Europ. J. Cancer, Vol. 15, pp. 1475–1483

0014-2964/79/1207-1475 $02.00/0

The Accumulation by Fibroblasts of Liposomally Encapsulated Vinblastine*

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Abstract—*Vinblastine, an anti-mitotic agent, was entrapped in liposomes prepared from phosphatidylcholine, cholesterol and stearylamine (mole ratio 7:2:1), and such liposomes incubated with cultured rat embryo fibroblasts. The fibroblasts accumulated the liposomally encapsulated vinblastine in a markedly different manner to that observed for the free agent. In the former case there was an initial high rate of drug uptake followed by a linear accumulation, which was parallel to that of a liposomal membrane marker and although the ratio of accumulated vinblastine to lipid was different to that pertaining to the original preparation prior to incubation, the ratio did remain constant over the linear phase of accumulation.*

The effect upon the accumulation of incubation temperature and of release experiments indicated that the mechanism of uptake of a liposomally encapsulated drug by fibroblasts was complex, possibly involving a combination of adsorption and endocytosis.

INTRODUCTION

THE DISCOVERY by Bangham *et al.* [1] that simple aqueous suspensions of phospholipids would form multilamellar 'onion skin' structures led to the great popularity of these structures, termed liposomes [2], as models for biological membranes. It was soon realized that since liposomes could entrap drugs in either their aqueous or lipid phase, that they had considerable potential as non-toxic biodegradable carriers for therapeutic use [3]. Thus to date a large variety of drugs, enzymes etc. have been encapsulated in liposomes, their surface charge and membrane fluidity varied, and their *in vivo* distribution monitored. A comprehensive review of the field has recently been published by Tyrrell *et al.* [4]. Recent reports that liposome encapsulation enhances the anti-tumor action of actinomycin D [5], cytosine arabinoside [6] and that methotrexate is protected against metabolic degradation [7], suggest that encapsulated drug formulations may have a role in cancer chemotherapy.

A number of chemicals that have the ability to arrest cells in metaphase apparently do so by combining with the microtubular protein vital for the formation of the spindle apparatus. Two such compounds, vinblastine and vincristine, are widely used in cancer chemotherapy. However, the therapeutic use of both, but especially vincristine, may be, according to Creasey [8], restricted by their neurological toxicity. For this reason we decided to study the potential for reducing their toxicity and hence increasing their utility by encapsulating vinblastine and vincristine in liposomes and firstly we investigated the uptake of encapsulated VBL by cultured fibroblasts, since the latter represent a good model for normal cells that has been well characterized [9].

Accepted 14 June 1979.

*This work was supported by Omnium Chimique S.A Bruxelles, and Caisse Générale d'Epargne et de Retraite (CGER), Belgium.

MATERIALS AND METHODS

Phosphatidyl choline (grade VE) and cholesterol (chromatography grade) were obtained from Sigma Chemical Co., St Louis, U.S.A. and stearylamine from Koch Light Labs Ltd, Colnbrook, U.K. Vinblastine sulphate (VBL) was purchased from E. Lilly Benelux SA, Brussels, Belgium. 26-^{14}C-Cholesterol (50 mCi/mmol, spec. act.) and G-^{3}H vinblastine sulphate (5–10 Ci/mmol, spec. act.), were purchased from the Radiochemical Centre, Amersham, U.K. The scintillation fluid, Aqualuma SB, was obtained from Lumac Systems AG, Basel, Switzerland. All other chemicals were reagent grade, and double distilled water from an all glass apparatus was used throughout.

Cell cultures

Primary cultures of fibroblasts were ob-

tained from 18–20-day old embryos (Wistar strain rats) and cultured at 37°C in Leighton tubes (5 cm^2, 5×10^5 cells in 2.5 ml) in Eagle Dulbecco medium supplemented with 10% calf serum by the method of Tulkens *et al.* [9].

Liposome preparation

Liposomes containing vinblastine were prepared according to methods described elsewhere [10]. Briefly, phosphatidylcholine, cholesterol and stearylamine, in a molar ratio of 7:2:1, dissolved in chloroform, were evaporated under vacuum onto the walls of a glass flask, Vinblastine, being predominantly lipophilic (octanol–water partition coefficient 92.5 [11]), was dissolved in chloroform and mixed with the lipids prior to evaporation, the radioactive labels, ^{14}C-cholesterol and ^{3}H-VBL being similarly incorporated. Liposomes were formed by adding physiologically isoosmotic phosphate buffered saline (PBS) (0.8% (w/v) NaCl, 27 mM KCl; 7.9 mM Na_2HPO_4. 2 H_2O; 1.47 mM KH_2PO_4, pH 7.3) to the dried film, the mixture was then dispersed by shaking and subsequent sonication. Sonic disruption was carried out using a Branson Sonifier (75 W, 20 kHz, 27.5 mm probe) for 15 sec at 1 min intervals for a total sonication time of 2 min. The sonication was performed under a nitrogen atmosphere at 25°C. After sonication the suspension was kept under N_2 at 37°C for at least 2 hr to allow liposomes to form and seal [12].

As discussed by Bangham [13], vesicles prepared by this method without sonication are large multilamellar structures (1 μm) whilst extensive sonication produces small vesicles bounded by a single bilayer (25 nm) [14]. Whilst the liposomes prepared for this study almost certainly do not conform to the latter category, light scattering measurements indicated a mean diameter of 80 nm suggesting that these liposomes were small vesicles bounded by a few bilayers.

Liposomes containing encapsulated VBL were separated from the free drug by filtration on a Sephadex G25 column (40 × 3.5 cm) maintained at 20°C. The void volume fraction was found to contain all the lipid, as measured by ^{14}C-cholesterol plus incorporated ^{3}H-VBL, whilst the retarded volume fractions contained only free drug (Fig. 1). The absence of ^{14}C-cholesterol from the latter fractions does not, of course, indicate the concomitant absence of phosphatidylcholine or stearylamine since these were originally present in unlabelled form. The phospholipid content of a fraction was measured by the method of Van Gent and Roseleur [15], 2.0 ml of butanol dimethylsulfoxide (1:1 v/v) being found necessary to stop the colorimetric reaction for aqueous suspensions of lipids. Such an assay failed to show any phospholipid in the retarded volume fractions (Fig. 1), as neither did thin layer chromatography of the free drug peak fractions. Recoveries of some 90% of lipid (by assay), cholesterol (^{14}C) and VBL

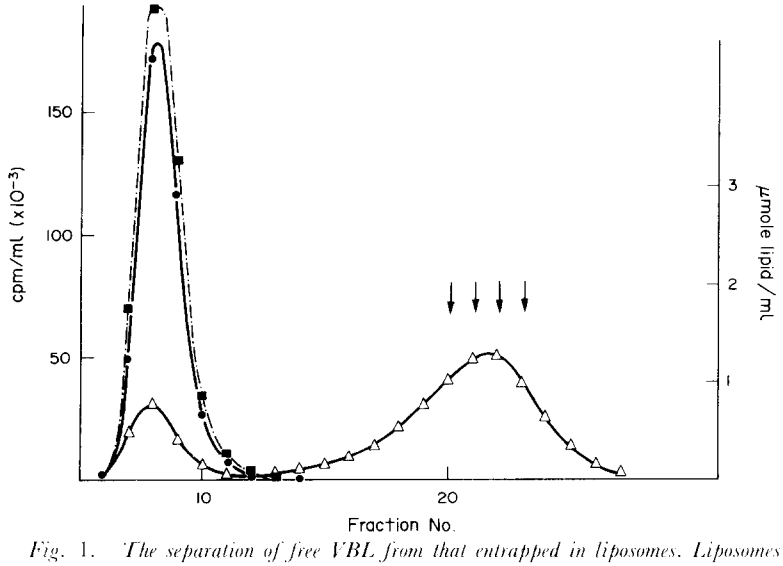

Fig. 1. The separation of free VBL from that entrapped in liposomes. Liposomes were formed from 35 μmole *phosphatidylcholine*, 10 μmole *cholesterol*, 10 μCi ^{14}C-*cholesterol*, 5μmole *stearylamine in* 5 ml 1.09 mM *VBL*, 50 μCi ^{3}H-*VBL as described in text; they were eluted on Sephadex G25 with PBS* pH 7.3 *at* 20ml/hr. △—△ ^{3}H-*VBL* counts/min, ●—● ^{14}C-*cholesterol* counts/min; ■ · ■ *phospholipid* [15]. *Fractions* 20–23 *were further assayed for phospholipid by thin-layer chromatography.*

(^{3}H) were routinely obtained, both phospholipid and VBL being known to adsorb slightly to the gel. The efficiency of entrapment was defined as the percentage of the original amount of drug which remained associated with the liposomes after passage on G25, and usually was in the range 8–11%, little or no variation being observed with increasing sonication time (up to 10 min) as might be expected for a predominantly lipophilic compound [11].

The liposomes thus prepared were sampled and examined for lipid oxidation by the method of Klein [16], none was detected within the sensitivity limits (0.01%) of this method. Further processing by pressure filtration (Amicon cell, XM 100A and UM2 membranes) followed by centrifugation at 10,000 rev/min for 20 min, or by reapplication to G25 column, did not show any breakdown or material loss as indicated by any of the above evaluation techniques.

Stability of the liposome–drug complexes

The rate of leakage of the entrapped drug from the liposomes was determined by dialysis [17] at 37°C, both in the presence and absence of 50% serum. VBL is readily adsorbed onto glass etc. and was found to bind to dialysis and filtration membranes, fortunately this binding is rapid, relatively irreversible and hence can be corrected for. The leakage showed two distinct phases, a rapid loss of 25–30% during the first 8–10 hr, followed by a slow linear loss of less than 1%/hr (Fig. 2). The initial loss probably represents desorption of weakly bound drug molecules. Dialysis at 4°C also showed two phases, the initial larger loss being however extended over a longer time scale, but at the onset of the slow linear phase of leakage (after 30 hr), the total leakage was almost identical to that observed for dialysis at 37°C.

Vinblastine is also known to bind to serum proteins [18], and it was necessary therefore to determine whether serum *per se* affected the leakage rate. From the comparison of leakage in the presence and absence of 50% serum (Fig. 2) it is evident that the leakage rates are very similar. By using simple equilibrium equations it was found that not only was the leakage of VBL from the liposomes unaffected by the presence of serum, but that of the free drug in the serum some 56% of it would be bound to the proteins, which is in agreement with the results of Donnigan and Owellen [18].

Incubation of liposomes with fibroblasts

Liposomes, to be used for incubation, were dialysed overnight at 20°C against PBS, this being sufficient time to reach the linear loss phase, concentrated by ultrafiltration, then resuspended. The fast large loss did not reoccur, only the slow leakage (<1%/hr) remained.

Fibroblasts were incubated for varying times in the presence of liposomes of known lipid and VBL content, after incubation the cells were washed thrice with PBS, dried and finally frozen at −18°C. For protein and radioactivity measurements the cells were re-

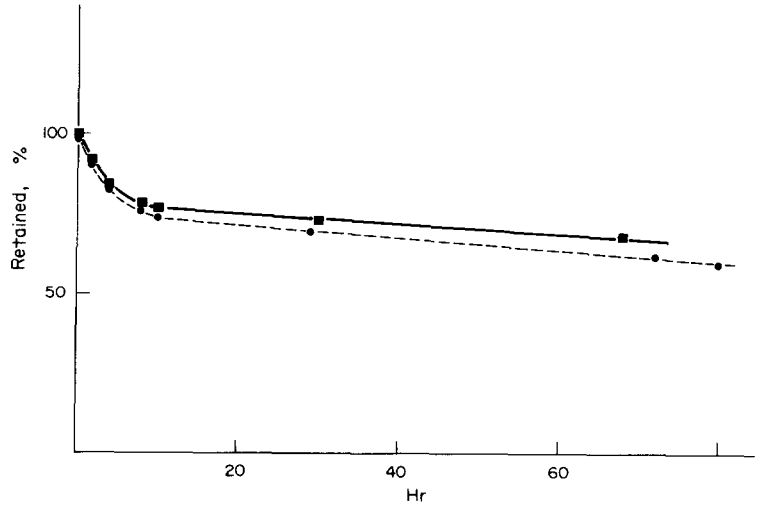

Fig. 2. Leakage of VBL from liposomes as determined by dialysis. Liposomes were prepared exactly as for Fig. 1, after separation 20.2 pmole *of VBL were initially entrapped by* 49.3 μmole *of total lipid in* 5 ml. *The liposomes were dialysed at* 37°C *against* 1.0 l *of PBS* ■—■, *or* 50% *serum* ●- - -●, *the external medium was changed after each measurement.*

suspended in 1% (w/v) deoxycholate pH 11.3. Aliquots were added to 9 ml of Aqualuma and samples counted for ^{3}H and ^{14}C in a Packard Tricarb scintillation counter. Protein determination was by the method of Lowry *et al.* [19].

RESULTS

Free VBL is rapidly taken up by fibroblasts, and incubation of some 6 hr results in a saturation of this uptake (Fig. 3a). This represents, calculated on the basis of an intracellular volume of 4–5 μl/mg of the cells, a 60–70-fold intracellular accumulation over the initial extracellular concentration. There is no evidence that the uptake (or efflux) of VBL is by any pump mechanism and hence the accumulation must proceed until some equilibrium is attained between the extracellular and the intracellular media and the binding to cellular components. The equilibrium level shows a linear dependence on the initial concentration (up to 5 μM), and may itself exhibit saturation, however reliable demonstration of such is difficult because of the problem of cell toxicity by concentrations of VBL above 10 μM. Such a saturation would, of course, indicate complete occupancy of the intracellular binding sites.

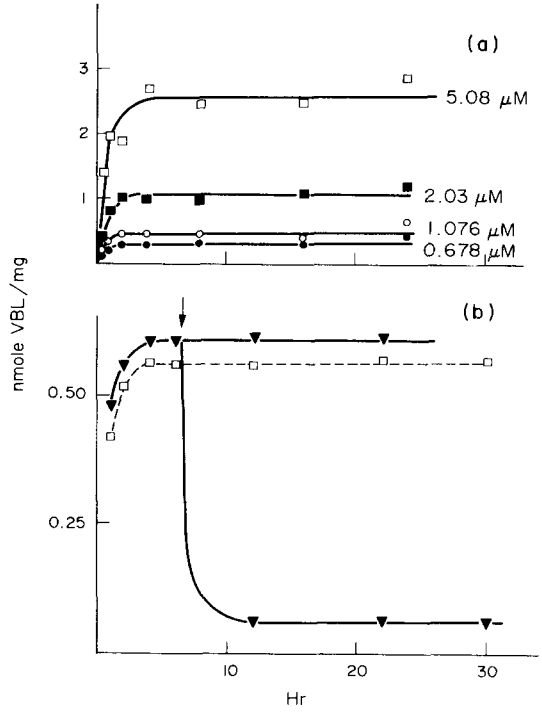

Fig. 3. The accumulation of free VBL by cultured rat embryo fibroblasts. (a) Samples of 5×10^5 *fibroblasts in* 2.5 ml *of Eagles medium containing* ^{3}H-*VBL at the concentrations indicated were incubated at* 37°C *for the times shown; the cells were then washed* 3 *times with PBS, dried and resuspended in* 1% (w/v) *deoxycholate* pH 11.3, *aliquots were taken for radioactivity and protein measurements. (b) Ten samples of* 5 $\times 10^5$ *cells in* 2.5 ml *of* 1.76 μM ^{3}H-*VBL in Eagles medium were incubated for the times shown* ▼—▼, *after* 6 hr *the medium of* 5 *samples was exchanged for one free of VBL. Seven samples of* 5×10^5 *cells were pre-incubated in* 2.5 ml *of* 1.096 μM *VBL in Eagles medium, the medium was then exchanged for* 1.76 μM ^{3}H-*VBL and the uptake of VBL measured* □- - -□.

As can be seen from Fig. 3b, pre-incubation with unlabelled VBL has no effect on the uptake of ^{3}H-VBL; this was to be expected since the concentrations being used are well within the linear range for uptake. That the intracellular binding is reversible is also shown in Fig. 3b, replacement of the external medium, in either case (once saturation has been reached), by one free of any VBL, results in a re-equilibration to a new saturation level. It is not surprising that the new level is comparatively low, since on exchange of the medium the accumulated VBL has to be redistributed over a volume 2×10^3 times larger (the external medium). Assuming the intra- and extracellular concentrations of free VBL to be identical at equilibrium, a 60-fold original accumulation, and an intracellular volume of 4–5 μl/mg protein [20], one may calculate that the new equilibrium level, for such an exchange of medium, will be at 1/20th of the former level; a level of 1/17th of the original was observed.

The uptake of VBL loaded liposomes by fibroblasts incubated at 37° and 4°C is shown in Fig. 4, also shown is the uptake of free VBL at the same initial concentration as is contained in the liposome solution. The VBL and lipid scales have been related such that they are in the same ratio as was VBL to cholesterol in the original preparation. The accumulation of the liposome label (^{14}C-cholesterol) is linear over the range of incubation times, and that of VBL (^{3}H-VBL) becomes linear after the first hour. Even after incubation for 22 hr the total accumulated VBL is still below the plateau level observed for free VBL. The uptake of empty liposomes, at the same lipid concentration, was identical, in terms of ^{14}C-cholesterol, to that of loaded liposomes (not shown). Whilst over the complete range of incubation time 0.399 pmole VBL/nmole lipid were taken up as compared with a ratio of 0.387 pmole/nmole in the original preparation, it is evident from the inset of Fig. 4, that, initially, there was a marked change in rapport. This change could represent a loss of 25–30% of the originally entrapped VBL from the liposomes which is

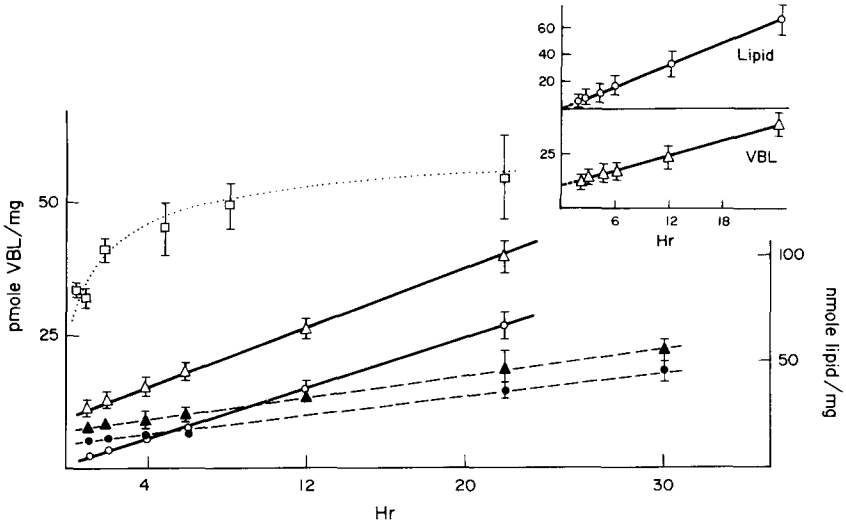

Fig. 4. The accumulation of VBL entrapped in liposomes by fibroblasts. Liposomes, prepared as for Fig. 1, *were incubated with* 5×10^5 *cells in* 2.5 ml *of medium, final concentration of VBL* 174 nM (5 μCi ^{3}H-*VBL*), *and of total lipid* 454 μM (10 μCi ^{14}C-*cholesterol*). △—△ *uptake of* ^{3}H-*VBL at* 37°C; ○—○ *of* ^{14}C-*cholesterol at* 37°C; ▲- - -▲ *of* ^{3}H-*VBL at* 4°C; ●- - -● *of* ^{14}C-*cholesterol at* 4°C. *For fibroblasts incubated with free VBL* 171 nM (7 μCi ^{3}H-*VBL*) □· · ·□. *The VBL and lipid uptake scales have been related on the basis of the original rapport as described in the text, the inset shows the uptake at* 37°C *on unrelated scales. Indicated values represent the mean and range of* 5 *samples at each time point.*

then taken up in its free form by the fibroblasts. After this initial change, the rapport between VBL and lipid remains constant, as shown by the uptake of both labels being parallel. The fact that the lines are not coincident in Fig. 4 reflects the initial change in rapport. The uptake of liposomes by fibroblast incubated at 4°C shows the same form as at 37°C but with much reduced rates, however, there was in this case an initial faster uptake of both VBL and cholesterol which was then followed by a slow parallel accumulation of both labels. Over the complete range of incubation time, 0.396 pmole of VBL were taken up per nmole of lipid, whereas the initial ratio was 0.387 pmole/nmole. Similarly, as at 37°C, there is an initial change in rapport resulting in an increased uptake of VBL, as indicated by the two lines being non-coincident.

It is not, of course, possible from these results, to infer that VBL loaded liposomes are taken up into the interior of the fibroblasts in their intact form, but the consistency of the VBL to cholesterol ratio, after the initial interaction, when the rapport is changed, does suggest that the interaction with, or incorporation by, fibroblasts is with intact liposomes. Since the kinetics of the accumulation process for free and encapsulated VBL are different (cf. Figs. 3 and 4), indicating that the routes are probably different, and that the uptake of free VBL exhibits saturation, it is pertinent to try to determine firstly whether the routes are independent and secondly whether the accumulation of VBL via liposomes shows a similar eventual equilibration. The latter is rather difficult to demonstrate since long incubation is required for the slow uptake of VBL delivered via liposomes to approach the level where free VBL shows equilibrium.

In Fig. 5, fibroblasts, with a somewhat lower accumulation of feee VBL (45-fold compared with the usual 60–70-fold) may, after long incubation, be showing just such an equilibrium of liposomally delivered VBL. Once again the accumulation of the liposome label (^{14}C-cholesterol) is linear over the range of incubation time, the VBL label exhibits an initial faster uptake followed by a slow linear accumulation parallel to that of the liposome label, however the latter reaches a plateau whilst the former shows no such effect within the limits of the incubation times being used. The fact that this equilibrium level is not identical to that observed for free VBL (at the same concentration) is to be expected since it merely reflects the differing equilibrium situations. In the former case equilibrium must be reached with the extracellular drug which is in an encapsulated form (although 25–30% of the originally entrapped drug could have

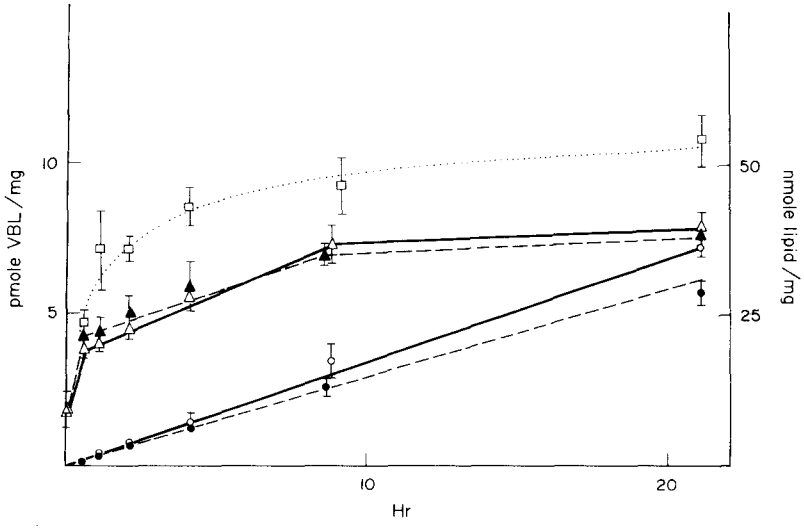

Fig. 5. The accumulation of VBL entrapped in liposomes in the presence of free VBL. Fibroblasts were incubated at 37°C for the times indicated under the same conditions as in Fig. 4, in the presence of free VBL 44.9 nM □· · ·□; of encapsulated VBL 46.6 nM in 228.3 μM total lipid, △—△ ^{3}H-VBL, ○—○ ^{14}C-cholesterol; and after preincubation for 1.5 hr with free VBL 44.9 nM with the same concentration of liposomally encapsulated VBL (46.6 nM VBL, 228.3 μM lipid), ▲---▲ ^{3}H-VBL, ●---● ^{14}C-cholesterol. Indicated values represent the mean and range of 5 samples at each time point.

been liberated during the initial change in rapport); whilst in the latter case equilibrium is simply with the free extracellular VBL. Figure 5 also shows that the initial linear uptake of liposomes is at the most only slightly affected by the presence of free VBL, which is itself equilibrating by its own pathway. As was shown in Fig. 3, the uptake of free VBL by fibroblasts incubated at 37°C proceeds until equilibrium is reached, this equilibrium involves intracellular binding which is reversible. In Fig. 6, the accumulated VBL, delivered via liposomes, is examined in the same way; after 22 hr of incubation the external medium is replaced by one free of any VBL and the subsequent redistribution examined. The liposomes and fibroblasts were incubated at 37°C (Fig. 6a) and also at 4°C (Fig. 6b) and in both cases free VBL samples were monitored as controls. The uptake of both ^{3}H-VBL and ^{14}C-cholesterol were linear and parallel between 6 hr and 22 hr of incubation. The level of VBL from liposomes decreased on exchange of medium at a similar rate for both incubation temperatures, but in neither case was the decrease as large as was observed for the redistribution of free VBL. In the latter, the new level is that due to a simple recquilibration over a large extracellular volume. Whilst the decrease in VBL has a similar time course at both 37° and 4°C, that for the liposomal membrane marker (^{14}C-cholesterol) is markedly different at the two temperatures.

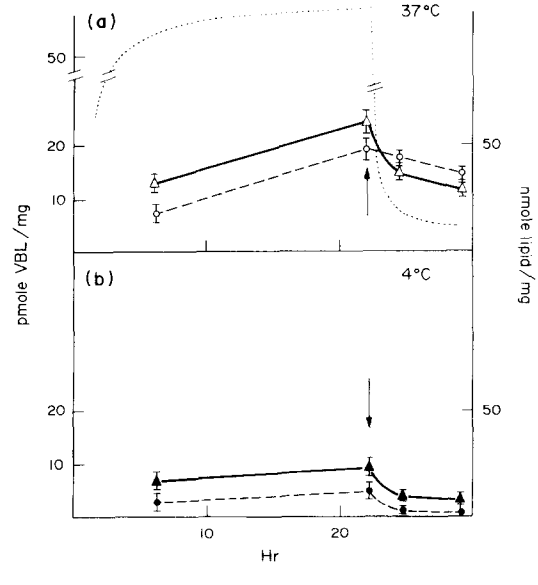

Fig. 6. a and b. The effect of exchange of incubation medium for one without VBL on the accumulation of loaded liposomes by fibroblasts incubated at different incubation temperatures. Incubation conditions as described in Fig. 4.
(a) Incubation at 37°C, . . . free VBL, △—△ encapsulated VBL, ○- - -○ lipid; after 22 hr of incubation the incubation medium was exchanged for one without any VBL.
(b) Incubation at 4°C, . . . free VBL, ▲—▲ encapsulated VBL, ●- - -● lipid. As before the uptake of VBL and lipid was determined from measurements of the radioactive labels ^{3}H-VBL and ^{14}C-cholesterol respectively. Indicated values represent the mean and range of 4 samples at each time point.

At 4°C the decrease in ^{14}C-cholesterol follows a similar time course to that of VBL, but at 37°C a slow linear decrease is observed with the same rate constant as for the uptake. The possibility that incubation at 4°C causes structural changes in either, or both, the liposomes and the fibroblasts can be discounted since changing the incubation temperature from 4° to 37°C, or vice versa, merely results in the uptake rate changing to that of the new temperature. This would support the view that the interaction of liposomes by fibroblasts is by a temperature dependent pathway.

The uptake of free VBL at 4°C, as shown in Fig. 6B, is very much slower than at 37°C, subsequent redistribution on exchange of the external medium did not occur. In other experiments some redistribution was observed, but even in these cases the new equilibrium level was several times higher than that which would be observed for complete redistribution of the accumulated drug, as was observed for incubation at 37°C. This is of interest since it may imply that intracellular binding is not only preferred but is relatively irreversible at low temperature and in turn be directly related to the state of polymerization of tubulin [21]. The effect of incubation at different temperatures between 4° and 37°C on accumulation is shown in Fig. 7; the uptake of both labels is very similar, between 4° and 22°C there is a slow change with temperature, whereas between 22° and 37°C a much greater increase in uptake per degree rise in incubation temperature is observed. The accumulation of both labels shows a marked discontinuity around 22°C.

DISCUSSION

The uptake of liposomally entrapped VBL is markedly different from that of the free form of VBL, is sensitive to the temperature of incubation (Figs. 3 and 4), and appears to proceed independently of that of free VBL. It has been shown that microtubule disruptive drugs such as VBL have little or no effect on uptake, nor on subsequent formation of phagolysosomes [22, 23], but do cause a reduction of membrane associated transport processes after endocytosis [24].

Poste and Papahadjopoulos [25] have shown that VBL has no effect on the uptake of any class of liposomes. The finding that the uptake of VBL loaded vesicles by fibroblasts is not affected by the parallel uptake of free drug supports this and may in turn be a reflection on the different interaction mechanisms for free and encapsulated VBL.

The question as to whether the observations described here are the result of endocytosis or fusion, or alternatively merely due to surface adsorption is difficult to answer unequivocally.

According to Hoff *et al.* [26] and Rabinovitch [27] endocytosis only proceeds if the incubation temperature exceeds some critical threshold (18–20°C), below that temperature there is very little uptake. Whereas Steinman *et al.* [28] describe much of the

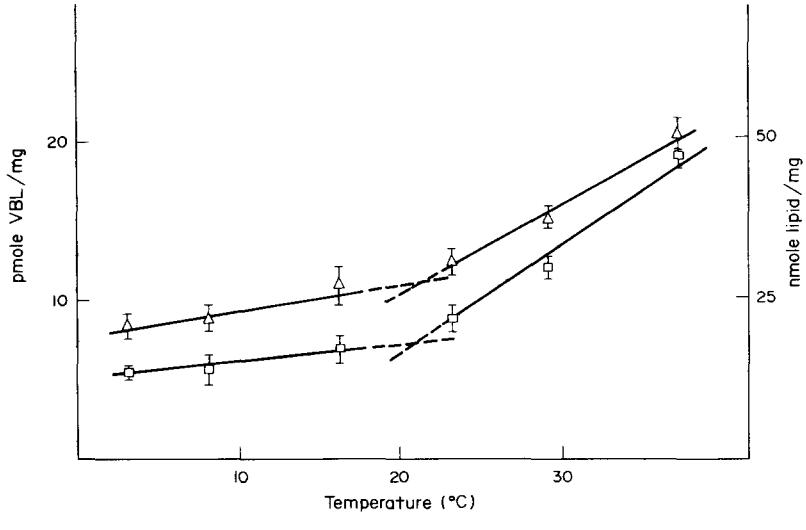

Fig. 7. The effect of incubation temperature on VBL loaded liposome accumulation by fibroblasts. Incubation conditions as in Fig. 4, for 6 hr at temperature indicated. △—△ ^{3}H-VBL uptake, □—□ ^{14}C-cholesterol uptake. Indicated values represent the mean and range of 4 samples at each temperature point.

uptake by fibroblasts as a fluid form of endocytosis which is linearly dependent on incubation temperature between 2° and 38°C with some uptake still occurring at 4°C. The data of Fig. 4 and Fig. 7 which show firstly an uptake at 4°C and secondly a marked increase in uptake above 22°C indicates that an uptake linearly dependent on temperature does not apply here, neither does it fit to a simple form of endocytosis. The data could also be in general agreement with the concept of liposome–fibroblast fusion as proposed by Poste and Papahadjopoulos [25]. Certainly from studies of the phase transition of lipid mixtures [29–31] we would conclude that the liposomes used here would be in a fluid condition over the range of incubation temperature and hence capable of fusion with the fibroblast plasma membrane.

The release experiments (Figs. 6a and 6b), clarify the interaction mechanism problem. At 4°C (Fig. 6b), the exchange of the medium results in a rapid and parallel release of both VBL and the liposomal membrane marker. This indicates that the interaction at this temperature is predominantly, if not entirely, one of surface adsorption and that probably this mechanism applies to incubation temperatures up to about 22°C.

When the release experiment is performed at 37°C (Fig. 6a), the situation is complex; the liposomal marker shows similar kinetics of efflux to that of uptake, whereas a part, but a part only, of VBL is released with similar efflux kinetics to that of free VBL. It is probable, therefore, that part of the accumulated VBL is in a free form, the origin of which could either be from lysosomally degraded liposomes or equally from a fraction released during the initial incubation which resulted in the disproportionally higher uptake of VBL. The efflux kinetics of the remainder of VBL which appears to follow that of the liposomal membrane marker, are different to those attributed to surface desorption at 4°C, and may be due to the slow degradation of the accumulation liposomes with the concomitant release of the entrapped VBL.

In conclusion, the ability of liposomes to entrap reasonable quantities of VBL, to retain it with only a small leakage even in the presence of serum has been demonstrated. The uptake of liposomally encapsulated VBL by fibroblasts is very different from that for the free drug. There is an immediate change in rapport (which may involve cell-liposome contact) but thenceforth the rapport remains constant. The effects of temperature and of release indicate that the mechanism of uptake at 37°C is complex and may involve a combination of adsorption and endocytosis or fusion, rather than a single process, but the results presented here do not allow an unambiguous model to be proposed.

Acknowledgements—We wish to thank Drs. E. Mayhew and P. Tulkens for helpful discussions, and M. C. Cambier for provision of primary fibroblast cultures.

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