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THE UPTAKE OF DISTEAROYLPHOSPHATIDYLCHOLINE/CHOLESTEROL LIPOSOMES BY RAT INTESTINAL SACS IN VITRO

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Summary

The uptake of free and liposome-entrapped ^{125}I-labelled polyvinylpyrrolidone was measured in an intestinal sac preparation from adult rats. At an equal concentration of ^{125}I-labelled polyvinylpyrrolidone, the rate of uptake of the liposome-entrapped macromolecule by the tissue was over 4-times that of the free macromolecule. The quantity of ^{125}I-labelled polyvinylpyrrolidone present in the serosal fluid of gut sacs, cultured for 2 h, was 1.8-times greater when the macromolecule was entrapped in liposomes than when it was free in the culture medium. When gut sacs were cultured with liposome-entrapped macromolecule, approx. 50% of the total ^{125}I-labelled polyvinylpyrrolidone present in the serosal fluid was associated with a 100 000 × *g* liposomal pellet.

Introduction

Recently liposomes have received much attention as potential vehicles for the oral administration of therapeutic agents which would not normally survive or be absorbed in the gastrointestinal tract. While there is general agreement that liposome-entrapped orally administered insulin can cause a decrease in blood glucose in diabetic rats [1—4], there is some uncertainty over the extent to which liposomes remain intact in vivo [2,4—10]. The reported absorption of intact liposomes from the gastrointestinal tract [2] has not been confirmed [8]. Furthermore, it was shown in a previous in vitro study [11] that a bile salt solution simultating that of human intestinal content caused almost total release of an entrapped macromolecule from most of the liposomes used in

various in vivo experiments. Only liposomes composed of distearoylphosphatidylcholine and cholesterol were capable of retaining the majority of an entrapped macromolecule in extremes of pH and in the presence of bile salts and pancreatic lipase [11]. As these distearoylphosphatidylcholine/cholesterol liposomes appeared to have the qualities required of a protective carrier it was decided to determine whether such liposomes could be absorbed by the gut.

The present paper compares the uptake of non-degradable free ^{125}I-labelled polyvinylpyrrolidone (^{125}I-labelled PVP) with that of ^{125}I-labelled PVP entrapped in distearoylphosphatidylcholine/cholesterol liposomes by everted rat gut sacs in vitro. The serosal fluid from the gut sacs was also studied to determine whether any liposomes had crossed the gut wall.

Materials and Methods

Liposome preparation. Multilamellar liposomes containing ^{125}I-labelled PVP were prepared from 54 mg of distearoylphosphatidylcholine and 7.6 mg of cholesterol as described previously [11]. The final liposome preparations contained 1.19 ± 0.04 μg ^{125}I-labelled PVP/mg lipid (mean ± S.E. of six separate liposome preparations). 'Empty' liposomes were prepared by the method described previously [11] but in the absence of ^{125}I-labelled PVP.

Everted gut sac culture. The preparation and method of culture of everted gut sacs of adult rat intestine has been described previously [12]. Experiments were performed with substrate conditions as follows: (i) free ^{125}I-labelled PVP; (ii) ^{125}I-labelled PVP entrapped in liposomes; and (iii) free ^{125}I-labelled PVP in the presence of 'empty' liposomes. The overall medium concentration of ^{125}I-labelled PVP was kept constant at 0.2 mg/l in all experiments, whether it was entrapped in liposomes or not. Liposome concentration was maintained at 168.76 mg lipid/l medium. Uptake from the medium into the tissue and from the medium into the serosal fluid was calculated as pg ^{125}I-labelled PVP/mg tissue protein and was plotted against time. From the slope of a best fit line, calculated by regression analysis, the rate of uptake of ^{125}I-labelled PVP in pg/mg tissue protein/h was determined.

Study of the serosal fluid. Eight gut sacs were cultured for 2 h with either (a) free ^{125}I-labelled PVP, (b) liposome-entrapped ^{125}I-labelled PVP or (c) free ^{125}I-labelled PVP + 'empty' liposomes. The contents of the eight sacs were pooled and 0.5 ml samples of the serosal fluid and culture medium were counted for radioactivity. The remaining serosal fluid and samples of the medium were centrifuged at 100 000 × *g* for 10 min and then 0.5 ml samples of the supernatant were counted for radioactivity. In this way the percentage of the total ^{125}I-labelled PVP that was free in the medium and serosal fluid after 2 h culture could be determined. The percentage of the total ^{125}I-labelled PVP that was free in the medium and serosal fluid from cultures containing liposome-entrapped ^{125}I-labelled PVP was also determined, by the same method, before and after incubation with 1.25% (v/v) Triton X-100 at 37°C for 30 min.

Results

Uptake of free and liposome-entrapped ^{125}I-labelled PVP by rat gut sacs

Figs. 1 and 2 show that both free and liposome-entrapped ^{125}I-labelled PVP are taken up by the everted rat gut tissue. The uptake of liposome-entrapped ^{125}I-labelled PVP by the tissue and its appearance in the serosal fluid was lower than for the free macromolecule over the first 20 min culture. However, from 30—120 min the uptake of ^{125}I-labelled PVP by the tissue and its appearance in the serosal fluid was increased when the macromolecule was entrapped in liposomes. As the uptake of both free and liposome-entrapped ^{125}I-labelled PVP appeared to be linear with time, the overall rate of uptake of the macromolecule was calculated by regression line analysis of the graphs in Figs. 1 and 2. It can be seen that the rate of uptake of ^{125}I-labelled PVP by the tissue was increased 4.7-fold when the macromolecule was entrapped in liposomes (Table I). The rate of appearance of the macromolecule in the serosal fluid was increased 2.45-fold. The final quantities of ^{125}I-labelled PVP accumulated by the tissue and the serosal fluid after 2 h were 2.75 and 1.84-fold greater respectively when the macromolecule was entrapped in liposomes (Table I).

The presence of 'empty' liposomes had no effect on either the rate of uptake of free ^{125}I-labelled PVP by the tissue or its rate of appearance in the serosal fluid (Figs. 1 and 2, Table I). Furthermore, the presence of 'empty' liposomes had no effect on the final quantity of ^{125}I-labelled PVP accumulated by the tissue and serosal fluid after 2 h (Table I).

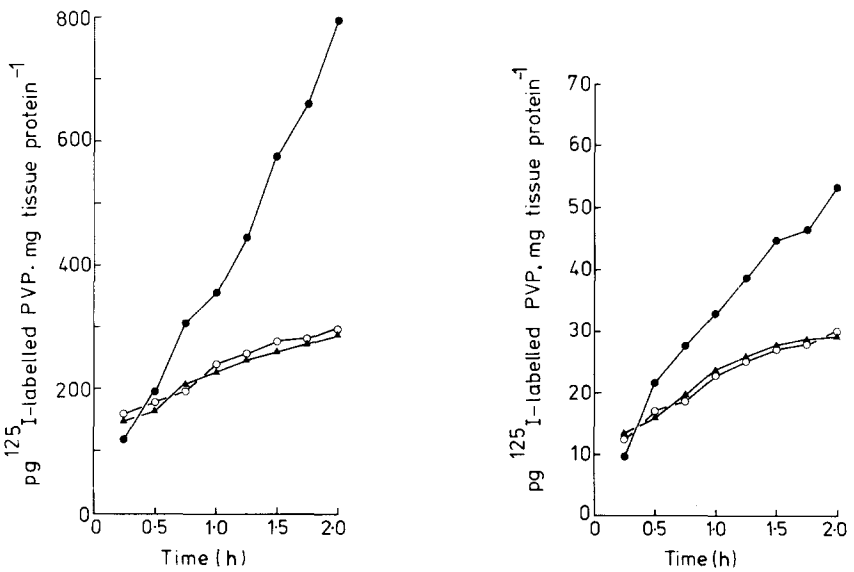

Fig. 1. Uptake of ^{125}I-labelled PVP by gut sac tissue. Each line represents the mean of six experiments. ▲———▲, free ^{125}I-labelled PVP; ●———●, liposome-entrapped ^{125}I-labelled PVP; ○———○, free ^{125}I-labelled PVP + 'empty' liposomes.

Fig. 2. Appearance of ^{125}I-labelled PVP in the serosal fluid. Each line represents the mean of six experiments. ▲———▲, free ^{125}I-labelled PVP; ●———●, liposome-entrapped ^{125}I-labelled PVP; ○———○, free ^{125}I-labelled PVP + 'empty' liposomes.

TABLE I

RATE OF UPTAKE OF ^{125}I-LABELLED PVP BY EVERTED GUT SACS FROM ADULT RATS

Uptake is expressed as pg/mg tissue protein/h ± S.E., computed from the slopes of the graphs shown in Figs. 1 and 2. The final quantity of ^{125}I-labelled PVP accumulated at 2 h is expressed as pg/mg tissue protein ± S.E., calculated from values of six sacs. Liposome concentration = 168.75 mg lipid l^{-1}.

Substrate (0.2 mg l^{-1})	Uptake by tissue	Tissue accumulation at 2 h	Appearance in serosal fluid	Serosal fluid accumulation at 2 h
Free ^{125}I-labelled PVP	76.4 ± 4.5	289.2 ± 20.8	9.4 ± 1.5	28.9 ± 2.3
Liposome-entrapped ^{125}I-labelled PVP	360.9 ± 16.0	795.4 ± 76.0	23.1 ± 2.8	53.1 ± 5.3
Free ^{125}I-labelled PVP + 'empty' liposomes	73.8 ± 12.0	286.2 ± 22.1	9.3 ± 1.2	29.3 ± 2.4

Study of the serosal fluid

When either the medium or the serosal fluid from sacs which had been cultured in free ^{125}I-labelled PVP for 2 h was centrifuged at 100 000 × *g* for 10 min, 98% and 95% respectively of the total ^{125}I-labelled PVP was present in the supernatant. Similarly, while a small white pellet, presumably of liposomes, was found following the centrifugation of medium or serosal fluid from sacs cultured in the presence of free ^{125}I-labelled PVP plus 'empty' liposomes, a negligible amount of the total ^{125}I-labelled PVP was associated with this pellet (Table II). This suggests that there was no association of free ^{125}I-labelled PVP with either tissue or medium components, or with 'empty' liposomes, in such a way that the macromolecule became sedimentable at 100 000 × *g*.

However, when medium containing liposome-entrapped ^{125}I-labelled PVP was centrifuged at 100 000 × *g* after 2 h culture with gut sacs, only 12% of the total ^{125}I-labelled PVP was found in the supernatant. This suggests that most of the ^{125}I-labelled PVP had remained entrapped in liposomes during culture. This was supported by the fact that incubation of 2 h liposome medium with Triton X-100 released 97% of the ^{125}I-labelled PVP into the 100 000 × *g* supernatant (Table II). When the serosal fluid from gut sacs which had been cultured in

TABLE II

THE PERCENTAGE OF FREE ^{125}I-LABELLED PVP PRESENT IN THE MEDIUM AND SEROSAL FLUIF OF GUT SACS CULTURED FOR 2 h

The percentage of the total ^{125}I-labelled PVP that was free in the 100 000 × *g* (10 min at 20° C) supernatant was determined for the medium and the serosal fluid of gut sacs cultured for 2 h with the various substrates. The figures in brackets refer to the percentage of the total ^{125}I-labelled PVP that was present in the 100 000 × *g* supernatant following incubation of serosal fluid and medium with Triton X-100 (1.25% v/v) at 37° C for 30 min. Each value represents the mean ± S.E. of five separate experiments.

Substrate	Medium	Serosal fluid
Free ^{125}I-labelled PVP	98.3 ± 0.4	95.6 ± 1.6
Liposome-entrapped ^{125}I-labelled PVP	11.8 ± 0.7	46.9 ± 0.5
(After treatment with Triton X-100)	(97.3 ± 0.5)	(98.7 ± 0.3)
Free ^{125}I-labelled PVP + 'empty' liposomes	98.1 ± 0.6	96.3 ± 0.5

medium containing liposome-entrapped ^{125}I-labelled PVP for 2 h was centrifuged, 47% of the total ^{125}I-labelled PVP present was found in the supernatant. The remaining 53% of the ^{125}I-labelled PVP was associated with a small white, presumably liposomal, pellet. That this pellet consisted of relatively intact liposomes was suggested by the fact that incubation of such serosal fluid with Triton X-100 resulted in 99% of the total ^{125}I-labelled PVP present being found in the 100 000 × *g* supernatant (Table II).

Thus, following culture of gut sacs with liposome-entrapped ^{125}I-labelled PVP, ≃ 50% of the total ^{125}I-labelled PVP present in the serosal fluid was free in solution and ≃ 50% was associated with relatively intact liposomes.

Discussion

The present study confirms the previous report by Bridges et al. [13] which showed that uptake of a macromolecule, ^{125}I-labelled PVP, by adult rat gut could be demonstrated in vitro. Furthermore, the present study also confirms that entrapment of the macromolecule in liposomes can increase this uptake [12]. However, it was reported earlier [11] that most of the liposomes used in previous in vivo studies, and those liposomes used by Bridges et al. [12], released the majority of an entrapped macromolecule in the presence of bile salts in vitro. This suggests that these liposomes would not protect their contents from degradation in the gastrointestinal tract in vivo. The distearoylphosphatidylcholine/cholesterol liposomes used in the present study were shown earlier [11] to be 'stable' in the presence of extremes of pH, bile salts and pancreatic lipase. The demonstration that the uptake of ^{125}I-labelled PVP by rat gut can be increased by entrapment of the macromolecule within these 'stable' distearoylphosphatidylcholine/cholesterol liposomes suggests that these liposomes have the qualities required of a protective carrier for the oral administration of therapeutic agents which would not normally survive or be absorbed in the gastrointestinal tract.

The present study also shows that liposomes can cross the rat gut wall in vitro and can reach the serosal fluid in a relatively intact form. The presence of liposomes in the serosal fluid was not demonstrated by Bridges et al. [12].

The mature small intestine has been shown to absorb proteins and other macromolecules in the intact form [14]. Similar studies of lipid absorption [15,16] have shown evidence of the absorption of intact fat particles by intestinal epithelial cells. Macromolecule uptake under these conditions was thought to be by an endocytic mechanism.

The composition of liposomes used in both the present study and that of Bridges et al. [12] would favour endocytosis rather than membrane fusion [17–21] since at 37°C both dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine are below their transition temperatures of 41°C and 58°C respectively [22]. Uptake of free ^{125}I-labelled PVP by rat yolk sac is by fluid-phase endocytosis [23]. This is also the case in rat intestine (Bridges, J.F., personal communication). Liposomes, however, are thought to adsorb to cell membranes [20,24]. If liposomes were taken up both by fluid-phase endocytosis and by absorption to the membrane of an endocytic vacuole, then the uptake of the liposome-entrapped macromolecule would be greater than that

of the free macromolecule [23,25]. Such as mechanism of uptake could explain the increased uptake of liposome-entrapped ^{125}I-labelled PVP by gut tissue. It is thought [24] that liposomes may initially bind weakly to cell membranes and at this stage they could be removed by washing or they may spontaneously desorb. Later, the liposomes may become more strongly bound to the cell membrane, possibly via a trypsin-sensitive component on the cell surface [26]. The lower rate of uptake of liposome-entrapped ^{125}I-labelled PVP by gut tissue over the first 15—20 min of culture may be due to loss of the loosely bound liposomes during washing of the sacs. This may explain the failure of Whitmore and Wheeler [27] to detect significant uptake of liposome-entrapped ^{22}Na by everted gut sacs over 20 min of culture.

The uptake of free macromolecule was not altered by the presence of 'empty' liposomes, suggesting that liposomes neither stimulate endocytosis nor compete with free macromolecules for uptake. The absence of competition would be expected at low substrate concentrations if the majority of free PVP was taken up by fluid-phase endocytosis, as suggested earlier.

The demonstration of ^{125}I-labelled PVP in the serosal fluid of cultured gut sacs suggests that both free and liposome-entrapped macromolecules are capable of crossing the intestinal wall. The increase in the appearance of ^{125}I-labelled PVP in the serosal fluid when gut sacs were cultured with the liposome-entrapped macromolecule refelcts the increase in uptake by the tissue. The fact that approx. 50% of the ^{125}I-labelled PVP present in the serosal fluid was associated with the $100\,000 \times g$ pellet, and that this ^{125}I-labelled PVP could be released into the supernatant on treatment with Triton X-100, suggests that approx. 50% of the ^{125}I-labelled PVP present in the serosal fluid was entrapped in liposomes.

If a liposome is endocytosed by gut tissue it may, as a phagosome, fuse with a lysosome or it may not. If an endocytic vacuole does not fuse with a lysosome, the contents could be exocytosed intact into the extracellular fluid (in this case the serosal fluid). Since a process is known to occur in endothelial cells [28]. Alternatively if the endocytic vacuole containing the liposomes fuses with a lysosome, the liposomes could be either (a) totally disrupted, releasing their contents into the cytoplasm where it may diffuse to the serosal fluid or (b) partially disrupted, loosing some of their outer layers and releasing some of their contents, before being exocytosed to the serosal fluid eomewhat reduced in size.

Thus, the free ^{125}I-labelled PVP present in the serosal fluid could have arisen from the total or partial disruption of some of the liposomes, while the liposome-entrapped ^{125}I-labelled PVP present in the serosal fluid could be in intact or partially disrupted liposomes. It is not possible to distinguish between these alternatives in the present study.

In conclusion liposomes composed of distearoylphosphatidylcholine/cholesterol appear to have the qualities required of a protective carrier for the oral administration of therapeutic agents which would not normally survive or be absorbed in the gastrointestinal tract i.e. they are stable in the presence of extremes of pH, bile salts and pancreatic lipase [11], can be taken up by adult rat intestine in vitro and can pass to the serosal space in a relatively intact form. These liposomes are at present being tested in the everted gut sac system to

determine whether they can protect a degradable macromolecule during their passage through the gut tissue.

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