Cell-Induced Leakage of Liposome Contents†

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ABSTRACT: Using the principle of relief of self-quenching of carboxyfluorescein [Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R., & Hagins, W. A. (1977) *Science 195*, 489-492] upon leakage of the dye from the interior of lipid vesicles, we investigated the integrity of sonicated small unilamellar vesicles in the presence of isolated hepatocytes, Zajdela ascites hepatoma cells, and plasma membranes of either cell type. We observed that cells as well as plasma membranes induce leakage of carboxyfluorescein from vesicles. Two parameters (initial rate and maximal level of induced leakage) were determined to quantitate the leakage events and were found to depend on cell density, vesicle concentration, and vesicle lipid composition. The magnitude of both parameters is shown to increase with cell density and to decrease with increasing vesicle lipid concentration and seems to be proportional to the number of vesicles found in close contact with the cell. For vesicles made of phosphatidylcholine and cholesterol, the degree of induced leakage increases steeply with cholesterol contents increasing from 30 to 40 mol %. In the case of simultaneous presence of 10 mol % phosphatidylserine, induced leakage can be observed at cholesterol contents exceeding 20 mol %. We show that leak-inducing activity resides in the plasma membrane and that it can be considerably reduced by treatment of the plasma membranes with neuraminidase or trypsin, suggesting the involvement of cell-surface glycoprotein(s). Release of activity from intact cells and isolated plasma membranes into the medium occurs spontaneously (at a slow rate) but can be facilitated by freezing and thawing; the activity can subsequently be recovered in a soluble form from the medium.

Lipid vesicles[1] are currently under study as potential carriers for various water-soluble substances to be introduced into mammalian cells [for reviews see Pagano & Weinstein (1978) and Tyrrell et al. (1976)]. Yet, many of the details of vesicle–cell interaction, particularly at the level of the cell surface, are unknown. Recent studies from our laboratory (Van Renswoude et al., 1979; Hoekstra et al., 1981) as well as from others (Szoka et al., 1979) called attention to the probability of leakage of vesicle-entrapped substances as a result of contact between vesicles and cell surfaces. Szoka et al. (1979) recognized that the presence of cells enhanced leakage of the fluorescent dye carboxyfluorescein from vesicles. In studying the interaction of vesicles with Zajdela ascites hepatoma cells (Van Renswoude et al., 1979) we obtained evidence of CF[2] release from vesicles in presence of cells, superimposed on bulk leakage, and we arrived at the conclusion that this superimposed CF leakage most likely occurs from a fraction of vesicles that is in persistent or transient physical contact with the cells. A similar superimposed leakage of CF was observed by Hoekstra et al. (1981) in their study of in vitro interaction of isolated hepatocytes and lipid vesicles. Since this leakage phenomenon was observed with various cell types as well as with vesicles of various lipid composition, we considered it important to have more insight into the nature of its mechanism. We used CF as an entrapped substance mainly for three reasons: first, leakage of CF from vesicles can easily be monitored continuously; second, there is no need for separation of vesicles from released solute; third, because of the high sensitivity of CF fluorescence measurement, the use of this dye offers the possibility to investigate the effect at relatively low vesicle concentrations.

The CF method has been described in detail (Weinstein et al., 1977; Blumenthal et al., 1977). Briefly, the dye is entrapped inside lipid vesicles in a high, self-quenched concentration (100 mM). Escape of CF from the inner aqueous compartment of a small unilamellar vesicle results in a vast dilution of the dye into the surrounding medium, causing an instantaneous fluorescent signal (F_{direct}). The total amount of CF within the test sytem can be assessed by the addition of a detergent (e.g., Triton X-100) which causes immediate and complete disintegration of the vesicles, concomitant dilution of all CF into the total system volume, and a maximal fluorescent signal ($F_{indirect}$). Thus, leakage of CF from vesicles can be expressed in terms of F_d/F_i. Details on the quantitative treatment of CF leakage data are given elsewhere (Van Renswoude et al., 1979).

Materials and Methods

Materials. Egg yolk lecithin, cholesterol (CH-S grade) and dicetyl phosphate were from Sigma Chemical Co. Phosphatidylserine was purified from bovine brain extract (Sigma) by preparative thin-layer chromatography. *N-methyl*-^{3}H-labeled egg yolk lecithin (49 μCi/μmol) was prepared essentially as described by Stoffel (1975). All lipids used exhibited one spot on TLC in $CHCl_3$–CH_3OH–H_2O (65:25:4).

Horse radish peroxidase (HRPO), trypsin (from bovine pancreas), and the diammonium salt of 2,2′-azinodi(3-ethylbenzothiazoline)sulfonic acid (ABTS) were bought from Boehringer, Mannheim, G.F.R. Neuraminidase (*Clostridium perfringens*), specific activity 5.4 units/mg of protein using

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[1] In this report, the terms (lipid) vesicle and liposome are used as synonyms.

[2] Abbreviations used: CF, carboxyfluorescein; PC, phosphatidylcholine (egg); PS, phosphatidylserine; DCP, dicetyl phosphate; HRPO, horse radish peroxidase; ABTS, 2,2′-azinodi(3-etfiylbenzothiazoline)-sulfonic acid; NAN-lactose, *N*-acetylneuraminosyl-D-lactose; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FITC-dextran, fluorescein isothiocyanate tagged dextran; Hanks' BSS, Hanks' basal salt solution; TLC, thin-layer chromatography (with silica gel).

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NAN-lactose, was obtained from Sigma. Trypsin inhibitor (from soybean) was purchased from Merck. Carboxyfluorescein was from Eastman Kodak and purified as described by Blumenthal et al. (1977). FITC–dextran (average M_r 3000) was purchased from Pharmacia.

Preparation of Phospholipid Vesicles. Small unilamellar vesicles of various lipid compositions and molar ratios (given in the appropriate figure or table), containing either 100 mM carboxyfluorescein (in doubly distilled water, adjusted to pH 7.4 with NaOH) or horse radish peroxidase (50 mg/mL) in 0.135 M NaCl–0.01 M Hepes, pH 7.4, were generated as described elsewhere (Van Renswoude et al., 1979; Hoekstra et al., 1978). Nonentrapped substance was removed by column gel chromatography (Sephadex G-100 for CF-containing vesicles and Sepharose CL-6B for those containing HRPO). Vesicle lipid concentrations were assessed by lipid phosphorus determination (Chen et al., 1956).

Cells. Hepatocytes and Zajdela cells were isolated as described previously (Hoekstra et al., 1978; Van Renswoude et al., 1979) and kept in calcium-free Hanks' balanced salt solution before use in the experiments.

Isolation of Plasma Membranes. The isolation of plasma membranes, derived from isolated hepatocytes and Zajdela cells, was carried out according to the method of Ray (1970). Purity of plasma membrane fractions (d = 1.16–1.18 g/cm^3) was assessed by enzyme determination of 5′-nucleotidase (Aronson & Touster, 1974), glucose-6-phosphatase (Swanson, 1955), and acid phosphatase (Gianetto & De Duve, 1955). Specific activities of 5′-nucleotidase in hepatocyte and Zajdela plasma membrane fractions were 10- and 15-fold enriched, respectively, when compared to cell homogenates. In plasma membrane fractions of neither cell type acid phosphatase activity could be detected, whereas only trace amounts of glucose-6-phosphatase were present.

Measurements of Vesicle Leakage Induced by Cells or Plasma Membranes. Continuous monitoring of leakage of CF from vesicles was carried out with a flow chart recorder coupled to a Perkin-Elmer MPF43 fluorescence spectrophotometer, set at excitation and emission wavelengths of 490 and 520 nm, respectively.

Incubations in which CF leakage from vesicles was continuously monitored were carried out in 1 cm light path quartz cuvettes fitted in a thermostated (37 °C) sample holder inside the fluorescence spectrophotometer. Calibration of the instrument was done as described elsewhere (Van Renswoude et al., 1979).

HRPO-containing vesicles were incubated in 0.135 M NaCl–0.010 M Hepes, pH 7.4, at 37 °C (thermostated shaking water bath), in polystyrene tubes. At suitable time intervals 0.5-mL samples were withdrawn from the incubation mixtures and centrifuged for 30 s in an Eppendorf table-top centrifuge. The clear supernatants were assayed for enzyme activity in the absence and presence of Triton X-100, essentially according to Steinman & Cohn (1972), with ABTS as an oxygen acceptor.

Other Methods. Protein was determined by the method of Lowry et al. (1951). Cell viability, monitored by exclusion of Trypan Blue (0.25%), was >95% throughout the experiments. Cell-associated radioactivity, after incubation with *N-methyl*-^{3}H-labeled egg PC containing vesicles, was determined as described before (Hoekstra et al., 1978; Van Renswoude et al., 1979).

Results and Discussion

Description of the Test System. Figure 1 shows the typical time course of the F_d/F_i ratio (in the following to be designated as Λ) recorded on small unilamellar vesicles, composed of PC, cholesterol, and PS (molar ratio 5:4:1), containing 100 mM carboxyfluorescein. Section A represents basal (or bulk) leakage of carboxyfluorescein from vesicles in the absence of cells. Curve B results from the addition (arrow) of either hepatocytes or Zajdela cells. The intersection C then represents the jump in fluorescence quantum yield, $\Delta F_d/F_i = \Delta\Lambda$, due to the presence of cells during a certain time and expresses the induced leak of carboxyfluorescein from vesicles. For all vesicle lipid compositions as well as for all cell densities and vesicle lipid concentrations employed, curve B levels off with time and after ~10 min attains the same slope as line A. The intersection C then equals $\Delta\Lambda_{max}$. If after any time (<10 min) of incubation the cells are removed from the incubation mixture by centrifugation, the values of F_d, F_i, and $\Delta\Lambda$ remain essentially unchanged, indicating that at any incubation time and at the vesicle concentration employed the great majority of dye molecules, either outside or inside (self-quenched) the vesicles, are not associated with the cells. Hence, the value of $\Delta\Lambda$ can be taken to represent the net increase in the number of dye molecules released from the vesicles into the medium as a result of the presence of cells. For incubation times between 3 and 10 min, plots of $(\Delta\Lambda)^{-1}$ vs. time^{-1} yield straight lines and thus allow precise determination of $\Delta\Lambda_{max}$. The inital rate of induced leakage (i.e., immediately after addition of cells to the system) is obtained by construction of the tangent to curve B at t = 0 (addition of cells) and will be denoted as $\Delta\Lambda_i$ (fractional leak per minute).

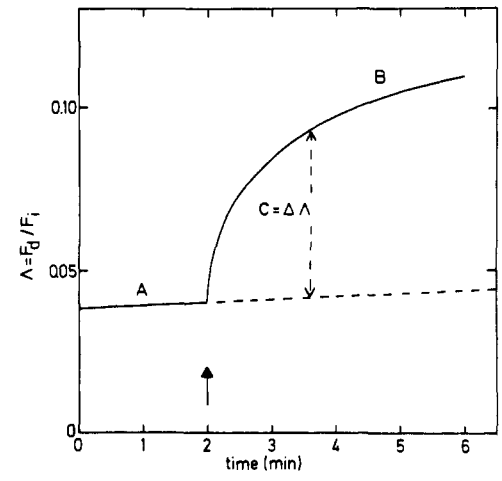

FIGURE 1: Schematic representation of a recorder fluorescence tracing of carboxyfluorescein leakage from vesicles. Section A: basal (bulk) leakage of dye from vesicles in the absence of cells. Curve B: development of the fluorescent signal upon addition of cells (arrow). Intersection C: increase of fluorescence quantum yield due to the presence of cells, as a function of time. The units along the Y axis were determined after addition of Triton X-100 [final concentration 1% (v/v)]. Symbols: F_d, (direct) fluorescence (arbitrary units) in the absence of detergent; F_i, (indirect) fluorescence (arbitrary units) in the presence of detergent. Λ, ratio of F_d to F_i. For further details see the text.

Influence of Cell Type and Cell Density. Hepatocytes and Zajdela cells give similar patterns of induced leakage, although it requires 6 (3) times as many Zajdela cells as hepatocytes to produce a certain $\Delta\Lambda_{max}$ ($\Delta\Lambda_i$).

For a fixed concentration of vesicles, the values of both $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ depend on the density of cells in the incubation mixture, as is shown in Figure 2. $\Delta\Lambda_{max}$ tends to level off with increasing cell density, indicating that only a fraction of the total vesicle-associated dye can be released from the total vesicle population in the incubation mixture. At the same fixed vesicle lipid concentration, however, the initial rate at which these dye molecules are released apparently increases linearly

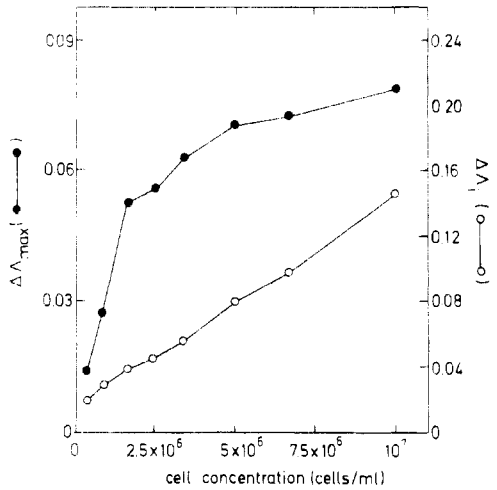

FIGURE 2: Cell-induced leakage of carboxyfluorescein from vesicles as a function of cell density. Different numbers of Zajdela cells in Hanks' BSS were added to a fixed final lipid concentration of 0.01 mM vesicles, consisting of PC, cholesterol, and PS (molar ratio 5:4:1), containing 100 mM CF. The final volume was 3 mL. For each cell density studied, development of fluorescence was recorded as described in the legend of Figure 1; the resulting $\Delta\Lambda$ values were plotted against the density of cells in the incubation mixture. For incubation conditions see Materials and Methods. Symbols: $\Delta\Lambda_i$ (○) represents initial rate of dye leakage; $\Delta\Lambda_{max}$ (●) represents the maximal value of $\Delta\Lambda$ (see the legend of Figure 1).

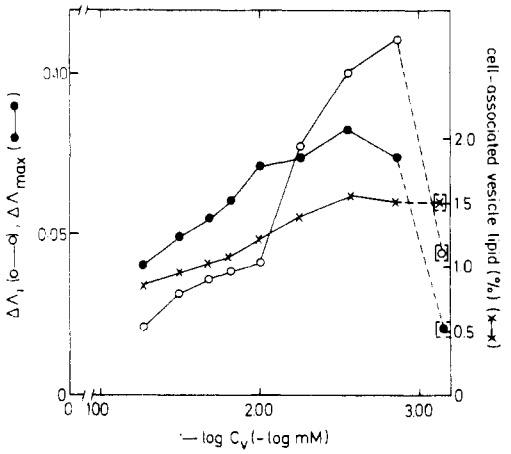

FIGURE 3: Cell-induced leakage of carboxyfluorescein from vesicles as a function of vesicle lipid concentration. 10^7 Zajdela cells in Hanks' BSS were added to different final concentrations of 100 mM carboxyfluorescein containing vesicles, consisting of *N-methyl*-^{3}H-labeled egg PC, cholesterol, and PS (molar ratio 5:4:1), in a final volume of 3 mL. For each vesicle lipid concentration used, fluorescence development was followed as described in the legend of Figure 1 and in the text; the resulting $\Delta\Lambda$ values [$\Delta\Lambda_i$ (○); $\Delta\Lambda_{max}$ (●); for symbols see the legend of Figure 2] were plotted against the vesicle lipid concentration. After $\Delta\Lambda_{max}$ had been attained (10 min), the incubation mixtures were transferred to polystyrene tubes. Non-cell-associated vesicles were removed by repeated centrifugations and washings (NaCl–Hepes buffer, pH 7.4). The washed cells were then assayed (see Materials and Methods) for cell-associated ^{3}H-labeled PC radioactivity (×). C_v: vesicle lipid concentration (mM).

with the number of cells in the incubation. For all cell densities tested, $\Delta\Lambda$ always was found to reach a maximum within 10 min of the incubation time.

Influence of Vesicle Lipid Concentration. At a fixed cell density, $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ also depend on the vesicle lipid concentration, as is depicted in Figure 3. $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ have maximal values at vesicle lipid concentrations of 0.0014 and 0.0028 mM, respectively. With increasing vesicle lipid concentration, $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ are found to decrease. At vesicle lipid concentrations below 0.0014 and 0.0028 mM, the values of the leakage parameters are consistently found to be submaximal (dashed lines) but although their determination is sufficiently reliable, considerable interexperimental variation is seen. The fraction[3] of vesicles which, after 10 min of incubation time, is found to be cell associated, based upon the amount of cell-associated vesicle-derived radiolabeled lecithin (Van Renswoude et al., 1979), depends on the vesicle lipid concentration in much the same way as $\Delta\Lambda_{max}$ (Figure 3, symbol × and filled circles, respectively). This similarity suggests that the amount of dye released via induced leakage is correlated to the number of vesicles bound by the cells. Quantitatively, the fraction of vesicles which have become cell associated is ~5 times smaller than the fraction of dye molecules that have been induced to escape from the interior of the vesicles. It has to be kept in mind, however, that in order to assess the amount of cell-associated vesicle lipid, bulk (i.e., non-cell-associated) vesicles have to be meticulously removed by several washings. This procedure may cause loosely cell associated vesicles to detach from the vesicle–cell complex, resulting in an underestimation of the amount of cell-associated vesicles.

In an attempt to further evaluate the similarity mentioned above, we have to ask whether the observed fluorescence increase ($\Delta\Lambda$) is caused by (1) a release of all entrapped dye from a small fraction of all vesicles or by (2) a release of a minor fraction of the total vesicle carboxyfluorescein from the majority of the vesicles. Since medium that has been in contact with cells for 10 min at 37 °C itself does not contain significant leakage-inducing capacity (see also below), we assume that for the initiation of the leakage induction a transient or lasting physical contact[4] between vesicles and cells is required. Our consistent observation that $\Delta\Lambda$ always attains a maximal value ($\Delta\Lambda_{max}$) as a function of incubation time (Figure 1; see also the text above) and that at this maximal value of $\Delta\Lambda$ always $F_d/F_i \ll 1$ argues against possibility 2. If, during a fast hit-and-run traffic of vesicles between medium and cell surface, the majority of vesicles would undergo leakage induction, we would expect $\Delta\Lambda$, as a function of time, to increase gradually until $F_d = F_i$. The quantitative similarity between carboxyfluorescein and a much larger molecule, horse radish peroxidase (see below), with respect to cell-induced release from vesicles, further disfavors the choice of possibility 2 to explain our results. We therefore assume that, most likely, a small fraction of all vesicles releases a major fraction of its contents into the medium upon interaction with cells.

The most obvious explanation for the observation that $\Delta\Lambda_{max}$ reaches to a maximal value (Figure 3) is given on the assumption of an average limited leakage-inducing capacity per cell. At the minimal vesicle concentration required to saturate the leakage-inducing capacity, a maximal fraction of vesicles releases its contents. Both at higher and at lower vesicle concentrations a smaller fraction of vesicles would then be

[3] Assuming an average of 4000 lipid molecules per vesicle (Hauser et al., 1973), we calculate from our radioactivity data that after 10 min of incubation time and at 0.01 mM vesicle lipid concentration on the average ~5500 vesicles must have become associated with one cell.

[4] The assumption implies that the process of leakage induction would be governed by vesicle–cell collision kinetics, at least initially. The vesicle–cell collision frequency should inter alia be directly proportional to the product of vesicle concentration and cell density. This criterion seems to be fulfilled by the roughly linear dependence of the initial rate of induced leakage ($\Delta\Lambda_i$) on cell density (Figure 2) and vesicle lipid concentration (Figure 3, after converting fractional leak into absolute leak).

involved in cell-induced leakage: i.e., $\Delta\Lambda_{max}$ would then be lower. At the lowest concentration, however, this fraction is obviously not related to total cell-associated lipid. At present we have no explanation for this discrepancy. Apparently, the initial rate of induced leakage reaches a maximum at a lower vesicle concentration than $\Delta\Lambda_{max}$. Although we have no clear explanation for this difference in optimal (for leak induction) concentration, it could be suggested that at vesicle lipid concentrations > 0.0014 mM the leak-inducing "sites" become progressively less accessible to other vesicles binding to the cell surface, while at the same time the degree of saturation of the remaining (fully exposed) leak-inducing sites increases, i.e., at least up to a vesicle lipid concentration of ~0.0028 mM.

On double-reciprocal plotting of the data of Figure 3, after converting the values of $\Delta\Lambda_{max}$ into absolute maximal leak values by multiplying for each vesicle lipid concentration the values of $\Delta\Lambda_{max}$ with the corresponding vesicle lipid concentration, we obtained a straight line (not shown). Extrapolation of this line to infinite vesicle lipid concentration yields the maximal number of vesicles (expressed as vesicle lipid) that could be taken subject to leakage induction by 3.33×10^6 Zajdela cells: 4.5 nmol.

The results described above reveal that at relatively high vesicle lipid concentrations (e.g., within the range of 0.1–1.0 mM, which has been studied most extensively in many investigations) leakage of vesicle-entrapped substances due to vesicle–cell interactions may go unnoticed, as the assay methods usually average the solute release from all vesicles present in an in vitro incubation with cells. For vesicles at the cell surface, however, the magnitude of the leakage induction phenomenon is not negligible [cf. Van Renswoude et al. (1979)].

In order to show that the phenomena are not merely associated with the use of carboxyfluorescein as an entrapped compound, we also encapsulated HRPO and FITC–dextran and measured parameters of leakage.

Influence of Species and Concentration of Entrapped Substances. If, in a 10-min incubation with cells, instead of carboxyfluorescein, horse radish peroxidase (M_r 32000) is used as a vesicle-entrapped marker (Figure 4), the latency of the enzyme inside vesicles appears to decrease with decreasing vesicle concentration in much the same way as the $\Delta\Lambda_{max}$ is found to increase when using carboxyfluorescein-containing vesicles (cf. Figure 3). Unfortunately, the sensitivity of the assay does not allow examination of the latency dependence at very low vesicle concentrations. With FITC-labeled dextran (average M_r 3000) as an encapsulated marker, a fluorescence jump similar to that observed with carboxyfluorescein is recorded upon addition of cells (results not shown).

Vesicles carrying 200 mM carboxyfluorescein yield ~3-fold higher $\Delta\Lambda_{max}$ values than vesicles containing 100 mM dye. Presumably the 200 mM vesicles are less stable than the 100 mM vesicles, this lower stability partially being expressed at the level of vesicle–cell surface interaction. The higher $\Delta\Lambda_{max}$ values obtained with 200 mM dye containing vesicles also practically rule out the possibility that an increase of Λ is caused by an increase of the internal aqueous volume of the vesicles since there is no significant difference in the degree of self-quenching of 100 or 200 mM dye inside vesicles [cf. Van Renswoude et al. (1979)].

From the results described so far we tentatively conclude that, in the presence of either isolated hepatocytes or Zajdela cells, small unilamellar vesicles composed of PC, cholesterol, and PS (molar ratio 5:4:1) show a rapid loss of integrity, expressed by the release of their contents into the medium;

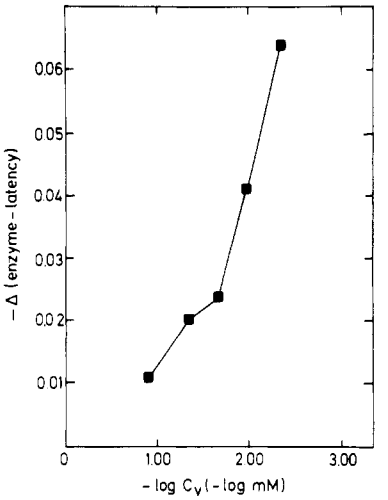

FIGURE 4: Cell-induced leakage of horse radish peroxidase from vesicles as a function of vesicle lipid concentration. 10^7 Zajdela cells in Hanks' BSS were added to different final lipid concentrations of vesicles, consisting of PC/cholesterol/PS (molar ratio 5:4:1) and containing horse radish peroxidase at a concentration of 50 mg/mL. The final volume was 3 mL. Leakage of the enzyme (expressed as $-\Delta$ latency; see below) was assessed after 10 min of incubation time. For a description of the assay of enzyme activity and incubation conditions, see Materials and Methods. Latency is defined as 1 − (activity in absence of detergent divided by activity in presence of detergent). The presence of detergent, Triton X-100, final concentration 0.1% (v/v), did not affect the enzyme activity. Within 10 min of incubation time cells did not release peroxidase activity into the medium. Values of $-\Delta$ (enzyme latency) were corrected for bulk leakage (i.e., in the absence of cells) of the enzyme from vesicles at different vesicle concentrations. C_V: vesicle lipid concentration (mM).

this phenomenon seems to be confined to a minor fraction of the total vesicle population. The magnitude of the leakage induction seems to be correlated to the vesicle fraction that is found to be cell associated and not to depend critically on the type of entrapped substance or its molecular weight.

Influence of Vesicle Lipid Composition. As there is a large variety in lipid composition of vesicles used by different investigators, we tested vesicles of various lipid compositions with regard to their integrity in the presence of cells. Under similar experimental conditions (with respect to incubation temperature, vesicle lipid concentration, and cell density) as used for the experiments with PC/cholesterol/PS (molar ratio 5:4:1), no significant fluorescence jumps ($\Delta\Lambda_{max}$ values <0.01) were observed with vesicles of the following lipid compositions: PC alone; PC/cholesterol, with cholesterol contents up to 30 mol %; PC/PS with a PS content up to 10 mol %; PC/cholesterol/PS comprising 10 mol % of PS and up to 20 mol % of cholesterol. Upon addition of cells, PC/cholesterol mixtures containing more than 30 mol % of cholesterol display a distinct fluorescence jump ($\Delta\Lambda_{max}$ > 0.06) which steeply increases with increasing mole fraction of cholesterol (Figure 5). This is a rather paradoxical result, since bulk leakage (i.e., in the absence of cells) at the same time is found to decrease from 0.24%/min at 30 mol % of cholesterol to 0.20%/min at 40 mol %. The decrease in the rate of bulk leakage was expected, since cholesterol is known to lower the passive permeability of lipid bilayers toward a variety of water-soluble substances, as a result of its condensing or rigidifying action on membranes that are in a liquid-crystalline state (Papahadjopoulos et al., 1973; Demel & De Kruijff, 1976). Several possibilities could be considered in attempting to explain the cell-mediated large increase in the permeability of vesicles with a high (>30 mol %) cholesterol content. For instance, an isothermal phase separation within the vesicle membrane might facilitate the interaction of the latter with cell surface proteins (see also

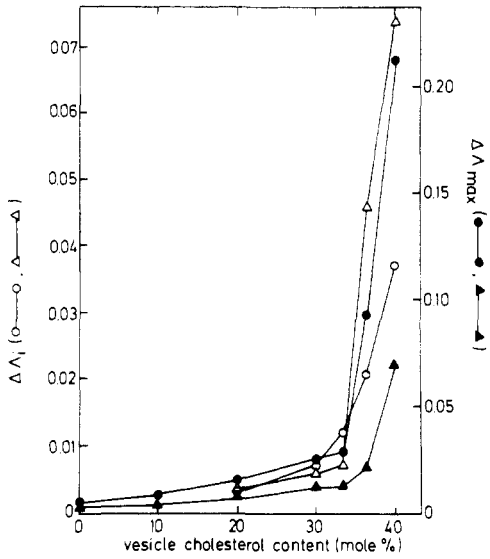

FIGURE 5: Influence of vesicle cholesterol content on cell-induced leakage. Vesicles consisting of PC and cholesterol in different molar ratios were incubated with either 2×10^6 hepatocytes (triangles) or 10^7 Zajdela cells (circles). Release of carboxyfluorescein from vesicles was monitored and evaluated as described in the legends of Figures 1 and 2. The initial rate of cell-induced leakage ($\Delta\Lambda_i$); open symbols; maximal leak ($\Delta\Lambda_{max}$), filled symbols. For further details, see Materials and Methods.

Concluding Remarks). Another possibility is that a net transfer of cholesterol between (initially) outer leaflets of vesicle and plasma membrane would cause a disproportion of the vesicle transbilayer cholesterol distribution, which might not be adequately compensated for by transmembrane movement of cholesterol (flip-flop) and lead to an increase of bilayer permeability or even rupture of the membrane. Further experimentation is required to gain insight into the role of cholesterol per se in the leak-induction process. An additional observation is that the presence of 10 mol % of net negatively charged lipid species (PS or DCP) in PC/cholesterol mixtures leads to a significant leakage induction ($\Delta\Lambda_{max} > 0.06$) even at cholesterol contents exceeding 20 mol %. Our results suggest, therefore, that the vesicle cholesterol plays an important role in the observed leak induction by cells, especially when the vesicle membrane simultaneously contains minor amounts of negatively charged lipid.

Preliminary results indicate that the phenomenon is not restricted to sonicated small unilamellar vesicles: reverse-phase evaporation vesicles (Szoka & Papahadjopoulos, 1978), made up from lipid compositions which for small unilamellar vesicles lead to significant $\Delta\Lambda_{max}$ values, show similar behavior in the presence of cells. All highly sonicated vesicle preparations used in this study were devoid of significant contamination with multilamellar liposomes, as judged by Sepharose CL-4B chromatography: on the average, more than 98% of lipid phosphorus elutes in the included volume. Centrifugation (100000*g*; 1 h) of sonicated preparations does not result in a change of the values of the leakage parameters. Thus, a possible small contamination of the preparations with multilamellar vesicles cannot per se be responsible for the occurrence of the induced leakage.

Tentative Characterization of Leak-Inducing Activity, with Isolated Plasma Membranes. The time-dependent kinetics of dye release observed with intact cells are similar to those seen with isolated plasma membranes (cf. Figure 1): i.e., a rapid fluorescence jump which levels off to a plateau value within ~10 min. Again, the results suggest that only part of the total vesicle carboxyfluorescein is induced to be released. As already discussed for whole cells (see above), the total amount of released dye presumably originates from a small fraction of all vesicles within the incubation mixture. The likelihood of the existence of such a small fraction is substantiated by the finding that sequential additions of small aliquots of plasma membranes lead to the same final $\Delta\Lambda_{max}$ value as is found when the sum of these aliquots is added at once (data not shown). The leak-induction capacity of a fixed amount of plasma membranes furthermore appears to be limited: upon sequential additions of aliquots of dye-containing vesicles, the final $\Delta\Lambda_{max}$ value is roughly equal to the corresponding value resulting from addition of all aliquots at once. Leakage induction is found to depend on vesicle lipid concentration in the same way as is observed for whole cells. For a given vesicle concentration, however, the value of $\Delta\Lambda_{max}$ generally is slightly higher than the one observed with cells. It was assumed (Ray, 1970) that plasma membrane protein accounts for ~3% of total cellular protein. We used this value to calculate the amount of plasma membrane equivalent to a certain number of cells.

Addition of dye-containing vesicles to plasma membranes which were premixed (during 5 min at 37 °C) with increasing amounts of empty vesicles results in a progressive and almost complete abolishment (at 2 mM empty vesicle lipid concentration) of leak-inducing capacity. Similar results are obtained, if, following a preincubation with empty vesicles, non plasma membrane associated empty vesicles are removed by centrifugation (100000*g*; 30 min) prior to incubation of the plasma membranes with dye-containing vesicles. Straightforwardly, these findings suggest that empty vesicles compete with dye-containing vesicles for the "leakage-inducing sites" at the cell surface and that these "sites" only become saturated at relatively high overall vesicle lipid concentration.

Influence of Various Treatments on Leak Induction. No significant loss of leakage-inducing capacity of plasma membranes is seen when they are preheated to 100 °C (boiling water bath) during 30 min. Hence, we consider it very unlikely that plasma membrane intrinsic enzymic activity (e.g., phospholipases) accounts for the induced leakage. Treatment of plasma membranes with trypsin (15 μg of enzyme/90 μg of protein weight of plasma membranes; incubation volume 3 mL; 10 min; 37 °C; reaction stopped by addition of 35 μg of trypsin inhibitor) decreased $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ 4- and 7-fold, respectively. Upon neuraminidase treatment (9 μg of enzyme/90 μg of protein weight of plasma membranes; incubation volume 3 mL; 30 min; 37 °C), $\Delta\Lambda_i$ and $\Delta\Lambda_{max}$ were reduced 3- and 4-fold, respectively. When either enzyme was added to plasma membranes immediately before addition of vesicles, or when vesicles were premixed with either enzyme, the values of the leakage parameters were unaltered. With intact cells neuraminidase treatment had a similar effect. From these results we conclude that induced leakage is mediated through one or more cell coat or intrinsic plasma membrane proteins, most likely glycoprotein(s).

Release of Leak-Inducing Activity from Cells and Isolated Plasma Membranes. Upon prolonged (up to 2 h) incubation of cells or plasma membranes, at 37 °C in Hanks' basal salt solution, we detected a gradual appearance of leak-inducing activity in the medium itself (assessed after removal of cells or plasma membranes by centrifugation), at the cost of the cell or plasma membrane associated activity (assessed after pelleting and resuspending the cells or membranes). After 1 h cells and plasma membranes gave 80 and 65% of their inititial (zero-time) $\Delta\Lambda_{max}$ values, respectively. With PC/cholesterol/PS (5:4:1) vesicles the medium itself then displayed

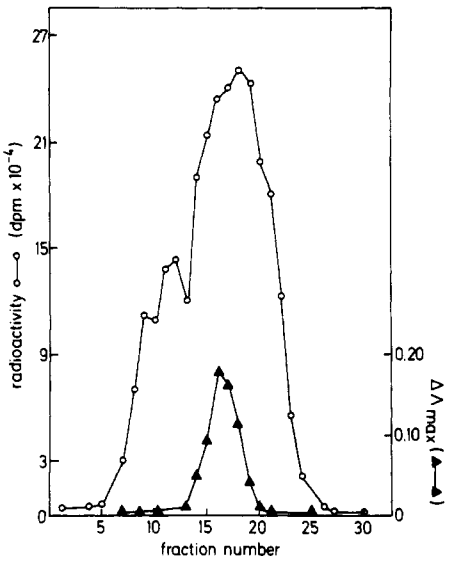

FIGURE 6: Gel chromatographic elution profile of leakage-inducing activity. Hepatocyte plasma membranes (suspended in NaCl–Hepes buffer, pH 7.4) were put through four freezing and thawing cycles and subsequently centrifuged at 100000*g* for 30 min (Spinco L2-65 B, Ti50 rotor). The supernatant was adjusted to pH 8.0 by addition of 0.15 M citrate. 500 μCi of carrier-free ^{125}I (The Radiochemical Center, Amersham) was then added to the supernatant, in order to label peptides and proteins according to Bocci (1969). After a reaction time of 30 min, the mixture was dialyzed overnight, against three changes of a 300-fold excess of NaCl–Hepes buffer, pH 7.4 to remove nonbound ^{125}I label and labeled materials with a M_r <10000. Subsequently, the dialyzed supernatant was chromatographed on Sepharose CL-6B (column 1.5 × 50 cm, equilibrated and eluted with NaCl–Hepes buffer pH 7.4). 3-mL fractions were collected; radioactivity (○) and leak-inducing activity [$\Delta\Lambda_{max}$ (▲)] were determined for each fraction. For measurement of leak induction, 0.01 mM PC–cholesterol–PS (5:4:1) vesicles, containing 100 mM carboxyfluorescein, were used.

low leakage-inducing activity ($\Delta\Lambda_{max}$ < 0.02) with kinetic characteristics (cf. Figure 1) similar to those of cell and plasma membrane induced leakage. Repeated freezing and thawing of isolated plasma membranes led to loss of almost all membrane-associated activity, while at the same time the medium gave rise to $\Delta\Lambda_{max}$ values > 0.06 (assessed after removal of plasma membranes by centrifugation). Such medium was found to contain no detectable protein by using the protein determination method according to Lowry et al. (1951) and was therefore incubated with carrier-free ^{125}I according to Bocci (1969). After dialysis overnight, the medium was subsequently chromatographed on Sepharose CL-6B (Figure 6). While ^{125}I-labeled material eluted throughout the column volume (fractions 5–26), leak-inducing activity was found only in fractions 14–19, indicating that, at that stage, it was no longer associated with membraneous structures, which would elute in the void volume. The leak-inducing activity in fractions 14–19 was almost completely abolished upon treatment with either trypsin or neuraminidase, according to the protocol described above. From the results obtained so far we tentatively conclude that most likely the leakage-inducing factor consists of one or more glycoproteins and that it is rather loosely associated with the cell surface.

Concluding Remarks. Attempts to give a general explanation for our observations meet with two important questions: (1) Which factor does, ultimately, initiate leakage? (2) Is any vesicle within the total vesicle population in principle prone to induced leakage or is this phenomenon restricted to a preexisting subpopulation of vesicles? Possibly, leakage starts upon interaction of cell coat protein(s) with the vesicle bilayer. There is a great deal of evidence that vesicles of a variety of lipid compositions readily associate with exogenous proteins [see the review by Tyrrell et al. (1976); Kimelberg, 1976; Zborowski et al., 1977; Hoekstra & Scherphof, 1979] and that such association may lead to enhanced release of solutes from the vesicle interior (Tyrrell et al., 1976; Zborowski et al., 1977). In addition, it has been shown, that cell surface protein from mouse spleen cells (Dunnick et al., 1976) and from erythrocytes (Bouma et al., 1977) is partly transferable to (bulk) vesicles during in vitro vesicle–cell incubations. In this report we demonstrate that the fraction of total vesicle carboxyfluorescein which is released is correlated to the number of vesicles found cell associated and that vesicles are subject to cell-induced leakage only when they contain more than 30 mol % of cholesterol or more than 20 mol % of cholesterol plus at least 10 mol % of negatively charged lipid. Our results with isolated plasma membranes indicate a direct involvement of cell surface protein(s) in the process of leak induction. We consider it conceivable that an isothermal phase separation within the vesicle membrane ultimately leads to the observed leakage. Such a phase separation could either be induced in situ upon interaction with cell surface proteins [cf. Papahadjopoulos (1977)] or preexist (i.e., present before the vesicle meets the cell surface) by virtue of the vesicle lipid composition [cf. Gebhardt et al. (1977)] or as a result of the influence of nonspecific interactive forces between the vesicle bilayer and cell surface [cf. Parsegian et al. (1979) and Sugâr (1979)]. A preexisting phase separation has been suggested to facilitate the interaction of the vesicle bilayer with high-density lipoproteins (Scherphof et al., 1979), phospholipase A_2 (Wilschut et al., 1978), and cell surface proteins (Szoka et al., 1979).

At present we cannot exclude the possibility of a structural and compositional heterogeneity within a vesicle population made up from a mixture of (phospho)lipids. It can, for example, be inferred from data provided by De Kruijff et al. (1976), Forge et al. (1978), and Carnie et al. (1979) that a vesicle population composed of PC and more than 30 mol % of cholesterol must be inhomogeneous, either with respect to the transbilayer distribution of lipids per individual vesicle or with respect to the molar ratio of the composing lipids per individual vesicle. Hence, it cannot a priori be ruled out that leak induction is limited to a preexisting structural subpopulation of vesicles.

We nevertheless consider the results of this study important both from a phenomenological and a methodological point of view, for two main reasons. First, cell-induced leakage may hamper the identification of different classes of vesicle–cell interaction sites (Blumenthal et al., 1977; Van Renswoude et al., 1979; Hoekstra et al., 1981). Vesicle–cell transfer of carboxyfluorescein does not seem to be affected by the occurrence of induced leakage: vesicles composed of PC/cholesterol/PS in a molar ratio of 5:4:1 or 7:2:1 (the latter composition not being subject to leak induction) display identical initial transfer rates. Also, pretreatment of hepatocytes with neuraminidase does not result in a change in transfer rate, whereas the amount of cell-associated, highly self-quenched vesicle carboxyfluorescein is found to be increased (D. Hoekstra, unpublished data). Second, use of the described on-line test system may contribute to the exploration of some of the characteristics of the complex interaction between vesicles and cell surface.

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Affinity Labeling of *Escherichia coli* Ribosomes with a Covalently Binding Derivative of the Antibiotic Pleuromutilin†

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ABSTRACT: Reaction of an alkylating pleuromutilin derivative with *E. coli* ribosomes led to the binding of the compound to both proteins and RNA. If ribosomes of the *E. coli* strain MRE600 were used, mainly S18 and L2 became labeled. Ribosomes from *E. coli* D10 bound the reagent to S18 and frequently to L27 instead of L2. Possibly a slight difference in the structure of these ribosomes exposes different, although closely neighboring, L proteins to the reagent. The simultaneous labeling of L and S proteins seems to reflect the presence of two binding sites for the antibiotic and indicates that the binding sites are located at the interphase region between large and small ribosomal subunits. Analysis of the RNA showed that the affinity label is mainly attached to the 23S species. These data are in good agreement with the known effects of pleuromutilin derivatives on ribosomal functions.

Tiamulin, a derivative of the antibiotic pleuromutilin, has been shown to inhibit *E. coli* ribosomes by blocking their assembly into functional initiation complexes (Hodgin & Högenauer, 1974; Dornhelm & Högenauer, 1978). The antibiotic binds very strongly, yet reversibly, to 70S ribosomes. The presence of two specific binding sites has been shown (Högenauer & Ruf, 1981), and at least one overlaps with that of the antibiotics chloramphenicol and puromycin. The binding sites for other protein synthesis inhibitors, like lincomycin, erythromycin, and thiostrepton, do not extend into the pleuromutilin-specific binding region (Högenauer, 1975; G. Högenauer, unpublished experiments). The fact that the nucleotides CpA and CpCpA reduce the binding of the pleuromutilin derivative is taken as evidence that the binding sites of the drug are located at or near the region for the attachment of the 3′-terminus of tRNA.

A further characterization of the binding sites for the antibiotic by identification of the proteins at this particular ribosomal location seemed desirable. This was achieved by the

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