-entrapped fluorophore would be diluted at once, either into the cytoplasm and/or into the surrounding medium ('leaky fusion'). Furthermore, small unilamellar vesicles are both physically better defined and geometrically more homogeneous than multilamellar vesicles [11]. In our experiments the uptake of vesicle-entrapped carboxyfluorescein was compared directly to uptake of vesicle-entrapped FITC-dextran, another highly water-soluble fluorescent compound with an average molecular weight of about eight times that of carboxyfluorescein and having fluorescent spectral properties nearly identical to those of carboxyfluorescein. This report describes the results of a series of incubations of cells with vesicles, in which special attention was paid to the concordance between uptake of vesicle lipid and uptake of either entrapped dye in the same experiment.

## Results

Introductory experiments showed us that incorporation of 40 mol% of cholesterol in phosphatidylcholine/phosphatidylserine mixtures resulted in a three-fold decrease of vesicle permeability towards carboxyfluorescein. All experiments in this study were therefore done with vesicles composed of phos-

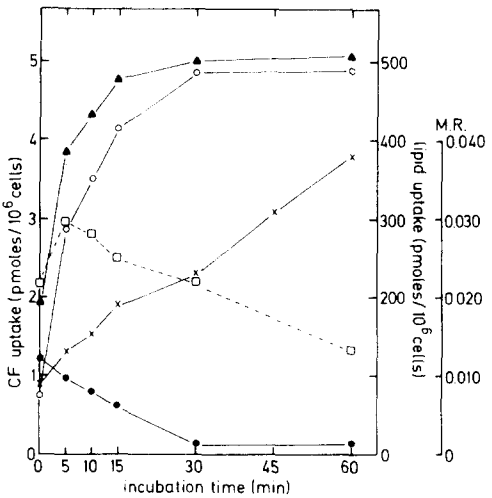


Fig. 1. Uptake of vesicle-entrapped carboxyfluorescein and of total vesicle lipid as a function of time. $1.75 \cdot 10^8$ Zajdela cells were incubated (37°C, shaking water bath) with 17.5 μmol of total vesicle lipid in a final volume of 17.5 ml in an open Erlenmeyer flask. Vesicles were composed of phosphatidylcholine/cholesterol/phosphatidylserine (5 : 4 : 1) with 5 μCi of [*Me*-$^{14}$C]phosphatidylcholine (spec. act. 2.2 Ci/mol) as a marker, and contained 100 mM carboxyfluorescein. At suitable times duplicate 1 ml aliquots were withdrawn from the incubation mixture and processed as described in the Methods section and Appendix. X———X, uptake of total vesicle lipid based on uptake of [*Me*-$^{14}$C]phosphatidylcholine; ▲———▲, total cell-associated carboxyfluorescein ($\Pi_i$; see Appendix); ○———○, carboxyfluorescein inside cells ($\Pi_d$; see Appendix); ●———●, 'adsorbed' carboxyfluorescein ($\Pi_a$; see Appendix). Each point represents the average of duplicate measurements, that agree within 5%. The accessory ordinate at the extreme right of the Fig. designates the molar ratio (M.R.) of total cell-associated carboxyfluorescein to total cell-associated vesicle lipid. Values for molar ratio were obtained by dividing the number of pmol of total carboxyfluorescein associated with $10^6$ cells by the number of pmol of vesicle lipid associated with $10^6$ cells. From fluorescence readings after addition of detergent and radioactivity determinations the molar ratio of total entrapped carboxyfluorescein to total vesicle lipid in the original vesicle preparation was calculated: 0.030. □- - - - -□, Molar ratio of total cell-associated carboxyfluorescein ($\Pi_i$) to total cell-associated vesicle lipid. CF, carboxyfluorescein.

phatidylcholine, cholesterol and phosphatidylserine in a 5 : 4 : 1 molar ratio.

Fig. 1 shows simultaneously measured uptake of vesicle lipid and vesicle-entrapped carboxyfluorescein as a function of incubation time. Both the amount of total cell-associated dye ($\Pi_i$), and the amount of dye inside the cells ($\Pi_d$) reach maximum values after about 30 min. The fraction of carboxyfluorescein which can be measured only after addition of detergent ($\Pi_a$) decreases from a maximum value at zero-time to a constant low value at longer incubation times. The uptake of vesicle lipid by the cells, based on $^{14}$C-labelled phosphatidylcholine, increases steadily with time, during the first hour of incubation. All uptake curves show relatively high zero-time * values, indicating that vesicle-cell contact occurs immediately upon addition of vesicles to cells. The molar ratio of total cell-associated carboxyfluorescein to total cell-associated vesicle lipid has a maximum value after 5 min of incubation and steadily

* It should be kept in mind, that the processing of samples from the incubation mixture takes approximately 3 min, so that 'zero-time' in our experiments is not identical to physical zero-time. In this report, zero-time refers to sampling.

decreases with time, suggesting a progressive loss of dye from vesicles and/or cells into the incubation medium. Since the carboxyfluorescein uptake values shown were corrected for leakage of dye from the bulk of vesicles in the incubation mixture (see Appendix), such loss of carboxyfluorescein could be considered to result from the contact between vesicles and cells. The magnitude of this phenomenon, as well as the absolute uptake values of carboxyfluorescein and vesicle lipid were found to vary within ±50% with the cell preparation used. The time-dependent decrease of the dye: lipid ratio, however, was observed in all experiments, and the shapes of dye and lipid uptake curves were also consistently similar throughout the experiments. The possibility of a time-dependent decrease in carboxyfluorescein quantum yield as a result of metabolic modifications of the molecule was excluded by the observation that there was no change in quantum yield of the dye during a 3 h incubation at 37°C in concentrated cell lysates.

Detailed examination (not shown) of the time interval between 0 and 15 min revealed that uptake of both carboxyfluorescein ($\Pi_i$) and of vesicle lipid is linear up to 5 min. We therefore measured uptake of dye and lipid as a function of vesicle lipid concentration after 5 min of incubation, as is shown in Fig. 2. Both total cell-associated carboxyfluorescein ($\Pi_i$), as well as the actually internalized fraction of carboxyfluorescein ($\Pi_d$), gradually increase with

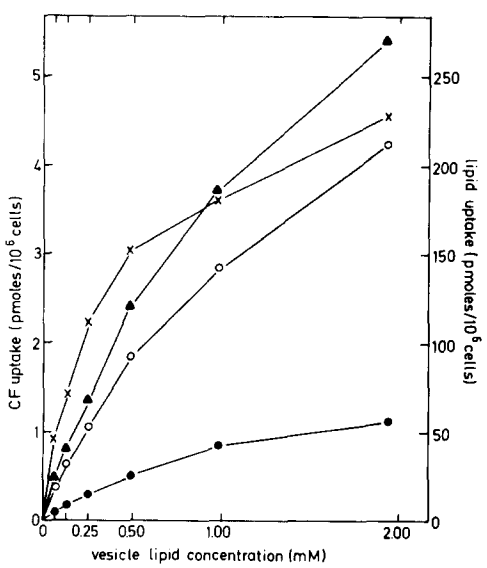


Fig. 2. Uptake of vesicle-entrapped carboxyfluorescein and of total vesicle lipid as a function of the total vesicle lipid concentration in the incubation mixture. For each vesicle lipid concentration studied, $5 \cdot 10^7$ Zajdela cells were incubated (37°C, shaking water bath) with suitable dilutions of one vesicle preparation, composed of phosphatidylcholine/cholesterol/phosphatidylserine (5 : 4 : 1) with 10 μCi of [*Me*-$^{14}$C]phosphatidylcholine (2.2 μCi/μmol) as a marker, in a final volume of 5 ml, in open Erlenmeyer flasks. After 5 min of incubation, duplicate 1 ml samples were withdrawn from each vessel and processed as described in the Methods section and Appendix. X———X, uptake of total vesicle lipid based on the uptake of [*Me*-$^{14}$C]phosphatidylcholine; ▲———▲, total cell-associated carboxyfluorescein ($\Pi_i$; see Appendix); ○———○, carboxyfluorescein inside cells ($\Pi_d$; see Appendix); ●———●, 'adsorbed' carboxyfluorescein ($\Pi_a$; see Appendix). Each point represents the average of duplicate measurements that agree within 5%. CF, carboxyfluorescein.

increasing vesicle lipid concentration. By contrast, the 'adsorbed' dye fraction ($\Pi_a$, see Appendix), saturates at high vesicle lipid concentrations. From a double reciprocal plot of 'adsorbed' carboxyfluorescein vs. vesicle lipid concentration we calculated that half of the maximum value of 1.5 pmol of 'adsorbed' dye per $10^6$ cells is reached at a vesicle lipid concentration of 0.82 mM. From Fig. 2 it is clear that the molar ratio of total cell-associated carboxyfluorescein (triangles) to total cell-associated lipid (crosses) increases with vesicle lipid concentration. The actual values of these ratios are presented in Table I, to be compared with the corresponding ratios in the entire incubation mixtures, which are fairly constant. Only at high vesicle lipid concentration there is good agreement between the bulk ratio and the cell-associated ratio. Apparently, the selective loss of dye, probably resulting, as discussed above from vesicle-cell contact, decreases with increasing vesicle lipid concentration. With FITC-dextran, to be described two paragraphs below as entrapped fluorophore, a similar phenomenon is observed.

When attempting to explain the observed changes in molar ratios three possibilities should be considered. First, vesicles plus contents are internalized, but contents (carboxyfluorescein) leak from the cells rapidly. Determination of leakage rates (average half-life of 14 min) of the dye from cells preloaded with free dye indicated that such a process may indeed contribute. Second, carboxyfluorescein leaks preferentially from vesicles attached to the cell surface. The observation that 'adsorbed' dye (Fig. 1, filled circles) rapidly decreases to a very low value is in line with such a view, as will be outlined in the Discussion. Third, uptake of $^{14}$C-labelled phosphatidylcholine is not representative of uptake of whole vesicles but, in part, reflects exchange of lipid between vesicle and cell membrane [5].

TABLE I

MOLAR RATIOS OF CARBOXYFLUORESCEIN : LIPID IN THE INCUBATION MIXTURES VS. IN THE CELLS

Data in this table are taken from the experiment described in Fig. 2. Middle column: Values were obtained as follows: to duplicate 50 μl samples from each incubation vessel Triton X-100 was added to a final concentration of 1% (v/v); fluorescence was read and radioactivity determined. The subsequently calculated number of pmol carboxyfluorescein was divided by the calculated number of pmol of total vesicle lipid per unit volume, yielding the initial molar ratio *. Right column: Values were obtained from the values shown in Fig. 2 as follows: the number of pmol of total cell-associated carboxyfluorescein was divided by the number of pmol of total cell-associated vesicle lipid, for each incubation vessel. Thus the molar ratio ** of cell-associated markers is obtained. For experimental conditions and statistics see the legend of Fig. 2. The molar ratio of entrapped carboxyfluorescein to total vesicle lipid in the original vesicle preparation was 0.027.

| Vesicle lipid concentration (mM) | Molar ratio * carboxyfluorescein/lipid | Molar ratio ** carboxyfluorescein/lipid |
|---|---|---|
| 0.059 | 0.029 | 0.010 |
| 0.119 | 0.029 | 0.011 |
| 0.238 | 0.026 | 0.012 |
| 0.475 | 0.027 | 0.016 |
| 0.950 | 0.027 | 0.021 |
| 1.900 | 0.027 | 0.023 |

* Molar ratio of entrapped carboxyfluorescein to total vesicle lipid in each incubation vessel.
** Molar ratio of total cell-associated carboxyfluorescein to total cell-associated lipid.

Since carboxyfluorescein leaks out of vesicles and cells fairly rapidly we set out to test a compound with similar fluorescence properties but higher molecular weight: FITC-dextran. In contrast to carboxyfluorescein, FITC-dextran at an intravesicular concentration of 100 mM, which is about the highest FITC-dextran concentration still allowing formation of vesicles, is only about 65% self-quenched. Hence, amounts of internalized or 'adsorbed' FITC-dextran cannot be calculated as accurately as was done for carboxyfluorescein, which is nearly 100% self-quenched in such conditions (also see Appendix). Yet, FITC-dextran has two major advantages over carboxyfluorescein: the overall leakage-rate of the dextran from vesicles in an incubation with cells is less than half that of carboxyfluorescein, and, in addition, FITC-dextran leakage is barely dependent on the environmental pH, in contrast to carboxyfluorescein, which at pH 5.8 leaks from vesicles about 3.5 times as fast as at pH 7.4.

Fig. 3 shows that, during incubation of cells with free FITC-dextran, cell-associated FITC-dextran fluorescence, as measured before addition of detergent, reaches a nearly constant level in about 10 min. In contrast, total cell-associated FITC-dextran fluorescence, as measured after addition of detergent, increases during up to 60 min of incubation time. Since, at the concentration

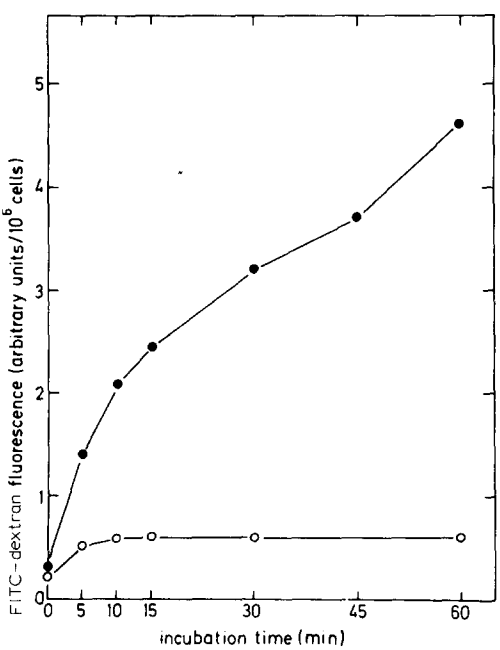


Fig. 3. Uptake of free FITC-dextran in the presence of empty vesicles as a function of time. $1.75 \cdot 10^8$ Zajdela cells were incubated (37° C, shaking water bath) with 17.5 μmol of total vesicle lipid plus 45 μM (final concentration) free FITC-dextran in a final volume of 17.5 ml in an open Erlenmeyer flask. Empty vesicles consisting of phosphatidylcholine/cholesterol/phosphatidylserine (5 : 4 : 1) had been previously mixed with free FITC-dextran. At suitable times duplicate 1 ml samples were taken from the incubation mixture and processed as described in the Methods section and Appendix. ●———●, cell-associated FITC-dextran fluorescence, as measured after addition of detergent; ○———○, cell-associated FITC-dextran fluorescence as measured before addition of detergent. Each point represents the average of duplicate measurements that agree within 5%. Filled circles: 1.5 arbitrary units/$10^6$ cells correspond to 1 pmol of FITC-dextran/$10^6$ cells.

added, FITC-dextran shows no significant self-quenching, the increasing discrepancy between fluorescence readings before and after addition of detergent must be due to accumulation of the dye to high intracellular concentrations or/and into subcellular compartment(s) of low average pH. Either possibility indicates, that most likely the free compound is endocytosed and ultimately accumulates in the lysosomes. This interpretation is supported by the observation that during prolonged incubation, after removal of free FITC-dextran, total cell-associated fluorescence remains constant, whereas fluorescence, as measured before addition of detergent, slowly decreases with time. We consider it unlikely that the dye is concentrated within secondary lysosomes to an extent which would give rise to significant self-quenching: even at high intralysosomal FITC-dextran concentrations a marked residual fluorescence ($\Lambda_0$, see Appendix) would remain. Therefore the ratio of fluorescence values between upper and lower curve in Fig. 3 can be considered as a measure for the fraction of FITC-dextran molecules that is exposed to low (intralysosomal) pH. In this view it is interesting to see the results of others, who used high molecular weight FITC-dextran for the measurement of the intralysosomal pH [12].

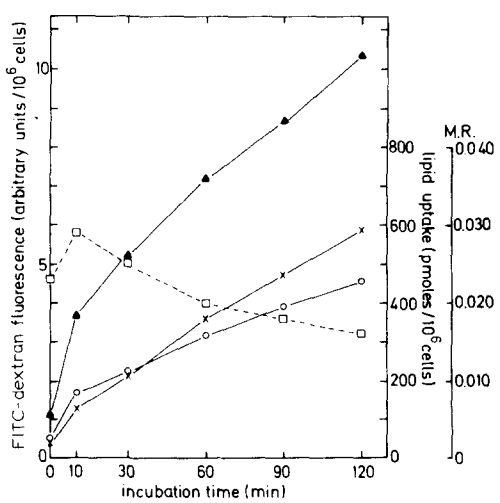


Fig. 4. Uptake of vesicle-entrapped FITC-dextran and of total vesicle lipid as a function of time. $1.4 \cdot 10^8$ Zajdela cells were incubated (37°C, shaking water bath) with 14 μmol of total vesicle lipid in a final volume of 14 ml in an open Erlenmeyer flask. Vesicles were composed of phosphatidylcholine/cholesterol/phosphatidylserine (5 : 4 : 1) with 5 μCi of [*Me*-$^{14}$C]phosphatidylcholine (spec. act. 2.2 Ci/mol) as a marker, and contained 100 mM FITC-dextran. At suitable times duplicate 1 ml samples were taken from the incubation mixture and processed as described in the Methods section and Appendix. X———X, uptake of total vesicle lipid based on the uptake of [*Me*-$^{14}$C]phosphatidylcholine; ▲———▲, cell-associated FITC-dextran fluorescence, as measured after addition of detergent; ○———○, cell-associated FITC-dextran fluorescence, as measured before addition of detergent. Each point represents the average of duplicate measurements, that agree within 5%. The accessory ordinate at the extreme right of the Fig. designates the molar ratio (M.R.) of total cell-associated FITC-dextran to total cell-associated vesicle lipid. Values for molar ratio were obtained by dividing the number of pmol of FITC-dextran associated with $10^6$ cells by the number of pmol of vesicle lipid associated with $10^6$ cells. From fluorescence readings after addition of detergent and radioactivity determinations the molar ratio of total entrapped FITC-dextran to total vesicle lipid in the original vesicle preparation was calculated: 0.034. □- - - - -□ molar ratio of total cell-associated FITC-dextran to total cell-associated vesicle lipid. Filled triangles: 10 arbitrary units/$10^6$ cells correspond to 9.34 pmol of FITC-dextran/$10^6$ cells.

When, by contrast, vesicle-entrapped FITC-dextran is offered to the cells, cell-associated FITC-dextran fluorescence, as measured before addition of detergent, steadily increases with time (Fig. 4). Total cell-associated FITC-dextran fluorescence, as measured after addition of detergent, matches the uptake of vesicle lipid slightly better than in case we used vesicle-entrapped carboxyfluorescein (Fig. 1). Yet, the molar ratio of dye to lipid still tends to decrease after reaching a maximum at short incubation time. The difference in FITC-dextran uptake characteristics between Figs. 3 and 4 suggests, that vesicle-entrapped FITC-dextran is, at least partly, delivered directly into the cytoplasm: since for the experiment of Fig. 4, FITC-dextran inside vesicles was 67.5% self-quenched, the increase in quantum yield of the dye upon addition of detergent (ranging from 2.0—2.3-fold) is too low to indicate mere adsorption of vesicles to the cell surface. In the latter case we would expect to measure a 3.1-fold increase in quantum yield.

In order to test to what degree cells are able to retain vesicle lipid and vesicle-entrapped carboxyfluorescein or FITC-dextran, we incubated cells for 45 min with vesicles containing either type of dye, removed non cell-associated vesicles,

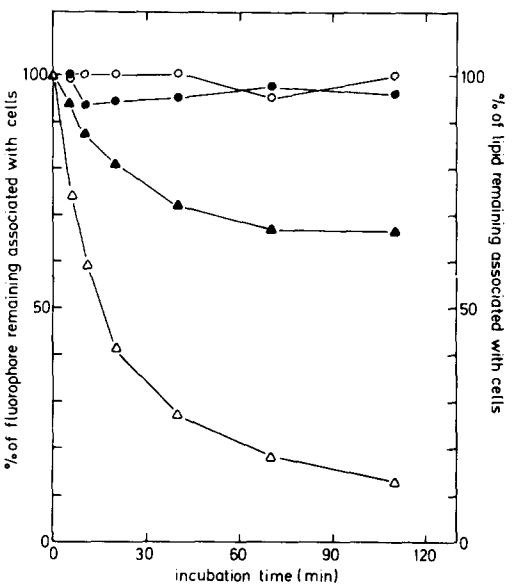


Fig. 5. Release of vesicle lipid and vesicle-transferred fluorophore (FITC-dextran or carboxyfluorescein) from cells as a function of time. $2.5 \cdot 10^8$ Zajdela cells were incubated in a final volume of 25 ml with 25 μmol of total vesicle lipid containing either 100 mM carboxyfluorescein or 100 mM FITC-dextran. The vesicles were composed of phosphatidylcholine/cholesterol/phosphatidylserine (5 : 4 : 1), and carried 5 μCi of [*Me*-$^{14}$C]phosphatidylcholine (spec. act. 3 μCi/μmol) as a lipid marker. Incubations were carried out at 37°C (shaking water bath) in open Erlenmeyer flasks. After 45 min of incubation the cells from each incubation vessel were centrifuged (600 × *g* for 30 s) and washed twice to remove non cell-associated vesicles. Subsequently, 25 ml of fresh, vesicle-free medium was added to the cells in each vessel, and the incubation was continued. At suitable times duplicate 1 ml aliquots were taken from the incubation mixtures and processed as described in the Methods section and Appendix. Lipid and fluorophore amounts remaining associated with the cells are expressed as percent of values determined immediately after medium-change (zero-time). ▲———▲, total cell-associated FITC-dextran; △———△, total cell-associated carboxyfluorescein; ●———●, total cell-associated vesicle lipid from vesicles containing 100 mM FITC-dextran; ○———○, total cell-associated vesicle lipid from vesicles containing 100 mM carboxyfluorescein. Each point represents the average of duplicate measurements that agree within 5%.

and continued incubation for another 60 min in vesicle-free, fresh medium. The results are shown inFig. 5. Lipid from both types of vesicles remains firmly cell-associated, indicating that neither intact vesicles nor water-soluble metabolites of the lipid marker are released into the medium. Vesicle-transferred carboxyfluorescein leaks from the cells almost completely, with a half-life of approx. 15 min. By contrast, vesicle-transferred FITC-dextran is released only to a limited extent: about 65% of vesicle-transferred molecules remain associated with the cells. These findings are in line with what we observed in Figs. 1 and 4: a higher degree of concordance between uptake of FITC-dextran and lipid (Fig. 4) than between carboxyfluorescein and lipid (Fig. 1). Cell-associated carboxyfluorescein, as measured before addition of detergent, follows the same decay pattern as total cell-associated carboxyfluorescein. This means that, at any time, all cell-associated carboxyfluorescein molecules show the same change in quantum yield upon addition of detergent. Thus, there is no unidirectional transport of the dye between subcellular compartments during the continued incubation. Most likely, carboxyfluorescein readily diffuses out of the cytoplasm. With FITC-dextran, only a 10% decrease in fluorescence can be measured directly (i.e. before addition of detergent) during continued incuba-

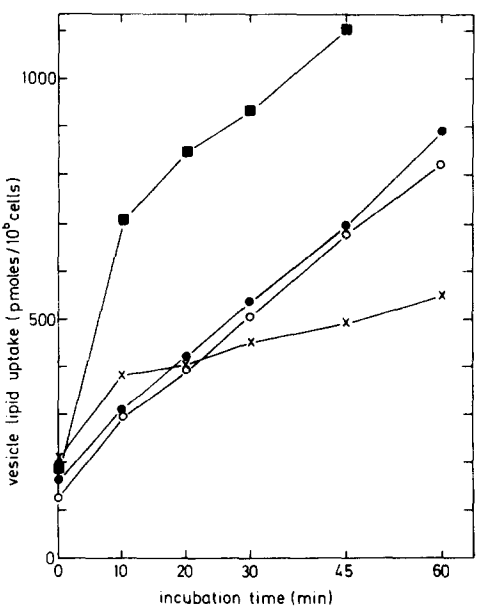


Fig. 6. Uptake of empty vesicles, based on three different lipid radioactive markers, as a function of time. For each type of vesicle preparation $1.875 \cdot 10^8$ Zajdela cells were incubated with 15 μmol of total vesicle lipid in a final volume of 15 ml. The incubation was carried out in open Erlenmeyer flasks at 37° C (shaking water bath). At suitable times duplicate 1 ml samples were taken from the incubation mixtures and processed as described in the Methods section. ■———■, phosphatidylcholine/cholesterol/phosphatidylserine (5 : 4 : 1) with 5 μCi of [1α,2α(n)-$^3$H]cholesterol (spec. act. 43 mCi/μmol) as a marker. ●———●, phosphatidylcholine/cholesterol/cholesteryl oleate/phosphatidylserine (5 : 3.87 : 0.13 : 1) with 3 μCi of [*Me*-$^{14}$C]phosphatidylcholine (spec. act. 3 μCi/μmol) as a marker. ○———○, phosphatidylcholine/cholesterol/phosphatidylserine (5 : 4 : 1) with 3 μCi of [*Me*-$^{14}$C]phosphatidylcholine (spec. act. 3 μCi/μmol) as a marker. X———X, phosphatidylcholine/cholesterol/cholesteryl oleate/phosphatidylserine (5 : 3.87 : 0.13 : 1) with 3 μCi of cholesteryl[1-$^{14}$C]oleate (15.4 Ci/mol) as a marker. Each point represents the average of duplicate measurements that agree within 5%.

tion, whereas a 35% decrease is found after addition of detergent. This suggests that the greater part of FITC-dextran loss from the cells does not occur from within the cells, but rather from vesicles attached to the cell surface.

In view of the observed incubation-time dependent discrepancies between lipid uptake and fluorophore uptake we included two additional labels in our experiments: cholesterol and cholesteryl oleate, the former known to exchange readily between membranes [13], the latter being considered as non-exchangeable lipid. Empty vesicles, made of phosphatidylcholine, cholesterol, and phosphatidylserine (molar ratio 5 : 4 : 1), with or without a trace of cholesteryl oleate, were incubated with the Zajdela cells. The vesicles were labeled in the phosphatidylcholine, cholesterol, or cholesteryl oleate. Fig. 6 shows that the incorporation of 2.3 mol% of cholesteryl oleate did not significantly affect the uptake of phosphatidylcholine label (open and filled circles) by the cells. Transfer from both types of vesicles is linear for at least an hour and, when calculated as total vesicle lipid, 800—900 pmol/$10^6$ cells are taken up in this period. Based on $^3$H-labeled cholesterol, vesicle uptake values are more than twice as high as those based on $^{14}$C-labeled phosphatidylcholine. With $^{14}$C-labeled cholesteryl oleate the initial values of total lipid uptake are somewhat higher than those based upon phosphatidylcholine, but during continued incubation the cholesteryl ester values are progressively lagging behind those of phosphatidylcholine. At present we have no explanation for the higher initial values. Possibly, cholesteryl esterase activity, which reportedly is associated with rat liver cytosol and lysosomes [14], is present in the plasma membranes, leading to some transfer of free radiolabeled oleate to the cells. We are currently investigating this possibility.

TABLE II

INFLUENCE OF METABOLIC INHIBITORS ON THE UPTAKE OF [$^3$H]SUCROSE, FREE FITC-DEXTRAN AND VESICLE-ENTRAPPED FITC-DEXTRAN

$5 \cdot 10^7$ Zajdela cells were incubated (37°C, shaking water bath) for 60 min in open Erlenmeyer flasks, in a final volume of 5 ml, with either 5 μCi of [6,6′(n)-$^3$H]sucrose (spec. act. 1 Ci/mmol), 1 mM empty phosphatidylcholine/cholesterol/phosphatidylserine (5:4:1) vesicles plus 40 μM free FITC-dextran, or 1 mM phosphatidylcholine/cholesterol/phosphatidylserine (5:4:1) vesicles containing 100 mM FITC-dextran. For the latter preparation the trapping efficiency was such, that upon liberation of the entrapped FITC-dextran, a final concentration of 40 μM free FITC-dextran would result. The incubations were carried out in the absence (controls) or presence of 50 mM 2-deoxyglucose plus 5 mM sodium azide, added to the cells 30 min before starting the incubation. After 60 min of incubation 1 ml duplicate samples were withdrawn from each incubation, and processed as described in the Methods section and Appendix. Values represent the averages of duplicate measurements that agree within 5%.

| Addition | [$^3$H]Sucrose uptake in pmol/$10^6$ cells | Free FITC-dextran uptake in pmol/$10^6$ cells | | Vesicle-entrapped FITC-dextran uptake in pmol/$10^6$ cells * | |
|---|---|---|---|---|---|
| | | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| None (control) | 0.50 (100%) | 6.4 (100%) | 4.6 (100%) | 14.3 (100%) | 12.2 (100%) |
| 2-Deoxyglucose (50 mM) plus sodium azide (5 mM) | 0.24 (48%) | 2.2 (34%) | 2.0 (43%) | 13.9 (97%) | 10.2 (84%) |

* Total cell-association FITC-dextran.

Table II shows to what extent uptake of free and vesicle-entrapped materials depends on metabolic energy supply: uptake of vesicle-entrapped FITC-dextran is only slightly affected by the presence of 2-deoxyglucose plus sodium azide, a combination known to effectively inhibit energy-dependent endocytosis. By

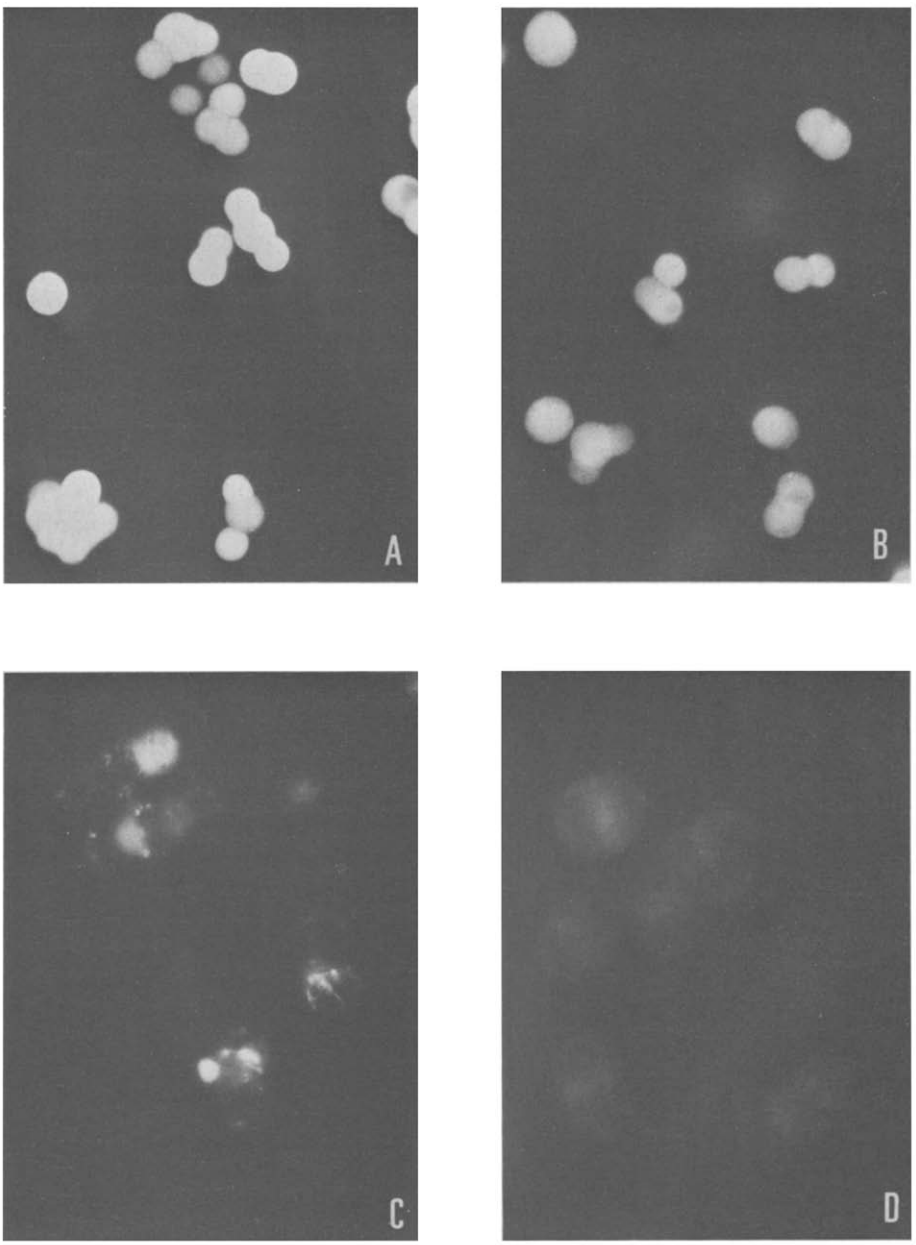


Fig. 7. Fluorescence micrographs of Zajdela cells incubated with free- and vesicle-entrapped fluorophores. Incubations (30 min) were carried out as described in the legends of Figs. 1, 3 and 4. Cells were incubated with (A) 1 mM empty vesicles plus 50 μM free carboxyfluorescein (375X); (B) 1 mM vesicles, containing 100 mM carboxyfluorescein (375X); (C) 1 mM empty vesicles plus 100 μM free FITC-dextran (600X); (D) 1 mM vesicles, containing 100 mM FITC-dextran (600X).

contrast, uptake of free [$^3$H]sucrose and of free FITC-dextran is inhibited more than 50% when compared to the corresponding controls. These findings confirm that energy-dependent endocytosis does not play a significant role in the uptake of FITC-dextran containing vesicles.

Fluorescence microscopic observations on viable cells following incubation with either free or vesicle-entrapped fluorophore agree with the results presented so far: after incubation of cells with free carboxyfluorescein (Fig. 7A), vesicle-entrapped carboxyfluorescein (Fig. 7B) or vesicle-entrapped FITC-dextran (Fig. 7D), an evenly distributed fluorescence is observed throughout the cytoplasm of most cells, the overall fluorescence intensity being highest in cells incubated with vesicle-entrapped carboxyfluorescein. Often the nuclei appear as relatively dark pits, indicating that fluorescence originates from within the cytoplasm. Occasionally, cells are found that show several brilliantly fluorescing spots. On morphological grounds (phase contrast microscopy) it was excluded that these cells are other than tumor cells. In case of vesicle-entrapped FITC-dextran (Fig. 7D) very faint fluorescent rims can be seen at the cell periphery. These most likely are caused by vesicles adsorbed to the cell surface (FITC-Dextran is not fully self-quenched inside vesicles, in contrast to carboxyfluorescein).

Generally, fluorescence intensity is quite heterogeneous throughout the cell population, with a small number of cells showing no fluorescence at all. When cells are incubated with free FITC-dextran (Fig. 7C), most cells show a large number of brilliantly fluorescing spots. The presence of metabolic inhibitors does not cause a significant change in the appearance of cells upon incubation with either free- or vesicle-entrapped FITC-dextran.

These observations confirm our conclusion, that vesicle-entrapped fluorophores end up diffusely distributed in the cells, i.e. in the cytoplasm. For FITC-dextran this is in sharp contrast to the fate of the free dye which is intracellularly recovered in distinct compartments, most probably endocytotic vacuoles and/or lysosomes.

## Discussion

The basic conclusion from the work presented in this report, is that Zajdela cells are capable of internalizing the contents of small unilamellar vesicles, and that the entrapped volume is released directly into the cytoplasm. Evidence is obtained using both quantitative spectrofluorimetry and fluorescence microscopy. Generally, a discrepancy between uptake of entrapped markers and uptake of vesicle lipid is observed: during vesicle-cell incubation the entrapped markers are taken up to a lesser extent than the vesicle lipid. This discrepancy is slightly smaller when FITC-dextran is used instead of carboxyfluorescein as a vesicle-entrapped marker; it increases with the duration of vesicle-cell incubation, and decreases with increasing vesicle lipid concentration in the incubation mixture. With carboxyfluorescein as entrapped fluorophore the discrepancy is likely to be due to a combination of leakage of vesicle-transferred carboxyfluorescein from the interior of the cell and from vesicles adhering to the cell surface. With FITC-dextran, on the other hand, the discrepancy is largely caused by leakage of the dye from vesicles attached to the cell surface. In both

cases an overestimation of vesicle lipid uptake caused by vesicle-plasma membrane exchange of $^{14}C$-labeled phosphatidylcholine cannot be excluded. Arguments against a major involvement of this mechanism are: first, when using FITC-dextran as a vesicle-entrapped marker the discrepancy is less than with carboxyfluorescein as a marker (Figs. 1 and 4) and second, others [15,16] have shown phosphatidylcholine-exchange between vesicles and cell surface, when compared to total vesicle lipid uptake, to be rather low at 37°C. Also, the use of serum-free medium excludes a contribution of lipoprotein- or albumin-mediated phosphatidylcholine transfer between vesicles and cell surface [17].

The absolute levels of uptake of vesicle-entrapped marker and of total vesicle lipid are of the same order of magnitude as those obtained by others, working with other in vitro systems [10,18—20]. Comparison with work from our laboratory on the ability of isolated hepatocytes in maintenance culture to take up lipid vesicles [21], shows that there is no major difference in the absolute levels of uptake of vesicles between Zajdela cells, which originate from hepatocytes [3], and isolated hepatocytes. Lymphosarcoma cells from the spleen of mice, however, show both in vivo and in vitro a fairly higher uptake level than Zajdela cells (Van Renswoude, A.J.B.M. and Konings, A.W.T., unpublished data). The fraction of the total vesicle population that is found associated with the cells at a given time, is very small: approx. 0.5% after 60 min of incubation. This, however, still represents a large number of vesicles associated with one single cell. If we assume one vesicle to be composed of 4000 lipid molecules [22] we can calculate that after 30 min of incubation some 35 000 vesicles will have become associated with one single cell. The experiments described offer circumstantial evidence that vesicles are primarily taken up by a vesicle-cell fusion-like mechanism. Arguments against a considerable involvement of endocytosis are found in the marked difference between uptake of free FITC-dextran and of vesicle-entrapped FITC-dextran (Figs. 3 and 4), in the relative insensitivity of vesicle-uptake to the presence of metabolic inhibitors (Table II), and in the fluorescence microscopic images of cells following incubation with free FITC-dextran and vesicle-entrapped FITC-dextran. With carboxyfluorescein as an entrapped marker, estimation of a contribution of endocytosis is difficult: carboxyfluorescein has a rather low molecular weight, and its permeation through vesicle membranes was found to be considerably accelerated on lowering the environmental pH (see Results section); hence a rapid passage of this dye through the lysosomal compartment cannot be excluded. For short incubation times, however, our data (Fig. 1) show, that not all vesicle-mediated carboxyfluorescein uptake could have occurred through an endocytotic route: the post- to pre-Triton fluorescence ratios (4.6, 2.4, 2.2 for 0, 5, 10 min of incubation time, respectively) are far too low as to indicate exclusive endocytosis of vesicles (in which case we would expect a ratio of 20—40).

A major problem is envisaged during observation of time-dependent uptake of vesicle lipid and vesicle-entrapped markers: apparently the interaction process is initiated so fast that, despite rapid processing of incubation mixture samples, relatively high zero-time values are obtained for uptake of both vesicle lipid and entrapped fluorophores. This is important in view of two observations: first, with either fluorophore the molar ratio of total cell-associated dye to total cell-associated vesicle lipid is consistently found to increase initially

between 0 and 5 min of incubation time, and then to decrease gradually with longer incubation times (Figs. 1 and 4), and second, the fraction of carboxyfluorescein, which becomes unquenched upon addition of detergent, is maximal at zero time (Fig. 1). One explanation for these observations is the occurrence of rapid leakage of both fluorophores (when compared to their leakage from the bulk of vesicles in the incubation mixture) from vesicles that approach to or make actual physical contact with the cell surface at the onset of the interaction process. Once internalization of (other) vesicles has started to proceed, this 'very leaky' fraction may become progressively masked. It is tempting to speculate on the existence of two major vesicle binding sites at the cell surface. Binding of a vesicle to one site would alter its structure such that entrapped markers would be released into the surrounding medium, whereas binding to the other site would result in an actual and complete capture of the vesicle lipid plus internalization of the vesicle contents. The results on concentration-dependent uptake of vesicle lipid and entrapped carboxyfluorescein (Fig. 2) lend support to this hypothesis: if the first binding site becomes nearly saturated, any further rise of the vesicle lipid concentration in the system is likely to result in a larger fraction of the interacting vesicles to be actually and completely internalized through the other binding site. Thus, the molar ratio of total cell-associated fluorophore to total cell-associated lipid would increase, as is observed. The amount of carboxyfluorescein, whose quenching is relieved upon addition of detergent ($\Pi_a$), reaches 75% of its maximal value at a vesicle lipid concentration of 1.9 mM (calculated from Fig. 2). By contrast, the amount of carboxyfluorescein, measurable before addition of detergent, does not simply saturate, as can be verified by constructing a double reciprocal plot. It would be of interest to see whether the site at which, in our hypothesis, complete capture of the vesicle lipid and contents occurs, would be kinetically (and may be physically) identical to the carboxyfluorescein transfer site, which was demonstrated to be present on the lymfocyte surface by Blumenthal et al. [2]. At present it is not known whether liposome-mediated carboxyfluorescein-transfer is the result of complete vesicle-cell fusion involving integration of the vesicle lipid into the lipid backbone of the cellular plasma membrane, or that transfer of the dye occurs through an alleviated permeability barrier consisting of two closely apposed bilayers (vesicle- and plasma membrane). Our results with FITC-dextran as an intravesicular marker clearly indicate that liposome-mediated carboxyfluorescein transfer is not a process intrinsically determined by the mere use of carboxyfluorescein as a vesicle-entrapped marker. To check the hypothesis outlined above, it would be important to gain a better insight in the kinetics of vesicle-cell interaction during the first few minutes of incubation. This, however, obviously requires a different experimental approach, involving instantaneous and complete separation of cells from bulk vesicles in an incubation.

An indication for the occurrence of a 'very leaky' fraction of vesicles adsorbed to the cell surface, following vesicle-cell incubation, is provided by the experiment described in Fig. 5. Only a limited fraction of the total amount of FITC-dextran that becomes cell-associated upon incubation of FITC-dextran-containing vesicles with cells is released during subsequent continued incubation. At the same time, no significant loss of cell-associated vesicle lipid, either

intact or metabolized, occurs. Free FITC-dextran, once taken up by the cells, remains firmly cell-associated, indicating that it is neither exocytosed nor released by diffusion across the plasma membrane. Hence it is very likely that the fraction of FITC-dextran leaving the vesicle-cell complex after preceding incubation of cells with FITC-dextran-containing vesicles, originates from vesicles adhering to the cell surface. It remains to be established, however, if and to what extent the size of this fraction depends on the duration of the preceding vesicle-cell incubation.

The observation that fluorescence intensity is quite heterogeneously distributed within the tumor cell population after incubation with dye-containing vesicles, might turn out to be of crucial importance in studying the antitumor drug carrier potential of liposomes. Laser-flow microfluorimetric [23] investigations on synchronized tumor cell populations, following vesicle-cell incubations at different cell cycle stages, will provide useful information in this respect.

## Appendix

The fluorescence reading obtained from the 2 ml of cell suspension remaining, after withdrawal of a 1 ml sample for radioactivity determination, is called $F_d$ (directly measurable fluorescence). After measuring $F_d$, 0.22 ml of a 10% (v/v) aqueous solution of Triton X-100 is added to the sample, followed by vortex mixing for several minutes. Then fluorescence is read again, and the value obtained is multiplied by 1.11, the dilution factor introduced by the addition of the detergent. This yields $F_i$ (indirectly measurable fluorescence). In this way values for $F_d$ and $F_i$ are obtained from duplicate measurements, and the averages, $\overline{F}_d$ and $\overline{F}_i$, are calculated. The total amount of carboxyfluorescein or FITC-dextran associated with $10^6$ cells and the amount of carboxyfluorescein inside $10^6$ cells, $\Pi_i$ and $\Pi_d$ respectively, are then calculated in pmol from the following equations:

$$\Pi_d = (\overline{F}_d - \overline{s}_d - \overline{f}_d)\,\mu\alpha k \tag{1}$$

$$\Pi_i = (\overline{F}_i - \overline{s}_i - \overline{f}_i)\,\mu k \tag{2}$$

$s_d$ and $s_i$ symbolize the autofluorescence plus light-scattering background from cells, treated in the same way as the samples from the incubation, but without exogenous fluorophore. Range of $s$: $0.03\,F < s < 0.30\,F$ for carboxyfluorescein, and $0.10\,F < s < 0.60\,F$ for FITC-dextran. $f_d$ and $f_i$ designate the fluorescence imparted to the cell sample by carboxyfluorescein, taken up as free dye after leakage from the vesicles during the incubation. If Zajdela cells are incubated with empty vesicles plus free carboxyfluorescein, the uptake of dye is linear with the concentration of free carboxyfluorescein in the incubation medium, for dye-concentrations up to 50 $\mu$M. From the leakage of carboxyfluorescein from the bulk of liposomes in the incubation medium (see below) the final concentration of free carboxyfluorescein in the incubation is known. Thus the amount of free dye taken up by the cells can be calculated, yielding $f_d$ and $f_i$. For accurate determination of $f_d$ and $f_i$, control incubations of cells with empty liposomes plus 25 $\mu$M free carboxyfluorescein were run in all

experiments described. Generally, $f < 0.05\ F$. When FITC-dextran was used as an entrapped fluorophore, $f$ proved to be negligible. Both $f$ and $s$ are introduced in Eqns. 1 and 2 as the average of duplicate measurements. The multiplication factor $\mu$, which corrects for leakage of carboxyfluorescein from the bulk of liposomes in the incubation, is introduced in order to determine, if desired, the number of vesicles taken up by or associated with the cells. Consequently, the values obtained for $\Pi_d$ and $\Pi_i$ from Eqns. 1 and 2, respectively, refer to idealized, non-leaky vesicles.

$$\mu = \frac{1}{1 - \Lambda} \tag{3}$$

In this equation $\Lambda$ stands for leakage of carboxyfluorescein from the bulk of liposomes (not associated with cells) in the incubation mixture.

$$\Lambda = \frac{1}{1 - \Lambda_0} (\Lambda_t - \Lambda_0) \tag{4}$$

Here $\Lambda_0$ designates the residual fluorescence, i.e. the ratio of fluorescence readings before and after addition of detergent in a liposome-sample immediately after collection of the void volume of the Sephadex G-100 column. $1 - \Lambda_0$ represents the degree of self-quenching of carboxyfluorescein inside the vesicles. Usually, $\Lambda_0 \approx 0.035$ (3.5%) and thus $1 - \Lambda_0 \approx 0.965$ (96.5%). The residual fluorescence is most likely the result of an incomplete self-quenching of the fraction of dye molecules facing the inner monolayer of the vesicle membrane. If $\Lambda_0$ is measured as a function of the carboxyfluorescein concentration inside the vesicles, $[CF]_i$, the relation $\Lambda_0 = k/[CF]_i$ (Eqn. 5) for 10 mM $< [CF]_i <$ 100 mM is obtained. Here $k \approx 5$ mM. $\Lambda_t$ stands for the leakage of carboxyfluorescein from the bulk liposomes at time $t$, and equals the ratio of fluorescence readings before and after addition of detergent in a sample taken from the incubation mixture. It should be noted that Eqn. 4 gives an approximation of the leakage of carboxyfluorescein from the bulk of liposomes at a given time $t$, since considerable leakage of dye from the vesicles causes a decrease in the self-quenching of carboxyfluorescein inside vesicles, thus increasing the value of $\Lambda_0$. As long as $\Lambda_t < 0.2$, however, which is the case in all the experiments described, this approximation is legitimate, as can be derived from Eqn. 5. The correction factor $\mu$ then ranges from 1 to 1.20. Differences in pH between medium and intracellular compartments to which carboxyfluorescein has access, require multiplication by $\alpha$. This is the factor by which, upon addition of detergent, fluorescence quantum yield increases in going from a certain intracellular pH to pH 7.4. When Zajdela cells are incubated with empty liposomes plus free carboxyfluorescein the dye is taken up by the cells, most probably mainly by diffusion across the cell membrane. After approx. 10 min of incubation the uptake of dye levels off, indicating that a steady state is established: influx = outflux. If cells are then washed several times in NaCl/Hepes buffer, and both $\overline{F}_d - \overline{s}_d$ and $\overline{F}_i - \overline{s}_i$ are determined, it is found that

$$1.7 < \frac{\overline{F}_i - \overline{s}_i}{\overline{F}_d - \overline{s}_d} < 2.0, \text{ for incubation times} > 10 \text{ min}.$$

Thus $1.7 < \alpha < 2.0$. Comparison of the value of $\alpha$ with the increase in fluores-

cence quantum yield of a carboxyfluorescein solution, which is brought from a pH = x to pH = 7.4 results in an x of 6.3—6.5. Thus, the average intracellular pH, at least for the compartment(s) to which the dye has access, is in the range of 6.3—6.5. FITC-dextran quantum yield depends in a similar manner on the environmental pH, but because the dye is not completely self-quenched inside vesicles, $\alpha$ cannot be applied with precision (Figs. 3 and 4). $k$, finally, is a composite factor, which includes the ratios of sample- and standard fluorescence readings, sample volume corrections, etc. When carboxyfluorescein is used as an entrapped marker, the amount of dye present in vesicles adsorbed to the cell surface and/or present in endocytotic vacuoles, can be calculated:

$$\Pi_a = \Pi_i - \Pi_d \quad (6)$$

Since $\Pi_i$ and $\Pi_d$ are calculated for idealized, non-leaky vesicles, $\Pi_a$ also can be converted easily into the number of vesicles 'adsorbed'.

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