UPTAKE OF FREE AND LIPOSOME-ENTRAPPED HORSERADISH PEROXIDASE BY RAT INTESTINAL SACS IN VITRO

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1. Introduction

Several groups have now shown that liposome-entrapped orally administered insulin can reduce blood glucose levels in diabetic rats [1–3]. However, there is some uncertainty over the extent to which liposomes remain intact and protect their contents in the gastrointestinal tract [2–9]. The reported absorption of intact liposomes [2] in vivo has not been confirmed [6]. Furthermore, while no significant uptake of liposome-entrapped material by everted rat gut sacs in vitro, was shown in [10], it was in [11] that the uptake of non-degradable polyvinyl-pyrrolidone could be enhanced in vitro by liposome-entrapment.

It has been shown [12] that both adult and neonatal rat gut sacs can absorb the enzyme horseradish peroxidase (HRP) in vitro and the presence of the enzyme in the serosal fluid can be measured enzymatically. In [8], only liposomes composed of distearoylphosphatidylcholine and cholesterol showed promise as protective carriers for oral use because they retained the majority of an entrapped macromolecule in the presence of extremes of pH, bile salts and pancreatic lipase. This study compares the quantity of HRP present in the serosal fluid of everted rat gut sacs cultured in vitro with free HRP or with HRP entrapped within these 'stable' liposomes. The results show that distearoylphosphatidylcholine/cholesterol liposomes containing HRP can cross the gut wall and can increase the quantity of enzymatically active HRP that reaches the serosal fluid.

2. Materials and methods

2.1. *Preparation of ^{125}I-labelled HRP*

HRP (type II, EC 1.11.1.7., Sigma) was labelled with ^{125}I by the chloramine T method [13] and the resulting solution was then dialysed (72 h at 4°C) in visking tubing against 10 changes of 1% (v/v) NaCl.

Low M_r reaction products and any remaining chloramine T or free iodide were removed by Sephadex G-150 chromatography. The specific activity of the HRP so obtained was 0.01 mCi/mg. The enzymatic activity of the HRP was not altered by the iodination procedure. Of the total radioactivity 98% was precipitated with 10% phosphotungstic acid (Sigma).

2.2. *Liposome preparation*

L-α-Distearoylphosphatidylcholine (Sigma) (27 mg) and 3.8 mg cholesterol (pure, Koch-Light Labs.) in 15 ml chloroform were rotary evaporated under nitrogen at 58°C and the lipid film was resuspended at 58°C, by hand agitation, with 2.5 ml 10 mM phosphate-buffered saline (pH 7.0) containing 150 mg cold HRP with or without 5.0 μCi ^{125}I-labelled HRP. Free HRP was removed by centrifuging the preparation at $10^5 \times g$ (10 min, 20°C) removing the supernatant and resuspending the pellet in fresh buffer. This process was repeated 3 times. It was calculated from radioactivity measurements that the final liposome preparations contained 54.0 ± 4.0 μg HRP/mg lipid (mean ± SE 8 preps.). Separate preparations of liposomes were pooled, centrifuged and resuspended in buffer. This final preparation was then diluted to give the various concentrations of liposomes required. In this way the lipid:HRP ratio remained constant over the

range of liposome concentrations used. The enzyme activity of HRP and the percentage of the total radioactivity that could be precipitated by phosphotungstic acid were unchanged by the liposome preparation procedure. 'Empty' liposomes were prepared by the above method but in the absence of unlabelled or ^{125}I-labelled HRP.

2.3. *Enzyme assay*

HRP was measured by a modification of the *o*-dianisidine method [14] proposed in [15] using 4-hydroxyphenylacetic acid (4-HPAA, Sigma) as fluorogen. The reaction mixture contained 1 ml H_2O_2 (0.3%, v/v), 0.8 ml 4-HPAA (1.522 mg in 10 ml methanol) and 98.2 ml 0.05 M phosphate buffer (pH 6.0). Dilutions of samples and standards were made in Tc 199 medium (Wellcome). A 0.1 ml sample of test solution was mixed with 2.9 ml of reaction mixture and allowed to stand for 10 min at room temperature. The reaction was stopped with 0.6 ml 3.5 M K_2CO_3 and fluorescence was determined in a Perkin-Elmer fluorimeter using an excitation wavelength of 319 nm and an emission wavelength of 410 nm.

2.4. *Liposomal latency*

Aliquots of liposomes containing both unlabelled and ^{125}I-labelled HRP were counted for total radioactivity and the total HRP content was calculated on the basis of an entrapment of 54 μg HRP/mg lipid. The percentage of the total radioactivity and HRP content that was present in a $10^5 \times g$ (10 min) supernatant was determined by γ counting and fluorimetry both before and after incubation of liposomes with Triton X-100 (1.25%, v/v) at 58°C for 30 min. The difference between the two sets of values represents the release of radioactivity and enzyme activity due to treatment of the liposomes with Triton X-100. The enzyme activity of HRP was shown in control experiments to be unaffected by incubation with Triton X-100 (1.25%, v/v) at 58°C or by the presence of 2.78 mg lipid/ml in the form of liposomes.

2.5. *Everted gut sac culture*

The preparation and method of culture of everted sacs of adult rat intestine has been described [11]. Gut sacs were cultured with either: (a) free unlabelled at 37.5, 75.0 and 150.0 μg/ml; (b) free unlabelled HRP at 37.5, 75.0 and 150.0 μg/ml in the presence of 'empty' liposomes at 0.695, 1.39 and 2.78 mg lipid/ml respectively; or (c) 37.5, 75.0 and 150.0 μg unlabelled HRP/ml entrapped in liposomes at 0.695, 1.39 and 2.78 mg lipid/ml, respectively.

After 2 h culture the serosal fluid from each sac was collected and the quantity of enzymatically active HRP present was determined fluorometrically both before and after incubation with 1.25% (v/v) Triton X-100 for 30 min at 58°C. The protein content of the gut sac tissue was determined as in [16]. The quantity of enzymatically active HRP in the serosal fluid was calculated as ng HRP/mg gut sac tissue protein.

3. Results and discussion

Assuming an original entrapment of 54 μg HRP/mg lipid, fluorometric assay of enzymatically active HRP showed that treatments of liposomes with Triton X-100 released 49.3 ± 0.8% (mean ± SE 16 expt) of the entrapped enzyme. The extent of the release was confirmed by γ counting which showed that the same samples released 48.6 ± 1.0% (mean ± SE 16 expt) of their entrapped radioactivity. A 61% release of HRP following detergent treatment of liposomes was reported in [17]. The total quantity of HRP present in a solution containing free and liposome-entrapped HRP can be calculated thus:

$$\text{Total HRP} = [100(Q_T - Q_F)/49.3] + Q_F$$

where Q_F and Q_T are the quantities of HRP measured before and after treatment with Triton X-100 respectively.

Table 1 shows that active HRP can be detected fluorometrically in the serosal fluid of gut sacs cultured with free enzyme and the quantity present increases with the substrate concentration. The presence of 'empty' liposomes in the culture medium containing free HRP had no effect on the quantity of enzymatically active HRP that reached the serosal fluid. Table 1 also shows that Triton X-100 did not affect the fluorometric assay of enzymatically active HRP. Only a small quantity of active HRP could be detected in the serosal fluid of sacs cultured with liposome-entrapped HRP, unless the serosal fluid was treated with Triton X-100 (table 1). Assuming the Triton X-100 released 49.3% of the entrapped enzyme, the total HRP content of the serosal fluid was calculated using the formula stated earlier. The corrected values (table 1) suggest that the majority (95–98%) of the HRP present in the serosal fluid was entrapped

Table 1
Comparison of the quantity of HRP present in the serosal fluid of gut sacs cultured for 2 h with free or liposome-entrapped HRP

	HRP (μg/ml)	Liposomes (mg lipid/ml)	ng HRP/mg tissue protein		
			Before Triton	After Triton	Corrected
Free HRP	37.5	–	1.16 ± 0.07	1.14 ± 0.06	–
	75.0	–	2.33 ± 0.12	2.34 ± 0.14	–
	150.0[a]	–	5.64 ± 0.20	5.60 ± 0.18	–
Free HRP + 'empty' liposomes	37.5	0.695	1.09 ± 0.04	1.11 ± 0.05	–
	75.0	1.390	2.29 ± 0.07	2.30 ± 0.08	–
	150.0	2.780	2.29 ± 0.07	2.30 ± 0.08	–
Liposome-entrapped HRP	37.5	0.695	0.16 ± 0.02	3.79 ± 0.12	7.52 ± 0.24
	75.0	1.390	0.26 ± 0.01	4.89 ± 0.13	9.65 ± 0.26
	150.0[a]	2.780	0.57 ± 0.03	5.78 ± 0.22	11.14 ± 0.45

The quantity of enzymatically active HRP present in the serosal fluid was determined fluorometrically both before and after incubation with Triton X-100 (1.25% (v/v), 30 min, 58°C). Each value represents the mean ± SE 8 expt ([a] 12 expt). The corrected value is derived from the fact that Triton X-100 releases only 49.3% of the entrapped enzyme (see text)

within liposomes. These liposomes had presumably crossed the gut wall in a relatively intact form without fusing with lysosomes. The composition of the liposomes would favour endocytosis rather than membrane fusion since at 37°C distearoylphosphatidylcholine is well below its transition temperature of 58°C [18].

Table 1 shows that entrapment of HRP within liposomes can increase the quantity of enzymatically active HRP that reaches the serosal fluid by up to 6.6-fold. However, the extent of the increase falls from 6.6 to 4.1 to 1.99-fold as the medium concentration of liposomes is raised from 0.695 to 1.39 to 2.78 mg lipid/ml. This suggests that the uptake of liposome-entrapped HRP involves an adsorptive component which gradually becomes saturated as the liposome concentration is raised [19]. The increased uptake of liposome-entrapped HRP was not due to disruption of the liposomes in the medium and re-association of the HRP with the lipid as empty liposomes had no effect on the uptake of free HRP (table 1). Entrapment of non-degradable ^{125}I-labelled polyvinylpyrrolidone in liposomes only increased the quantity of macromolecule that reached the serosal fluid by 2-fold [11], even though the liposome concentration was considerably lower than here. This is probably because free HRP is susceptible to degradation by both brush border and intracellular enzymes [20], while liposome-entrapped HRP is protected. This protection from enzymic degradation would not be apparent when using a non-degradable macromolecule.

In conclusion, distearoylphosphatidylcholine/cholesterol liposomes can cross the intestine wall and increase the quantity of a degradable macromolecule that reaches the serosal fluid in vitro. Given that these liposomes would probably protect their contents from intraluminal digestion in vivo [8], they appear to have the qualities required of a protective carrier for the oral administration of therapeutic agents.

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