# CHAPTER II

# DETECTION OF LIPOSOMES IN PORTAL BLOOD FOLLOWING ORAL ADMINISTRATION

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## II.1 Summary :

$^{125}$Iodine labelled human immunoglobulin G encapsulated liposomes were prepared. Glycosylated liposomes were made by incorporating asialoganglioside in liposomes. The presence of exposed galactose residues on the surface of asialoganglioside liposomes was checked by the aggregation of liposomes with the lectin Recinus Communis agglutinin. Rats were fed orally with the liposomes and at different time intervals distribution of radioactivity was checked in liver and in blood of the portal vein and heart. Blood from portal vein and heart was centrifuged and the plasma was passed through the Sepharose 2B column to check the integrity of liposomes and the encapsulated protein. Gel filtration studies showed the presence of intact liposomes and protein in blood plasma from portal vein. In contrast, neither liposomes nor protein was detected in cardiac blood but only lower molecular weight materials. The presence of liposomes in portal blood was further suggested from the experiments using 6-carboxyfluorescien entrapped liposomes. The level of liposomes in portal blood plasma was found to be lower in asialoganglioside liposomes. But this level could be increased almost to that in liposomes without asialoganglioside, by prior injection (i.v.) of a glycoprotein asialofetuin. Inhibition of liver uptake of asialoganglioside

liposomes was noticed by the injection (i.v.) of asialofetuin.

II.2 Introduction :

In recent years, liposomes have gained considerable interest for the possible use as vehicles for the transport of drugs and bioactive molecules to specific tissues (1-3). Among various routes of administration oral feeding is the simplest and has obvious advantages.

There are evidences that the normal adult gastrointestinal tract is permiable to macromolecules including proteins, in quantities large enough to be biologically active (4). It has been reported that microscopic particles can pass through intestinal mucosa (5,6). It was reported that insulin entrapped in liposomes and administered orally could reduce the blood glucose level in diabetic animals (7,8). Similarly, oral administration of blood coagulation factor VIII in liposomes to a hemophilic patient led to a higher rise in blood than did that of the free component (9). The applicability of liposomes as oral therapeutic carriers was further suggested by the in vitro results regarding the stability of different liposomes in the presence of bile salts, low pH, and pancreatic lipases (10). It was shown that liposomes made from synthetic phospholipids e.g., dipalmitoylphosphatidylcholine or distearoylphosphatidylcholine, impart greater stability than those made from natural phospholipids.

Here study has been carried out with $^{125}$Iodine

labelled HIgG encapsulated liposomes made from natural and synthetic phospholipid to investigate the possibility of liposomes as oral therapeutic carriers. The amount of liposomes and protein have been quantitated in the portal and cardiac blood. Glycosylated liposomes were also used to observe the effect of sugar coated liposomes on the uptake by the liver at different time after the oral administration.

## II.3 Materials and Methods

### II.3.1 Reagents :

Egg phosphatidylcholine (Pc), cholesterol (chol), and beef brain gangliosides were obtained from the CSIR Centre for Biochemicals, Delhi, India; fetuin Type III, DL-3--dipalmitoylphosphatidylcholine (DPPC), human immunoglobulin G (HIgG), and dicetyl p-osphate (DCP) were from Sigma chemical Company, St. Louis, Missouri; carrier-free Na-$^{125}$I was from BARC, Bombay, India; 6-carboxyfluorescein (CF) was from Eastman Kodak.

### II.3.2 Radioiodination of human immunoglobulin G :

Human immunoglobulin G was iodinated with $^{125}$I following the method of Hunter (11). Ten µl of 10 mg/ml HIgG (100 ug) was mixed with 0.5 mCi of Na$^{125}$I, 5 uL chloramine-T (10 mg/ml) and 10 µL of 0.5 M Sodium phosphate buffer pH 7.5 and allowed to react at room temperature. After 1.5 minutes, the reaction was terminated by adding 5 µL of sodium metabisulphite (20 mg/ml). One minute after the addition of sodium metabisulphite, 5 µL of KI (188 mg/ml) and 10 µL of BSA (100 mg/ml) were added. The $^{125}$I-

-labelled HIgG was separated from the free radioactive iodine by gel filtration on a Sephadex G50 column. The packed column (35 x 1 cm) was equilibrated with 0.01 M sodium phosphate buffer. PH 7.5, containing 0.15 M NaCl and 1 mg/ml BSA. The reaction mixture was then loaded on the column and eluted with equilibrating buffer. One ml fractions were collected and 10 uL aliquote were counted in a Packard Autogamma counter 5110 ( (70% efficiency). The $^{125}$I-HIgG had a specific radioactivity 10 x $10^5$ cpm/ugm.

II.3.3 Preparation of asialoganglioside and asialofetuin :

II.3.3.1 Preparation of asialoganglioside :

Asialoganglioside $GM_1$ was prepared by cleaving the N-acetyl neuraminic acid by incubating ganglioside $GM_1$ at 80°C in 0.1 N $H_2SO_4$ and the liberated N-acetyl-neuraminic acid separated by dialysis against water. The dialysate were freeze dried under vaccum (12).

II.3.3.2 Preparation of asialofetuin :

Twenty mg of Fetuin and 100 units neura minidase per ml were incubated at 37°C for 9 h in 0.1 M sodium acetate buffer pH 5.6 containing 0.15 M NaCl and 0.002 M CaCl2 in presence of 1% toluene. The neuraminidase-treated fetuin (designated as asialofetuin) was separated from neuraminidase by chromatography on DEAE-Sephadex A-25.

II.3.4 Preparation of liposomes :

Liposomes were made with a mixture of phospholipid/cholesterol/dicetyl phosphate in a 7 : 2 : 1 molar ratio according to the method of Gregoriadis and Ryman (13). In

brief, the lipid mixture was taken in chloroform-methanol (1 : 1, v/v). A thin film on the walls of the flask was formed by rotary evaporation of the solvents at 37° under reduced pressure. The flask was then kept in a vaccum dessicator overnight. The lipid film was then dispersed in 0.025 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 10 mg/ml HIgG, mixed with a trace amount of $^{125}$I--HIgG. The dispersion was completed by brief sonication for 30 sec at 4° in an MSE ultrasonicator. The liposomes suspension was kept for 2 hr at 4° after which it was centrifuged at 150,000 g for 60 min to separate the unencapsulated protein from the liposomes. The pellet was resuspended in buffer and washed further for a few more times. Liposomes containing DPPC were prepared according to the method of Gregoriadis and Ryman (13). Negatively charged DPPC liposomes were prepared from DPPC, cholesterol and Dicetyl phosphate in the molar ratio 7:2:1 and asialoganglioside DPPC liposomes were prepared from DPPC, cholesterol, asialoganglioside and Dicetyl phosphate in the molar ratio 7:2:2:1. The lipid mixture was taken in chloroform : methanol (1 : 1) and a thin film was formed on the walls of the flask after rotary evaporation at 37°C as described above. The film was dispersed in buffer containing $^{125}$I-HIgG by gently shaking for 10 min at 65° (which is above the phase Tc of DPPC). The suspension was kept at room temperature for 2 hr after a brief sonication for 30 sec and washed three times by ultracentrifugation as mentioned above.

For CF-entrapped liposomes, the dry film of lipids

was suspended in 0.1 M dye in PBS. The dispersion was completed by brief sonication (30 sec) with an MSE ultrasonicator. The liposomes with entrapped dye were separated from the unencapsulated materials by repeated washing in buffer by ultracentrifugation at 150,000 g for 60 min.

II.3.5 Animal experiments :

Male Swiss albino rats (IICB strain) weighing approx 100-110 g were used. Oral administration of liposomes was performed on the rats 30 to 40 min after they were being fed a standard diet. The liposomal suspension (0.5 ml, 1.0-1.5 mg lipids) containing 13-15 x $10^5$ cpm $^{125}$I-HIgG or CF-entrapped liposomes when appropriate was introduced intragastrically through a polythene tubing into each animal. As a control, free $^{125}$I-HIgG (13-15 x $10^5$ cpm) was also fed. After appropriate time intervals blood was collected in heparinized tubes from the heart and portal vein under ether anesthesia. The plasma was prepared by centrifugation at 2000 rpm for 15 min.

The same dose of liposomes was fed to another group of rats, and the animals were sacrificed at different times. The livers were removed and then washed with 0.9% NaCl solution and blotted with filter paper. The whole liver was digested in 30% KOH solution. The digested tissue (1.0 ml) and blood plasma (0.5 ml) were taken for radioactive counting. Total radioactivity in blood plasma was calculated by considering 6 ml of total blood plasma from each rat of 100 g body weight (14).

II.3.6 Inhibition studies with asialofetuin :

$^{125}$I-HIgG-encapsulated asialoganglioside liposomes and control liposomes (without asialoganglioside) were fed to rats. Asialofetuin (8-10 mg) was injected in the tail vein at different periods prior to the measurement of radioactivity in liver and blood from the portal vein.

II.3.7 TCA Precipitation of blood plasma :

Blood was collected at different time from the portal vein and heart of treated animals. Plasma was precipitated with cold TCA (Final conc 10%) following the treatment with Triton x 100 (1% final conc). The precipitate was washed twice with PBS and radioactivity was checked in the pellet.

II.3.8 Gel filtration of plasma :

The blood from the portal vein and heart was collected after 1.5 and 3 hr of feeding by which time radioactivity appeared in these tissues was analyzed by gel filtration on Sepharose 2B, and Sephadex G100 column (1 x 25 cm) which were prestandarized with $^{125}$I-HIgG and $^{125}$I-HIgG encapsulated liposomes. The eluted fractions were analyzed for $^{125}$I-radioactivity.

II.3.9 Assay of liposomal integrity in blood :

To measure the integrity of CF containing liposomes CF latency was measured. A spectrofluorimeter (Aminco Bowman) was set up with excitation and emission wavelength of 490 nm and 520 nm respectively and standardized by using

$10^{-5}$M CF. Free CF in one ml was measured by diluted to 3 ml with PBS. Total CF was measured by adding Triton x 100 (Final concentration 1%) which disrupts lipid bilayers to release all entrapped dye. Latency was calculated from

$$\% \text{ CF latency} = \frac{\text{dye t} - \text{dye f}}{\text{dye f}} \times 100$$

where 't' was total CF present measured after addition of Trition x 100 and 'f' was free CF measured before the addition of detergent.

CF-entrapped DPPC liposomes were fed to rats and blood from the portal vein and heart was collected at 1.5 and 3 hr respectively after the feeding. The latent CF in plasma was measured as above.

## II.4 Results

### II.4.1 Distribution of radioactivity :

Figure 1 depicts the pattern of radioactivity in blood plasma obtained from cardiac puncture after feeding rats orally with asialoganglioside and other liposomes. It can be seen that the radioactivity appeared early (1 h) in the case of free $^{125}$I-HIgG and was cleared from the blood at a rate quite fast compared to the protein encapsulated in liposomes. An appreciable amount of radioactivity (2.5-3.5% of total dose) appeared in plasma 3 h after feeding in the case of liposomal-encapsulated protein.

### II.4.2 The half-times ($t_{1/2}$) of radioactivity clearance from circulation :

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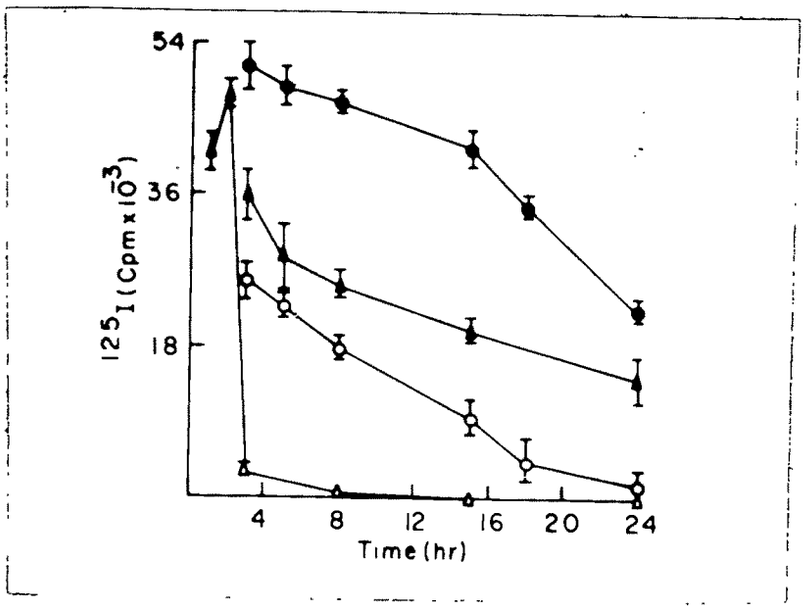


Fig.1 - Blood (cardiac) clearance of radioactivity in rats fed with various types of liposomes. Male Swiss albino rats (100-110 g ) were fed with $^{125}$iodine-labeled human immunoglobulin G (13-15 x $10^5$ cpm) entrapped in liposomes (1.0-1.5 mg lipids). The total volume of liposomal suspension in all cases was approximately 0.5 ml. At different time intervals blood was taken by cardiac puncture, and the amount of radioactivity was measured in 0.5 ml of blood plasma, and then total radioactivity per animal was calculated as described under Materials and Methods. In each case the vertical bars represent ± SD. asialcganglioside DPPC liposomes (●); DPPC liposomes (▲); PC liposomes (O); free HIgG (△).

circulation in rats fed with asialoganglioside DPPC liposomes, DPPC liposomes, and PC liposomes were 4.85, 4.81, and 2.1 h, respectively.

II.4.3 Amount of radioactivity in portal blood :

The radioactive count in blood plasma from the portal vein is shown in Fig.2. It can be seen that there is a sharp fall of radioactivity in the case of asialoganglioside liposomes while the count persists up to 16 h in DPPC liposomes (control liposomes).

We have compared the liver uptake of radioactivity after feeding of the liposomes with or without asialoganglioside (Fig.3).

II.4.4 Uptake of orally administered liposomes by liver :

Uptake of different types of liposomes by liver have been studied after oral administration of that liposomes. The differential uptake of various liposomes by liver have been studied with or without asialoganglioside (Fig.3). In the case of asialoganglioside liposomes, the rate was remarkably high in comparison with control liposomes.

II.4.5 Presence of liposomes in portal blood :

The blood from the portal vein and heart was collected after 1.5 and 3 h of feeding, by which time radioactivity appeared in these tissues. The plasma was passed through a Sepharose 2B column to establish the presence of liposomes or undegraded HIgG in the plasma. The radio-

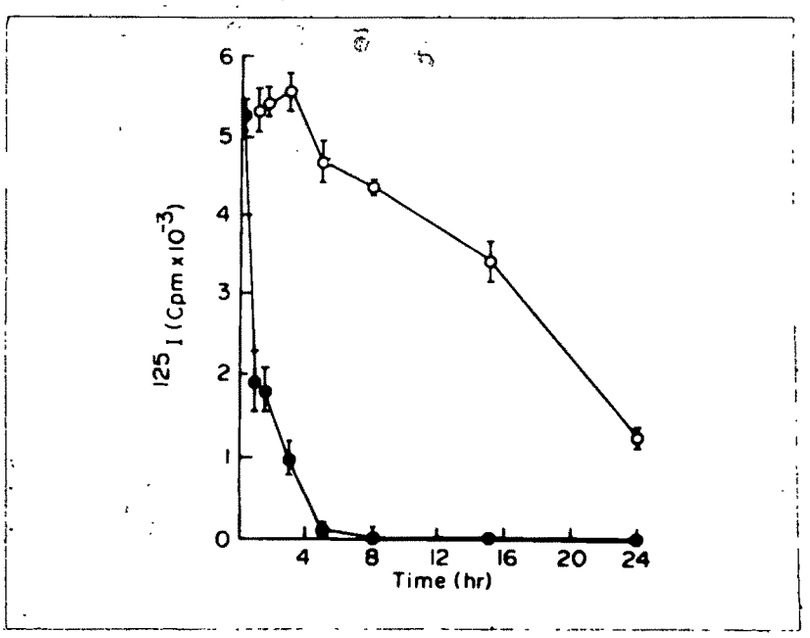


Fig.2 - Amount of radioactivity in 0.5 ml of portal venous blood plasma of rats fed with asialoganglioside DPPC liposomes (●) and DPPC liposomes (O). Following the conditions as described in Fig.1, blood was taken from the portal vein and the amount of radioactivity was measured in 0.5 ml blood plasma.

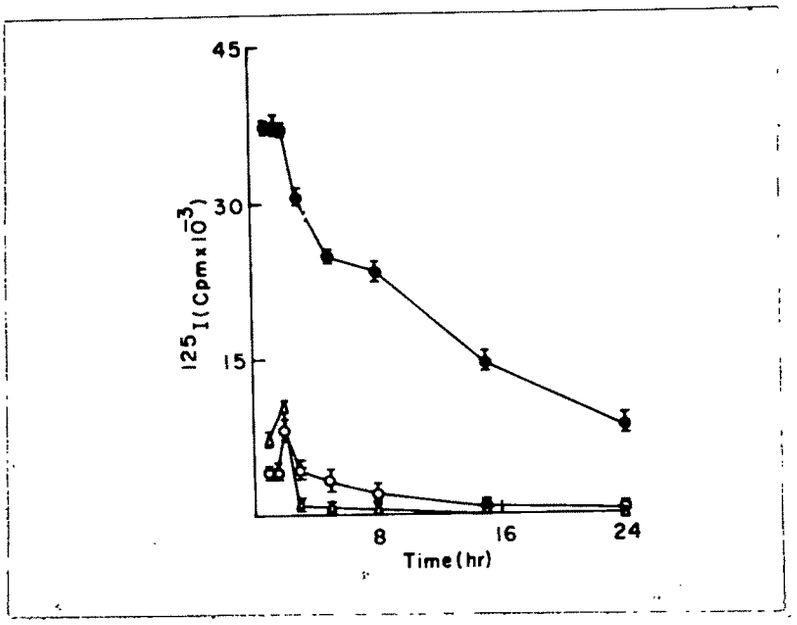


Fig.3 - Amount of radioactivity in livers of rats fed with asialoganglioside DPPC liposomes (●); DPPC liposomes (O); and free HIgG (△). At different time intervals whole livers were taken out, washed with 0.9% NaCl solution, blotted with filter paper and digested in 30% KOH solution, and the volume was made 20 ml with KOH solution. One milliliter of the digested liver was taken for counting.

activity was obtained in liposomal and protein fractions from the column loaded with plasma from the portal vein. The amount of radioactivity associated with these two peaks (Fig.4) was dependent on the types of liposomes used in oral feeding. For example, in the case of control liposomes about 50% of the radioactivity was in the first peak (liposomal peak), but only 15% of the radioactivity was obtained in this peak when asialoganglioside liposomes were administered. On the other hand, the presence of intact protein seems to be higher in the latter compared to control liposomes. It is interesting to note that undegraded protein was not found in portal blood from rats fed with free IgG (Fig.4d).

II.4.6 Characterisation of nature of radioactivity appeared in cardiac blood :

When plasma from the heart was passed through the Sepharose 2B column, neither liposome nor protein was detected (data not given). To characterize the nature of radioactivity in the cardiac blood, plasma was then fractionated on a Sephadex G-100 column through which liposomes or intact HIgG, if present, would appear in the void volume (Fig.5). The elution profile showed that there is neither intact protein nor liposomes present in cardiac blood. Asialoganglioside and control liposomes, as well as the free HIgG, behaved similarly, although there may be quantitative differences in their elution profiles.

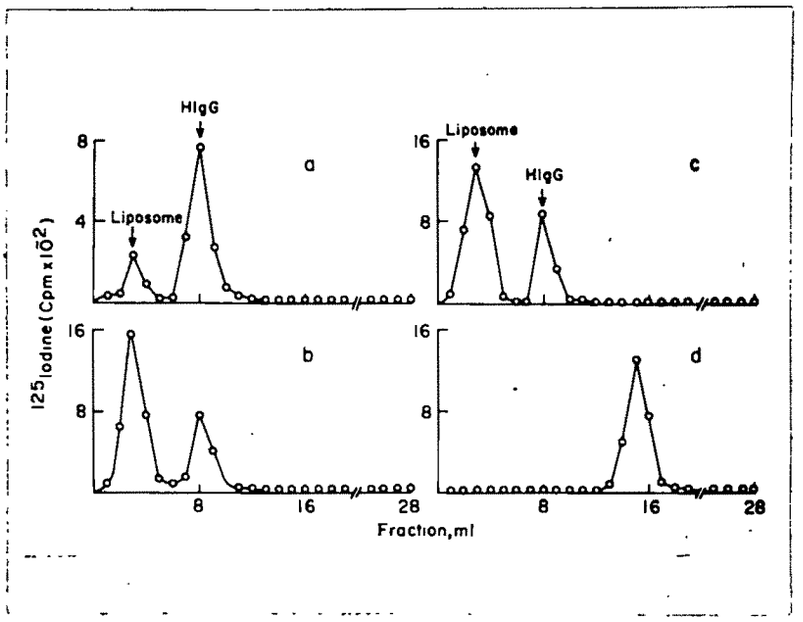


Fig.4 - Elution profile from Sepharose 2B column (1x25cm) of plasma taken from portal vein of rats 1.5 h after they were fed $^{125}$I-HIgG-encapsulated asialoganglioside DPPC liposomes (a); DPPC liposomes (b); asialoganglioside liposomes followed 80 min later with i.v. injection of asialofetuin (c); or free $^{125}$iodine-labeled HIgG (d). The elution positions of intact $^{125}$I-HIgG and $^{125}$I-HIgG-encapsulated liposomes are indicated with arrows. In each case 0.5 ml of blood plasma was loaded onto the column. Radioactivities associated with 0.5 ml of plasma were 2250 cpm (a); 5500 cpm (b); 5100 cpm (c); and 2500 cpm (d).

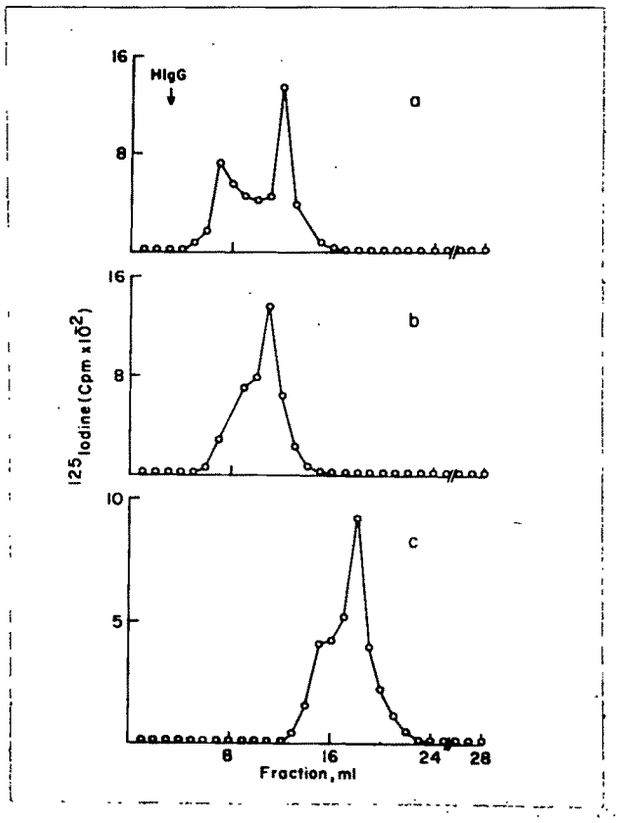


Fig.5 - Elution profiles from Sephadex G-100 column (1 x 25 cm) of blood plasma obtained from cardiac puncture of rats 3 h after feeding $^{125}$I-HIgG-encapsulated asialoganglioside DPPC liposomes (a); DPPC liposomes (b); and 1.5 h after feeding free $^{125}$I-HIgG (c). In each case 0.5 ml of blood plasma was loaded onto the column. The elution position of intact $^{125}$I-HIgG is indicated by the arrow.

II.4.7 Determination of latency values of CF in plasma from cardiac and portal vein blood :

In order to provide additional evidence for the integrity of liposomes during oral feeding, blood was collected from the heart and portal vein of rats fed with CF-entrapped liposomes. The latency values of CF in the plasma was determined. On taking the latency values as the index of liposomal integrity, it was observed that about 45% latent liposome was present in the portal blood but not particularly in the cardiac blood.

II.4.8 Inhibition with asialofetuin :

The effect of asialofetuin on liver uptake of asialoganglioside liposomes was checked. Since it was noted that radioactivity appeared in the liver around 1.5 h after the oral feeding (Fig.3), asialofetuin was administered to the liposome-fed rats at different times prior to 1.5 h. The $t_{\frac{1}{2}}$ of asialofetuin in the circulation in rats is known to be about 2 min (15). We found that the maximum inhibition of liver uptake was noticed when asialofetuin was injected at 80 min after the feeding, i.e., 10 min before the radioactivity is observed in the liver. But no effect on the DPPC liposomes was observed at any time (Table 1). A higher amount of radioactivity was noticed in portal blood from asialoganglioside liposomes in the presence of asialofetuin.

TABLE I

Effect of Asialofetuin Injection (iv) on the Uptake of Orally Administered Liposomes by Liver

| Type of liposome | Mean percentage of radioactivity in liver ± SD | | | | |
|---|---|---|---|---|---|
| | Without asialofetuin injection | Postfeeding time interval (min) | | | |
| | | 70 | 75 | 80 | 85 |
| Asialoganglioside | 2.2±0.08 | 2.25±0.14 | 1.05±0.05 | 0.92±0.03 | 0.95±0.06 |
| Control (without asialoganglioside) | 0.31±0.04 | 0.34±0.05 | 0.29±0.08 | 0.40±0.13 | 0.32±0.03 |

Note. Liposomes with entrapped $^{125}$I-HIgG (13-15 x $10^5$ Cpm) were introduced into rats which were treated with or without asialofetuin (8-10 mg in the tail vein of each animal) at different times after the feeding. Radioactivity was checked in the liver 1.5 h after feeding.

II.4.8.1 Plasma Fractionation on Sepharose 2B column :

Plasma fractionation on a Sepharose 2B column showed the amount of radioactivity associated with liposomes (Fig.4c). It is interesting to note that the amount of radioactivity associated with liposomes in portal blood now had become comparable to that found in DPPC liposomes.

II.4.9 TCA precipitation of blood plasma :

Blood plasma of treated rats was precipitated with TCA (10% final concentration) and the amount of radioactivity was checked in it (Table II). It has been observed that about 80% and 45% of the count in portal blood plasma was associated with the precipitate at 3h after the feeding of DPPC and PC liposomes respectively. The value decreased gradually with time. On the other hand, no detectable amount of radioactivity was observed at any time in the precipitate obtained from cardiac blood plasma of rats fed with DPPC or PC liposomes.

II.5 Discussion :

An attempt has been made in the present work to investigate the integrity of liposomes in oral feeding programs using rats as the experimental animal. Gel filtration of plasma from the heart and portal vein of rats fed with $^{125}$I-HIgG-entrapped liposomes was performed. Additional evidence for intact liposomes has been provided from latency values of CF-entrapped liposomes in plasma.

The latency values suggest the presence of liposomes

Table II.

TCA precipitation of blood plasma of rats fed with $^{125}$I-HIgG encapsulated PC and DPPC liposomes.

| Liposomes composition | Time of taking blood (h) | Radioactivity (%) | |
|---|---|---|---|
| | | Portal vein | Heart |
| PC/Cholesterol/DCP | 1.5 | - | - |
| | 3 | 45 | - |
| | 5 | 12 | - |
| | 8 | 6 | - |
| | 15 | - | - |
| DPPC/Cholesterol/DCP | 1.5 | 77 | - |
| | 3 | 80 | - |
| | 5 | 50 | - |
| | 8 | 45 | - |
| | 15 | 20 | - |

/ Results are expressed as percentage of total radioactivity in blood plasma /

in portal vein blood but not in cardiac blood. From gel filtration, it is estimated that about 75% of the radioactivity in portal vein blood is associated with either liposomes or free protein. The majority (50%) of the counts remains with the liposomal fraction for rats fed with DPPC liposomes. TCA precipitate data is in agreement with the view. But the amount of counts associated with liposomes was found to be low in the case of rats treated with asialoganglioside liposomes. An appreciable increase in this value could be achieved by asialofetuin injection and it reached almost the level of control liposomes. The gel filtration of plasma from cardiac blood, on the other hand, did not show the presence of intact liposomes or HIgG in the case of either the control or asialoganglioside liposomes. Interestingly, radioactive counts were associated with lower molecular weight fragments in cardiac blood as judged from the chromatographic pattern. Experiments with CF-entrapped liposomes also showed that liposomes were not present in cardiac blood in contrast to blood from the portal vein. These results can be explained by assuming that liposomes (or protein) present in portal blood were taken up by the liver, processed there, and then circulated through the heart. Such a view is further supported by the observation that the liver uptake of radioactivity is higher for asialoganglioside liposomes. This could be due to the involvement of a specific interaction of terminal $\beta$-galactose on the liposomal surface with the liver cells, as these cells are known to possess galactose-specific receptors (16-18).

As a corollary, marked inhibition on the liver uptake was noticed by asialofetuin. Previous work from this laboratory (19,20) had demonstrated that liposomes containing asialogangliosides or those covalently-coupled with p-aminophenyl-β-D-galactoside were able to facilitate the liver uptake significantly.

At present, the mode of absorption of liposomes from the gut is not clear. One report (21) claimed that intact liposomes could be absorbed from the intestine into the circulation. This observation is disputed by others (22,23). Results as presented here show the presence of liposomes in portal vein and indirectly suggest that intact liposomes can be absorbed from the intestinal tract into portal blood. The presence of undegraded HIgG in portal blood could be due to destabilization of liposomes by plasma factors, since the liposomes used in this study were cholesterol poor (24). After absorption, the liposomes are then taken up by the liver, which disrupts the integrity of both liposome and the encapsulated protein. The absence of liposomes or HIgG in cardiac blood is in agreement with this view. Since no undegraded HIgG was present in portal blood after oral feeding with free HIgG, in contrast to the results obtained with liposomal HIgG, it is concluded that liposomes could protect the encapsulated protein to some extent in the gut.

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