Effect of Entrapped Markers on the *in situ* Jejunal Uptake from Liposomal Systems

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Abstract: The effect of entrapped PEG-4000, inulin, and insulin on the *in situ* uptake from the rat jejunum of liposomes was determined. Each of these markers greatly reduced or completely eliminated jejunal uptake unless the liposomes were repeatedly "washed" to remove marker from the external bilayer surface. Addition of free marker to a suspension of empty liposomes resulted in complete loss of liposomal uptake. It appears that "inert" markers can profoundly affect the rate and extent of liposomal uptake from the rat jejunum and the preparative technique employed can greatly affect the behavior of the system.

Recent advances in medical research have offered some indication that liposomes may be able to enhance the oral bioavailability of certain drugs by protecting labile compounds from degradation or by enhancing the uptake of poorly absorbed compounds (1, 2). The broad spectrum of liposomal properties, together with the ability of many compounds to affect these properties renders the investigation of liposomes as an oral dosage form a complex and difficult task.

We have recently determined the *in situ* uptake from the rat jejunum of liposomes prepared from various saturated phosphatidylcholines in the presence or absence of cholesterol. Liposomes prepared from distearoylphosphatidylcholine, with or without cholesterol, were taken up at the fastest rate (apparent first-order uptake of intact liposomes with a half-life of approximately 20 minutes). The lipid components of liposomes could not be found in compartments where liposomes would be expected to accumulate, i.e., liver, spleen, lymph nodes, or thoracic lymphatic duct. When either non-absorbable markers or absorbable markers were entrapped within the liposome, all of the liposomes and markers that were taken up still remained associated with the jejunum and the liposomes remained intact (3).

Whereas there is compelling evidence, based on *in vitro* (4), *in situ* (3), and *in vivo* (1) data, that intact liposomes and their contents are readily taken up from the gastrointestinal tract, other studies have reported failures of entrapped markers (PEG-4000 or inulin) to be taken up from liposomal suspensions (5, 6). In our attempt to isolate and systematically study the important parameters which have been reported to affect the uptake and disposition of drugs entrapped in liposomes, we report on the dramatic effects of "inert" markers on the uptake of liposomes and their contents from the rat jejunum.

Materials and Methods

Materials

Distearoylphosphatidylcholine (DSPC), and cholesterol (CHOL) were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol was purified by recrystallizing three times from ethanol solution and DSPC was used as supplied, and stored below 0°C. Thin layer chromatography of the lipids revealed a single spot. Research grade crystalline porcine insulin with an activity of 28.5 units/mg, containing 0.6 % zinc was donated by Lilly Research Laboratories, (Indianapolis, IN). All other chemicals were reagent grade.

The following radiolabelled lipid and aqueous markers were purchased from Amersham (Arlington Heights, IL): *L*-α-phosphatidylcholine di[1-^{14}C]stearoyl (^{14}C-DSPC), [^{3}H]PEG-4000 and [^{3}H]inulin.

Preparation of Liposomes

Since the uptake rate constants of DSPC-containing liposomes are greater than those of the other systems tested (3), and since Rowland and Woodley (7) demonstrated that shorter chain length saturated and unsaturated phospholipids, even in combination with cholesterol, are unstable in the gastrointestinal environment, all studies on the effects of entrapped markers were limited to DSPC or DSPC/CHOL liposomes.

DSPC (32 μmole), with or without cholesterol (16 μmole), was dried from a chloroform solution at room temperature under nitrogen to form a thin film on the sides of a 20 ml glass vial. Residual chloroform was removed by placing the vial in a vacuum for at least 12 hours. The isotonic phosphate-buffered saline (PBS) used in these studies consisted of 67 mM sodium phosphate adjusted to pH 6.0 with sodium hydroxide.

Liposomes were formed by adding 1.0 ml PBS to the dried lipid vials followed by incubation for 30 minutes in a water bath at a temperature of 60°C. Compounds which were to be entrapped were dissolved in PBS before being added to the vials containing the dried lipids. Dispersion of the lipids was aided by agitation in a vortex mixer for 3–5 minutes during the incubation step. The vials were then sonicated for 30 seconds in a bath-type sonicator (Branson Cleaning Equipment Co., Shelton, CN) at the same temperature as the incubation, and allowed to equilibrate at room temperature for at least 4 hours.

Free and entrapped aqueous markers were separated by passing the liposome suspension through a 1 × 45 cm column of Sephadex G-50 (coarse) (Pharmacia Inc., Piscataway, NJ). Liposomes eluted (with fresh buffer) immediately after the void volume and the first 3 ml of this fraction were collected and further "washed" by centrifugation 3 times for 8 minutes at 8000 rpm. Each time the supernatant was removed and the liposome pellet resuspended in 2–4 ml of buffer. Liposomes not containing entrapped markers, i.e., "empty" liposomes were also subjected to this procedure. In another set of experiments, "empty" liposomes were incubated with various concentrations of markers for 4 hours at 37°C.

Quasi-Elastic Light Scattering

The liposomes were examined by quasi-elastic light scattering (QELS) in a Langley-Ford LSA-2 spectrometer (Langley-Ford Instruments, Amhurst, MS) containing a 5 mW helium-neon laser light source (λ_o = 632.8 nm) and a fixed scattering

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angle of 90°. Samples were examined at 37°C using a sample time of 2.9×10^{-5} seconds. Calculations of particle size diameter were performed by a Model 1096 CM64 autocorrelator using 64 channels.

An aliquot of the liposomal preparation was incubated at room temperature for 24 hours with either PEG-4000 (0.9 mg/ml), inulin (0.09 mg/ml), or PBS (control). The suspensions were diluted 1 : 15 with PBS immediately before QELS measurements were taken. Solutions of PEG or inulin in PBS at these concentrations showed no interference with the autocorrelation function.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) of liposomal suspensions was performed on a Perkin-Elmer DSC-2C calorimeter equipped with a Thermal Analysis Data Station (TADS) (Perkin-Elmer Corp., Norwalk, CN) for data analysis. The instrument was calibrated using an indium standard.

DSPC liposomes were prepared as described previously with the omission of the sonication step. This was done in order to obtain greater precision in the analysis of the gel to liquid crystalline phase transition of the lipids (8, 9). Liposomes containing entrapped PEG-4000 (10.0 mg/ml), inulin (1.0 mg/ml), insulin (0.5 mg/ml), or sucrose (10.0 mg/ml) were not separated from the unentrapped solutes. Thermograms of these solutes dissolved in isotonic phosphate buffered saline, pH 6.0, exhibited no deviations from the baseline (buffer alone).

Thermograms of liposomal suspensions were obtained from 15 μl aliquots (30 μmole lipid/ml) in sealed aluminum sample pans. An equal volume of buffer served as the reference. Heating of both sample and reference pans was performed over a temperature range of 20°C below to 20°C above the lipid phase transition temperature at a rate of 5°C/minute. The transition temperature, T_c, was obtained by extrapolation of the leading edge of the endothermic peak, as calculated by the TADS microcomputer.

In situ Rat Intestinal Uptake

A modified *in situ* Doluisio Method (10) was employed in these studies. Male Sprague-Dawley rats, fasted overnight, weighing between 250–300 gm were anesthetized with 20 % ethylcarbamate (Sigma Chemical COP., St. Louis, MO) solution by two 1 ml intraperitoneal injections 15 minutes apart. A midline abdominal incision was made from which the small intestine could be exposed. The proximal end of the jejunum was first cannulated with L-shaped tubing (4 mm o.d.; 2 mm i.d.). A second cannula was then placed 22 cm distal to the first and the segment was rinsed with 0.9 % sodium chloride at 37°C until clear. The segment was arranged in S-shaped curves free of constrictions, covered with gauze, and kept moist with 0.9 % sodium chloride solution at 37°C. The cannulas were attached to the barrels of plastic, disposable 5-ml syringes (Becton, Dickenson, and Co., Rutherford, NJ) which were, in turn, connected to a 2 directional piston air pump (Fluid Meter Inc., Oyster Bay, NY).

The jejunal segment was given a final rinse of PBS before the start of the experiment. Two ml liposome suspension or PBS solution were introduced into the lumen of the segment and oscillated back and forth twice before taking the "zero-time" sample. The fluid was pumped at 0.075 ml/sec, and the direction was reversed approximately every 10 seconds so that not more than 0.75 ml were displaced from the segment at any given time. Sample volumes of 25 μl were taken from the distal cannula every 5 minutes for 30 minutes.

Results and Discussion

In our previously published studies on the fate of liposomal lipids and entrapped compounds following their *in situ* uptake from the jejunum, we used PEG-4000 and inulin as non-absorbable markers and glucose as an absorbable marker (3). We noticed that if we did not take great caution in "washing" the liposomes containing entrapped PEG-4000 (and to a lesser extent, inulin) jejunal uptake was dramatically reduced. In fact, if no attempt was made to remove unentrapped PEG-4000, uptake of the liposomes (and PEG) was virtually eliminated.

The results of a more systematic study of the effects of various concentrations of PEG-4000 on liposomal uptake are shown in Table I. The values of the uptake rate constants, (k_u) shown in the table are determined from analysis of ^{14}C-DSPC as previously described (3). In a number of experiments, we used ^{3}H-PEG-4000 as a marker and found that there was a parallel decrease of both DSPC and the PEG entrapped marker (Fig. 1). Since PEG alone is not absorbed or adsorbed (Fig. 1), these results indicate that the uptake process involves intact liposomes, with no significant leakage of entrapped PEG.

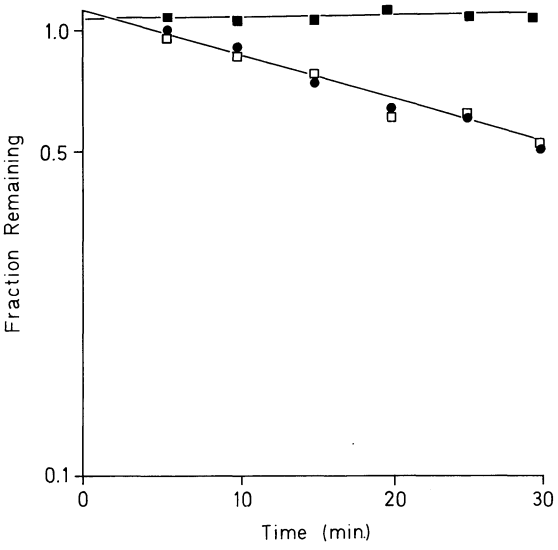

Fig. 1 The *in situ* uptake of DSPC-CHOL liposomes from the rat jejunum with entrapped or externally added PEG-4000. Key: ^{3}H-PEG-4000 (●) entrapped in ^{14}C-DSPC-CHOL (□) liposomes; ^{14}C-DSPC-CHOL (■) liposomes incubated with 13 μg/ml PEG-4000; 300 μg/ml ^{3}H-PEG-4000 (■) in the absence of liposomes. The latter two conditions gave identical results.

The liposomes containing PEG that were separated from free solute by column chromatography but not "washed" by further centrifugation all showed a significant reduction in the rate of uptake. Very careful "washing", as described in the Experimental Section, was needed for the uptake to approach control values. The importance of removing most of the externally adsorbed PEG is apparent when as little as 13 μg/ml of PEG added externally eliminated uptake completely (Table I and Fig. 1).

Table I. Effect of PEG-4000 and inulin on liposomal uptake from the rat jejunum *in situ* at 37°C

Experimental condition	Concentration[1] (μg/ml)	$k_u \times 10^4$/sec	Half-life (min)
Control	0	5.61 (0.42)[2]	21
Control incubated	13	0	>500
with PEG-4000	370	0	>500
Entrapped PEG-4000	7.1	0.92	130
(not washed)	23	0.52	220
	45	1.05	110
	132	0	>500
	138	0	>500
Entrapped PEG-4000	5.8	3.67	31
(washed)	11	2.68	43
Entrapped inulin	0.08	1.29	90
(washed)	0.11	1.97	59
	3.75	4.90	24
	4.93	1.53	76
	8.95	5.23	22

[1] Concentrations represent initial values of PEG-4000 or inulin used in the preparation of liposomes. Entrapment, as determined by the use of tritiated markers, was found to be between 3–5 % in all cases. For the control experiments, concentrations represent those of externally added PEG-4000.

[2] Represents the mean (±S. D.) for six experiments.

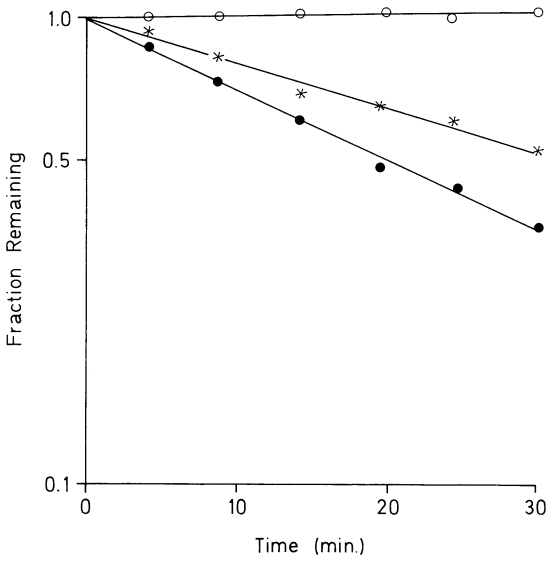

Fig. 2 The *in situ* uptake of DSPC-CHOL liposomes from the rat jejunum with entrapped or externally added insulin. Key: 240 μg/ml externally added insulin (○); 13 μg/ml entrapped insulin (✳); 13 μg/ml entrapped insulin, "washed" (●).

Recent reports in the literature (11, 12) have shown that polyethylene glycols interact with lecithin vesicles resulting in fusion and the formation of structural defects. Although these effects were observed at PEG concentrations much greater than those used in our studies, it is not known how small amounts of PEG externally bound to the bilayer would affect the various parameters involved in the uptake process.

As with PEG-4000, inulin is thought to adsorb to liposomal bilayers as has been reported with other dextrans and polysaccharides (13, 14). The effect to inulin on the uptake of washed liposomes containing various concentrations of entrapped inulin is also shown in Table I. Altough the results are not as clear cut as for the PEG-entrapped liposomes, reduction in uptake is observed in some instances. The effect on the rate constant appears to be independent of inulin concentration.

The uptake of washed liposomes containing entrapped inulin was monitored with ^{14}C-DSPC and ^{3}H-inulin. The parallel decrease of both markers again indicates that the uptake process involves intact liposomes. The extent of leakage of entrapped inulin was determined by removal of the liposomes after the experiment, centrifugation, and analysis of the supernatant and liposomal pellet. Over the course of the 30 minute experiment, less than 5 % inulin leakage was observed.

Preliminary experiments with insulin show that the uptake of liposomes was affected in a manner similar to that of PEG-4000. As shown in Fig. 2, externally added insulin (240 μg/ml) completely blocked liposomal uptake. Entrapped insulin (13 μg/ml) resulted in moderate uptake, whereas a washed suspension of entrapped insulin was taken up at a rate equal to that of the control.

Although the concentrations of PEG-4000 used in these studies were much lower than those reported in the literature to cause liposomal flocculation and structural changes, we determined if PEG-4000 or inulin affected the average diameter of the liposomes or the degree of polydispersity. Table II

Table II. Average liposome diameter and degree of polydispersity of liposomal suspensions in the presence of PEG-4000; inulin, or buffer as determined by quasi-elastic light scattering.

Additive	Diameter (μm) (S. D.)	Degree of Polydispersity (S. D.)
None	0.1562 (.0065)	0.4043 (.0135)
PEG-4000	0.1506 (.0056)	0.3846 (.0167)
Inulin	0.1584 (.0047)	0.3709 (.0079)

clearly shows that the markers, even at concentrations much higher than those used in the uptake studies, resulted in no detectable flocculation.

Differential scanning calorimetry (DSC) provides a relatively rapid and simple method of investigating the effect of the markers on the physico-chemical properties of liposomes, specifically the thermotropic behavior of the lipid bilayer. The thermograms of DSPC liposomes prepared in the presence of PEG-4000 (10 mg/ml), sucrose (10 mg/ml), inulin (1 mg/ml), or insulin (0.5 mg/ml) are essentially the same as those of the control liposomes. Neither the pre-transition or main transition peaks were significantly affected (<1°C difference) nor was the enthalpy of transition. These data indicate that the various markers used in this study do not significantly penetrate or disrupt the lipid bilayer. It should be pointed out that DSPC rather than DSPC/CHOL liposomes were used in this phase of the study since cholesterol obliterates the transition peaks at mole ratios higher than 0.33 (15).

Conclusions

Whereas ions such as sodium, chloride, and phosphate, and small hydrophilic molecules (glucose) have no apparent affect on liposomal uptake, larger hydrophilic compounds (PEG-

4000, inulin, and insulin) dramatically decrease the uptake, especially when extreme care is not taken to remove these compounds from the external environment. The latter compounds, although they do not form an integral part of the bilayer, apparently adsorb to some extent.

It is not clear at this point whether the adsorption of these markers blocks adsorption of the liposomes to the gut wall or blocks penetration into deeper layers. The important point is that "inert" markers can profoundly affect the uptake of liposomes as determined by this *in situ* procedure. This study also raises questions as to how materials normally found in the gastrointestinal environment, e.g., food, protein, etc., affects liposomal uptake. These observations may also offer some explanation for the divergent results reported in the literature on *in vivo* absorption of liposomally entrapped drugs.

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Relationship of Octanol/Water Partition Coefficient and Molecular Weight to Cellular Permeability and Partitioning in S49 Lymphoma Cells

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Abstract: We have used modified standard methods and derived new formulae to quantitate cell permeability (P), cell/media partitioning (λ), and intracellular sequestration or binding rate constants (m) for cultured S49 murine lymphoma cells in suspension. Using 15 standard compounds and anticancer drugs, we found quantitative relationships among log P, log P_o (octanol/pH 7.4 buffer partition coefficient), and molecular weight (MW) such that $\log P = -4.5 + 0.56\log(P_o(MW)^{-1/2})$. A good correlation among P, λ, and MW was also determined with $\lambda = 0.67 + 5890\,gm^{1/2}\,cm^{-1}\,sec\,(P\,(MW)^{1/2})$. These studies show that there is a strong partitioning (λ) dependence to molecular weight and permeability that can be predicted even for known carrier-transported and biotransformable compounds. Furthermore, results of this study show that the slope of the plot of permeability and lipophilicity is not necessarily unity as has been postulated from the results of other studies.

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The therapeutic efficacy of chemotherapy for solid tumors generally has been disappointing; this ineffectiveness is a reflection of the fact that frequently drugs cannot be delivered to cells at high enough levels to achieve a cytotoxic effect. We have modeled the effects of various physical factors on drug delivery to the extracellular environment around tumor cells that are various distances from tumor capillaries (1). We could not extend these observations to intracellular drug levels because, despite their importance, data on the permeability of chemotherapeutic agents and the rates of binding and sequestration in normal and transformed eukaryotic cells were not available. Therefore, we have measured the permeability and intracellular compartmental distribution and sequestration or binding of compounds with a wide range of molecular weights and lipophilicities, and have derived equations that correlate these data with the partitioning of a drug and the lipophilic nature of the cell membrane and cytoplasm. Measurements were made in the S49 murine lymphoma cell line using a modification of standard methods (2).

Materials and Methods

Radioisotopes and Chemicals

^{14}C-Labelled D-glucose, 2-deoxy-D-glucose, inulin, putrescine, and urea, and ^{3}H-labelled inulin and mannitol were