International Journal of Pharmaceutics, 7 (1980) 89–98

THE TOXICITY OF LIPOSOMAL CHLORAMPHENICOL FOR *TETRAHYMENA PYRIFORMIS*

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(Received September 1st, 1980)
(Accepted September 29th, 1980)

SUMMARY

The toxicity of suspensions of anionic and neutral liposomes containing chloramphenicol was compared with the toxicity of free drug solution for the protozoan, *Tetrahymena pyriformis*. The liposomal form of the drug was found to be markedly more toxic, anionic and neutral liposomes being equally effective in killing the cells. Cellular uptake and inhibition of uptake by cytochalasin B of diformazan liposomes and polystyrene beads was found to be similar. Uptake of [^{3}H]cholesterol-labelled liposomes was decreased in old cells and absent in killed cells. Quantitative aspects of the internalization of CAP in liposome form are discussed.

INTRODUCTION

Phospholipid vesicles have been used as carrier systems for drugs in vivo (Gregoriadis and Ryman, 1971; Rahman et al., 1974) and in vitro (Papahadjopoulos et al., 1973; Pagano and Huang, 1975). Some in vivo studies indicate that administration of certain cytotoxic agents entrapped in liposomes can enhance the activity of the drug 200 (Black et al., 1977) to 700 (Alving et al., 1978) times and in the case of entrapped antiviral antibody a 10^4 times enhancement of antibody activity has been reported (Magee and Miller, 1972). Such enhancement of drug activity may be due at least in part to the ability of the liposome formulation to alter the pharmacokinetics of the entrapped drug (Kimelberg and Mayhew, 1978).

A great many of the in vitro studies have concentrated on the mechanism of vesicle uptake by cells, great emphasis being placed on discriminating between fusion and endocytosis as the underlying phenomenon (Batzri and Korn, 1975), and on the role of vesicle characteristics, e.g. fluidity, size and charge in uptake (Tyrrell et al., 1976). Cells in culture have also frequently been used as models in the investigation of liposome as drug carriers in enzyme replacement therapy (Magee et al., 1974) and in cancer chemotherapy (Poste and Papahadjopoulos, 1976).

It would seem that an important analogy can be drawn between, on the one hand, the role of the liposome as a dosage form in the pharmacokinetics of the entrapped drug in the whole organism and on the other, the role of such a carrier in the micro- or cellular pharmacokinetics of the drug, cells in culture providing a useful model for the latter. At both the macro- and micropharmacokinetic level, the overt response to the drug may be significantly modified.

We compare here the cytotoxicity of free and liposomal chloramphenicol (CAP) on the protozoan *Tetrahymena pyriformis* which can ingest large quantities of particulate material by endocytosis through a specialized oral apparatus, the phagostome (Nilsson, 1976). The results indicate that this eukaryotic cell can by virtue of its endocytic activity internalize liposomes to an extent that their accumulated contents are cytotoxic although the extracellular concentration of drug, if in free form, is well below that toxic for this organism.

MATERIALS AND METHODS

Materials

Chloramphenicol, crystalline; DL-α-phosphatidyl choline, dipolmitoyl, crystalline; cholesterol, dicetyl phosphate; nitro blue tetrazolium, crystalline; β-nicotinamide adenine dinucleotide, reduced form, disodium salt; phenazine methosulphate; cytochalasin B from H. dematioideum; polystyrene latex beads, 1.1 μm mean diameter; 2,5-diphenyloxazole, crystalline (Sigma). Triton X-100; toluene, both scintillation grade (BDH Chemicals). [^{3}H]cholesterol 9.5 Ci mmol^{-1}, toluene solution (The Radiochemical Centre, Amersham). Sepharose 6B (Pharmacia). Dimethylsulphoxide (Fisons Scientific). Glutaraldehyde, E.M. grade, 25% (w/v) aqueous solution (Agar Aids). Neopeptone (Difco). Yeast extract (Oxoid). *Tetrahymena pyriformis*, strain GL (The Culture Centre, Cambridge).

Methods

Preparation of vesicles. Dried lipid films, molar composition dipamlimitoyl phosphatidyl choline : cholesterol : dicetyl phosphate (DPPC : CHOL : DCP; 7 : 2 : 1), anionic and DPPC : CHOL (7 : 2) neutral, were prepared in round-bottomed flasks by rotary evaporation from chloroform under reduced pressure at 37°C. The films were then hydrated for 2 h under N_2 at 47°C, typically 4 ml drug solution in 3.3×10^{-3} M, pH 7.2, phosphate-buffered 0.3% (w/v) saline (PBS) was used to hydrate 30–40 mg total lipid. Solutions of 2 mg ml^{-1} CAP or 2 mg ml^{-1} nitroblue tetrazolium plus 10^{-2} M β-nicotinamide adenine dinucleotide in PBS were used in the preparation of CAP and diformazan liposomes respectively.

Hydrated lipid suspensions were sonicated to optical clarity at 47°C under N_2 in a 20-ml tube using the 19 mm titanium probe of an MSE 150 W sonicator. In the case of diformazan liposomes, 0.1 ml 10^{-2} M phenazine methosulphate PBS solution was added to the suspension just prior to sonication. 4-ml aliquots of sonicated suspension were then fractionated over Sepharose 6B with PBS eluant using separate 40 × 2.3 cm columns for anionic and neutral vesicles and those 5 ml fractions containing vesicles (absorbance at 400 nm) pooled. The DPPC content of the pooled suspensions was determined as phosphate (Bartlett, 1959), the CAP content by a colorimetric method (Grove and Randall,

1955) and diformazan content by the method of Segal (1974).

For the preparation of labelled empty vesicles, 1 or 2 μl of toluene solution of [^{3}H]-CHOL (1 or 2 μCi) was added to the chloroform solution of DPPC/CHOL/DCP prior to film formation. Thereafter the method was as described above, although hydration of the lipid film was with PBS only.

Preparation of cell suspensions. *T. pyriformis* was maintained axenically under static conditions at room temperature in 500 ml Erlenmeyer flasks containing 100 ml medium comprising 2% (w/v) proteose peptone and 0.25% (w/v) yeast extract. 2–3-day-old cultures were centrifuged at 200 *g* for 5 min, the pelleted cells washed twice with PBS, and finally resuspended in this solution. Cells were used 18–20 h after centrifuging. The concentration of cell suspensions was determined directly, in a Helber counting chamber, cells fixed in 2.5% (w/v) glutaraldehyde or by Coulter counter (model D industrial) with a 200 μm counting tube, cells in a solution of 0.7% (w/v) NaCl, 1.05% (w/v) citric acid, 4% (v/v) formalin.

Toxicity experiments. The method is essentially that of Carter and Cameron (1973). To 3 ml liposomal CAP suspension, free CAP solution, empty liposome suspension or PBS was added 10–20 μl standardized cell suspension to give a final cell density of 6×10^3 ml^{-1}. Immediately after mixing, a small sample was taken and used to form duplicate lots of about 20 small droplets on the inner surface of a plastic petri dish which were then quickly covered with 8–10 ml light liquid paraffin. The number of cells in each droplet (1–10) was counted directly under the microscope (160X) at zero time and thereafter at time intervals of 1, 2, 3, 4, 5, 6 and 24 h and the number of living cells noted. Discrimination between viable and non-viable organisms was made on the basis of cell motility and ciliary movement. The fraction of cells surviving at each exposure time was calculated.

Exposure of cells to labelled liposomes. Volumes of cell suspension were added to labelled vesicle suspensions (around 60 nCi ml^{-1}) to give a final cell density of 6×10^4 ml^{-1} and at zero time, 1, 2 and 3 h, 1-ml samples were withdrawn into ice-cold PBS. The chilled suspensions were centrifuged (400 *g* for 10 min at 4°C), washed twice with ice-cold PBS and the cells resuspended in 1.1 ml cold PBS. Immediately after vortexing, 1 ml of the suspension was transferred to 10 ml counting cocktail comprising 0.5% (w/v) PPO in a toluene : Triton X-100 (2 : 1) solution by volume. Duplicate 10-min counts, corrected for background, were made on a Unilux II Liquid Scintillation Counter (Nuclear, Chicago) using external standards at an efficiency of 30%. 3-day-old, 8-day-old and formaldehyde-killed 3-day-old cells were all exposed to labelled vesicles.

Exposure of cells to diformazan liposomes. About 2 ml cell–liposome suspension containing 6×10^4 cells ml^{-1} was kept at room temperature and small samples, about 150 μl, removed into 2.5% (w/v) glutaraldehyde at 30-min intervals. The mean number of food vacuoles formed per cell was determined directly under the microscope (160 × magnification). Normal and cytochalasin B (CCB) pre-treated 3-day-old cells were used. CCB treatment consisted of exposing the cells for 30 min to a solution of 40 μg ml^{-1} CCB in 0.8% (v/v) DMSO.

These experiments were repeated with 1.1 μm diameter polystyrene bead suspension instead of liposomes.

RESULTS AND DISCUSSION

It was found (Fig. 1) that under the experimental conditions used here there was a concentration-dependent cytotoxic effect of free CAP on *T. pyriformis*. A concentration of >1000 $\mu g\ ml^{-1}$ free CAP was required to kill all the cells in a sample population and the LC_{50} (the concentration killing 50% of the cells) was 600 $\mu g\ ml^{-1}$. A linear relationship between cytotoxicity and drug concentration was found. The effective CAP concentration range found here is in accord with other reports in which similar, i.e. 10^2–10^3 $\mu g\ ml^{-1}$ CAP, concentrations were required to inhibit growth and morphology (Rohatgi and Krawiec, 1973), cell division (Gleason et al., 1974) and mitochondrial characteristics (Poole et al., 1971; Turner and Lloyd, 1971) in *T. pyriformis*.

Liposomal CAP (Fig. 2) by contrast was found to be markedly more active, the comparable concentrations being LC_{100} about 6 $\mu g\ ml^{-1}$ and LC_{50} about 1.5 $\mu g\ ml^{-1}$. In addition for liposomal CAP, the survivors' drug concentration curve was not linear which may reflect for this preparation a mode of entry into the cell different from that of free CAP. A linear relationship (Fig. 1) would be expected for a diffusional process, assuming that effect, in this case cell death was directly proportional to intracellular drug concentration.

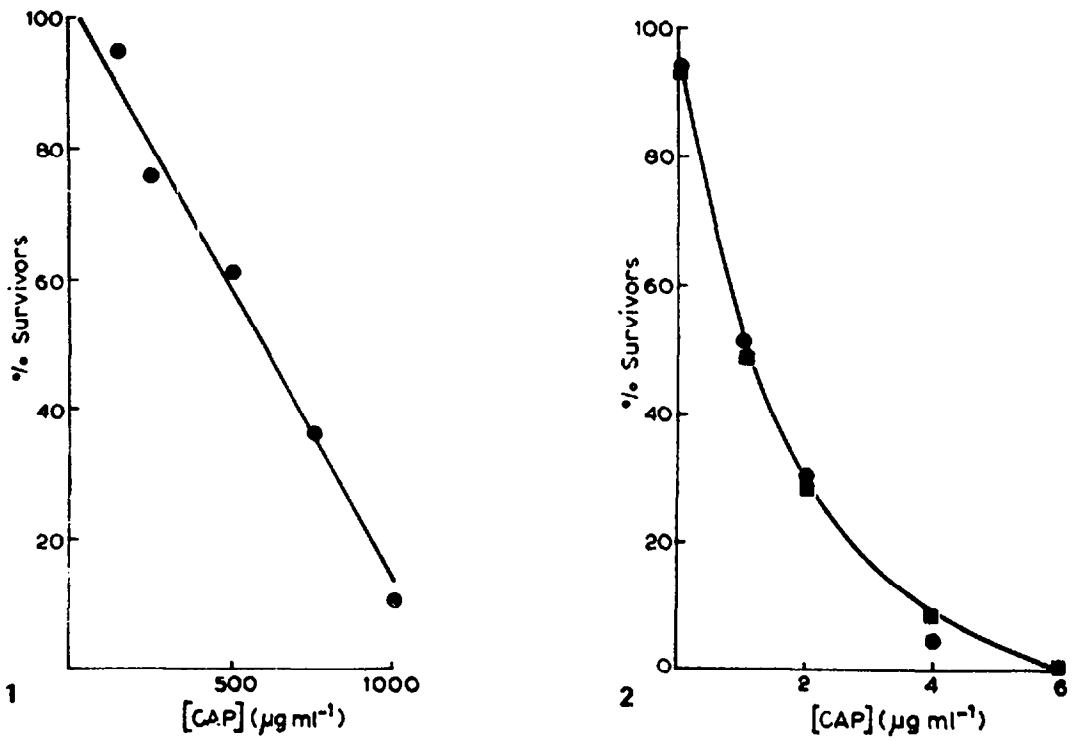

Fig. 1. The relationship between free chloramphenicol (CAP) concentration and the proportion of *T. pyriformis* cells surviving after 4 h incubation at room temperature. Each point is the mean of <4 experiments and the line shown is fitted by the least-squares method.

Fig. 2. The relationship between chloramphenicol (CAP) concentration and the proportion of cells surviving after 4 h incubation at room temperature in: •, anionic and ▪, neutral CAP-liposome suspensions. Each point is the mean of 3 experiments.

The non-linear relationship in Fig. 2 on the other hand indicates the presence of a saturable process which limits the access of liposomal drug to its site of action.

In terms of cytotoxicity, anionic and neutral liposomes (Fig. 2) appeared to be equally effective carriers for CAP in this system, a result similar to those of Pagano and Huang (1975) with mammalian cells that liposomes of different charge have similar uptake kinetics. Furthermore, Rasmussen and Modeweg-Hansen (1973) have demonstrated in the unimportance of the nett electric charge on particulate materials, which enhance phagocytosis and food-vacuole formation in Tetrahymena. Positively charged CAP liposomes have not been investigated. Suspensions of anionic and neutral liposomes used in this work were found to contain (mean ±S.E.M.); neutral 7.0 ± 0.25 μg CAP mg^{-1} DPPC, n = 7 and anionic 4.2 ± 0.13 μg CAP mg^{-1} DPPC, n = 4.

The effect of varying the time of incubation of cells with free and entrapped CAP (Fig. 3) confirms the results in Fig. 2 that the liposomal preparations are considerably more toxic than the free drug. Liposomal CAP was found to be more toxic than free CAP at all points within the time scale studied. The LT_{50} (time for 50% cell death) for 1000 μg ml^{-1} free drug was greater, LT_{50} = 90 min, than that for either 12 μg ml^{-1} or 4 μg ml^{-1} liposomal CAP with LT_{50} values of 30 min and 60 min respectively. It was also found (Fig. 3) that empty liposomes were not obviously cytotoxic to *T. pyriformis* which is probably similar in this respect to mammalian cells which can accumulate large quantities of empty liposomes with no significant cytotoxicity (Papahadjopoulos et al., 1974;

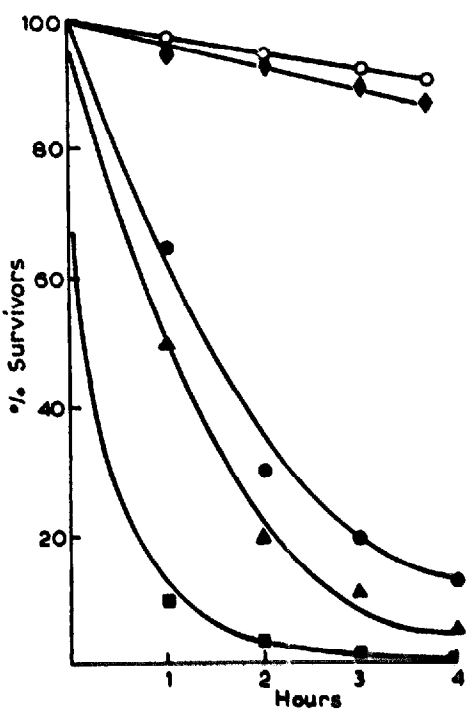

Fig. 3. The time course of *T. pyriformis* cell death during incubation at room temperature in: ○, phosphate-buffered saline; ●, free chloramphenicol (CAP) solution 1 mg ml^{-1}; ◆, empty liposome suspension; ▲, CAP-liposome suspension 4 μg ml^{-1}; CAP; ■, CAP liposome suspension 12 μg ml^{-1} CAP. (Neutral liposomes used throughout.) Each point is the mean of 4 experiments.

TABLE I

FOOD VACUOLE FORMATION IN STARVED 3-DAY-OLD *T. PYRIFORMIS* CELLS AFTER DIFFERENT TIMES OF EXPOSURE TO SUSPENSIONS OF 1.1 μm DIAMETER POLYSTYRENE BEADS, AND DIFORMAZAN LIPOSOMES

Time (min)	No. of cells counted		Total vacuoles counted		No. of vacuoles per cell	
	beads	liposomes	beads	liposomes	beads	liposomes
30	130	280	169	265	1.3	1.0
60	128	280	256	485	2.0	1.7
90	170	140	816	600	4.8	4.2
120	100	187	570	938	5.7	5.0
150	106	91	901	648	8.5	7.1

Entrappment equivalent to 48 μg diformazan mg^{-1} DPPC. 30 min pretreatment of the cells with cytochalasin B in 0.8% (v/v) dimethyl sulphoxide solution, 40 μg ml^{-1}, reduced the mean number of vacuoles per cell after 90 min incubation to 0.5 for beads and 0.6 for liposomes. Counting was done under phase-contrast microscopy at 160× magnification after cell fixation in 2.5% (w/v) glutaraldehyde.

Magee et al., 1974; Pagano et al., 1974). It would seem that phospholipids are non-toxic to Tetrahymena at room temperature and can be used to encourage the growth of fastidious species (Hill, 1972).

The extent of accumulation of diformazan in food vacuoles after exposure of cells to diformazan liposomes is similar to the intravacuolar accumulation of polystyrene latex beads (Table 1, Fig. 4A and B) which is used as a measure of phagocytic activity in this organism (Ricketts, 1971; Nilsson, 1976). Food-vacuole formation can also be observed in starved cells exposed to empty and CAP liposomes. Accumulation of diformazan and beads is inhibited by CCB (Table 1, Fig. 4C and D) which is known to inhibit phagocytosis in *T. pyriformis* (Rothstein and Blum, 1974; Nilsson et al., 1973) and in other cells (Allison, 1973; Malawista et al., 1971; Klaus, 1973). Further evidence for phagocytic uptake of liposomes is shown in Fig. 5 where it can be seen that on incubation of cells with labelled empty liposomes, the quantity of [^{3}H]CHOL found associated with older cells is, at all incubation times, less than that associated with younger cells, the latter being known to be more phagocytically active (Nilsson, 1976). There was no increase in killed cell-associated [^{3}H]CHOL with incubation time which indicates that vesicle adsorption onto cells or exchange of [^{3}H]CHOL between vesicles and cells was not responsible for the accumulation of label throughout the incubation period by the viable cells.

Exposure of cells to a mixture of empty liposomes and free CAP (10 μg ml^{-1} and 15 μg ml^{-1}) did not elicit obvious cytotoxicity which suggests that the relatively high toxicity of liposomal CAP for *T. pyriformis* is dependent on the carrier function of the liposomes rather than a synergistic action between them or their constituents and CAP. If it is assumed that free CAP enters a cell by diffusion and at cell death intra- and extracellular concentrations are in equilibrium, then at LC_{50} = 600 μg ml^{-1} free CAP (Fig. 1) the intracellular burden, based on a mean cell volume of 3×10^4 μm^3 (Ricketts, 1971), will be 1.8×10^{-5} μg per cell. Internalization of this amount of CAP in liposomal form by

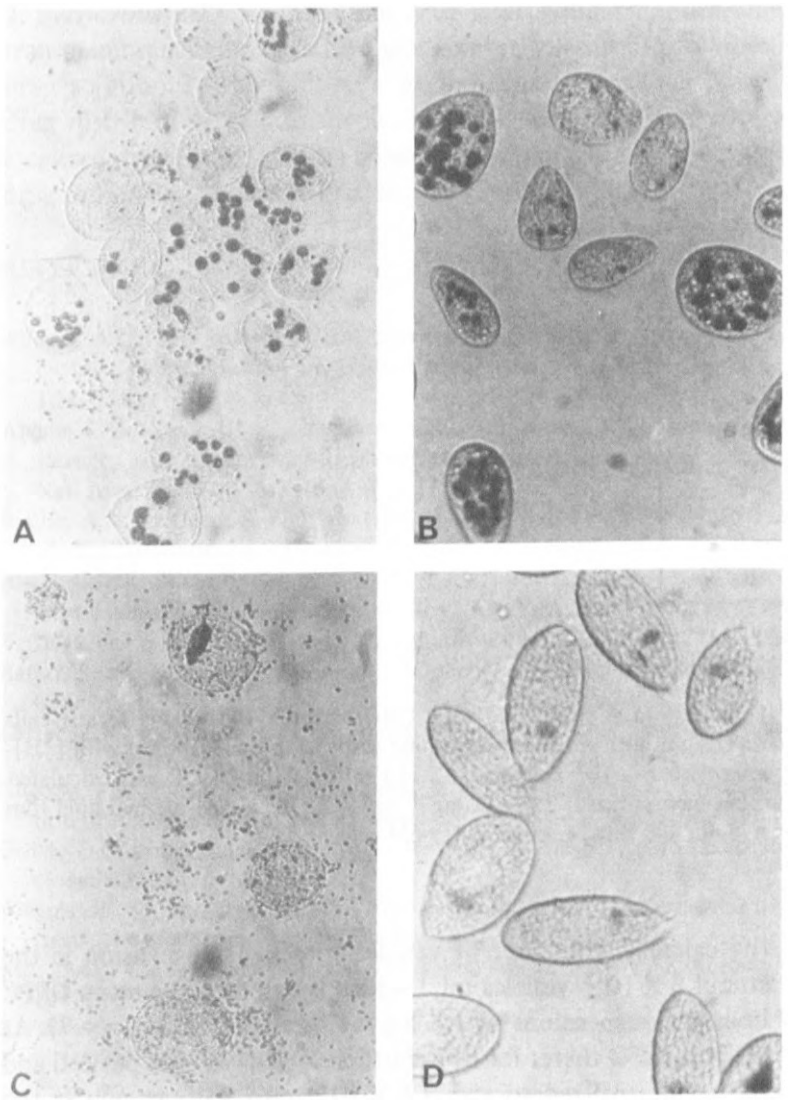

Fig. 4. Food-vacuole formation in *T. pyriformis* cells after incubation at room temperature for t min with: A, polystyrene latex beads, dia. 1.1 μm (t = 90) and B, diformazan liposomes (t = 150). Inhibition of bead C, and liposome D, vacuole formation by 30 min pretreatment with 40 μg ml^{-1} cytochalasin B in 0.8% (v/v) dimethylsulphoxide solution. Cells fixed in 2.5% (w/v) glutaraldehyde; magnification 160×.

each cell presumably then underlies the cytotoxicity of a comparable liposome preparation.

For a suspension of neutral liposomes the mean assay value of 7 μg CAP mg^{-1} DPPC is equivalent to 5.14 g CAP mol^{-1} DPPC. The mean radius of these liposomes is about 50 nm, by photon correlation spectroscopy (Baillie et al., 1979) and it is estimated that each comprises 5×10^4 DPPC molecules, so that each vesicle has a calculated CAP content of 4.2×10^{-13} μg. Therefore to attain the LC_{50} intracellular level of 1.8×10^{-5} μg per cell some 4.3×10^7 vesicles would have to be internalized by each cell. This number seems

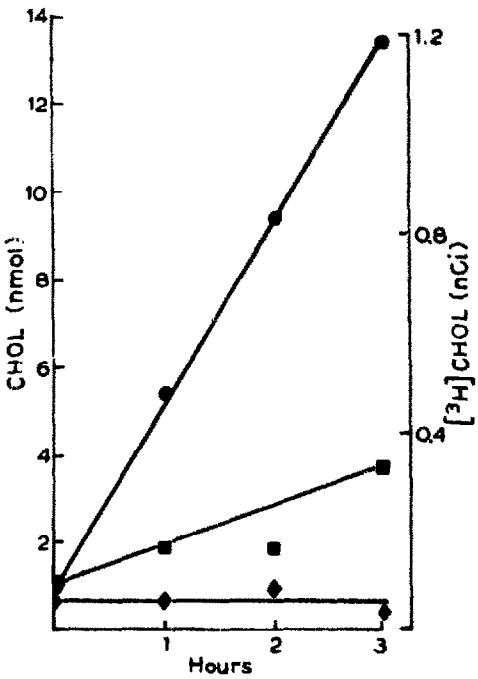

Fig. 5. Accumulation of [^{3}H]cholesterol (CHOL) and total CHOL by 6×10^4 *T. pyriformis* cells during incubation at room temperature with an anionic liposome suspension trace labelled with [^{3}H]-CHOL. Incubation mixture comprised 6×10^4 cells and 62 nCi ml^{-1}. Total CHOL was calculated from the cell-associated radiolabel incorporated, 233 nCi mg^{-1} total CHOL, in the original lipid film (Methods). •, 3-day-old cells; ▪, 8-day-old cells; ♦, killed 3-day-old cells.

large until compared to the calculated number of vesicles present in suspension in the toxicity experiments of around 3×10^{13} vesicles ml^{-1} which is based on the mean DPPC concentration of neutral liposome suspensions of 1.8 mg ml^{-1} (±0.09 S.E.M., n = 7). At a cell density of 6×10^3 ml^{-1} there is therefore in the order of 10^9 vesicles per cell and less than 1% of these need be internalized to give 1.8×10^{-5} μg CAP per cell. If the vesicles are regarded as 50 nm radius spheres packed into the cell with zero voids their total volume would be 2.25×10^4 μm^3 or 75% of the cell volume. Since metabolism of the vesicle constituents has not been considered this is probably not out with the phagocytic capability of *T. pyriformis* which may ingest in 150 min 21% of its volume in the form of latex beads (Ricketts, 1971) or 180% of its volume per hour when exposed to trypan blue (Seaman, 1961). Indeed even mammalian cells in culture are reported to easily incorporate up to 10^6 vesicles without impairing viability (Poste and Papahadjopoulos, 1976; Pagano and Huang, 1975).

If it is assumed that all of the cell-associated [^{3}H]CHOL was internalized in the form of vesicles, the 3-h value for 3-day-old cells of 13.4 nmol total CHOL (Fig. 5) can be used to calculate that each cell internalizes 9.5×10^6 vesicles in this period which we consider lends support to the calculated number of 4.3×10^7 vesicles per cell based on the toxicity data.

It would appear then that liposomes can be used to modify absorption of CAP by

T. pyriformis cells whereby the drug is accumulated intracellularly against an apparent transmembranal concentration gradient, as a result of phagocytosis of discrete packets of drug solution. It may be that a second aspect of cellular pharmacokinetics, intracellular drug distribution, is also involved. The liposome system offering a means of achieving localized depots of high drug concentration, in this case the food-vacuoles from which the drug can diffuse to its site of action.

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