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INTRAHEPATIC UPTAKE AND PROCESSING OF INTRAVENOUSLY INJECTED SMALL UNILAMELLAR PHOSPHOLIPID VESICLES IN RATS

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Small unilamellar vesicles consisting of sphingomyelin, cholesterol and phosphatidylserine in a molar ratio of 4:5:1 containing [^{3}H]inulin as a marker of the aqueous space or [*Me*-^{14}C]choline-labeled sphingomyelin as a marker of the lipid phase were injected intravenously into rats. After separation of the non-parenchymal cells into a Kupffer cell fraction and an endothelial cell fraction by elutriation centrifugation analysis of the radioactivity contents demonstrated that Kupffer cells were actively involved in the uptake of the vesicles whereas endothelial cells did not contribute at all. Uptake by total parenchymal cells was also substantial but, on a per cell base, significantly lower than that by the Kupffer cells. By comparising the fate of the [^{3}H]inulin label and the [^{14}C]sphingomyelin label it was concluded that release of liposomal lipid degradation products especially occurred from Kupffer cells rather than from parenchymal cells. In both cell types, however, substantial proportions of the ^{14}C-label accumulated in the phosphatidylcholine fraction, indicating intracellular degradation of sphingomyelin and subsequent phosphatidylcholine synthesis. Treatment of the animals with the lysosomotropic agent chloroquine prior to liposome injection effectively blocked the conversion of the choline-labeled sphingomyelin into phosphatidylcholine in both cell types. This observation indicates that uptake of the vesicles occurred by way of an endocytic mechanism.

Introduction

In recent years numerous reports have demonstrated the major role of the liver in the uptake of intravenously injected liposomes [1–6]. However, relatively little is known about the intrahepatic processing of the vesicles. We and others were able to show that large liposomes are mainly taken up by Kupffer cells, while substantial proportions of small unilamellar vesicles are associated with hepatocytes as well [2,5,7]. These small vesicles (diameter 25–80 nm) are considered to have direct access to this cell type by penetrating the fenestrations (diameter 30–200 nm) in the endothelial lining of the liver sinusoids.

For Kupffer cells we demonstrated with large liposomes that uptake occurs by way of endocytosis both in vitro and in vivo [8,9]. The uptake mechanism of the vesicles by hepatocytes, however, has not be clarified. One of the reasons is the relatively low capacity of this cell type to internalize liposomes. With hepatocytes in monolayer culture Hoekstra et al. [10] obtained evidence that multilamellar liposomes are taken up, at least in part, by way of an endocytic mechanism. After in vivo administration, however, the uptake mechanism of the vesicles by hepatocytes is still unknown. In addition, little information is available on the intracellular fate of the liposomal phospholipids.

In this study we injected small unilamellar vesicles containing sphingomyelin as the major phospholipid constituent. The small size would allow optimal access to the parenchymal cells; the labeled sphingomyelin would facilitate the observation of intracellular lipid processing. An investigation of the effect of chloroquine, finally, was intended to establish a possible involvement of an endocytic pathway.

Materials

Cholesterol, bovine brain sphingomyelin, L-α-phosphatidyl-L-serine and collagenase type I were purchased from Sigma. Pronase E was obtained from Merck; DNAase type I, grade II and chloroquine from Boehringer. [^{3}H]Inulin (904 mCi/mmol) was obtained from the Radiochemical Centre Amersham, U.K.; [*N-methyl*-^{14}C]sphingomyelin was prepared according to the method of Stoffel [11].

Methods

Small unilamellar vesicles consisting of sphingomyelin, cholesterol and phosphatidyl serine in a molar ratio of 4 : 5 : 1 were prepared as described previously [17]. When [*N-Me*-^{14}C]choline-labeled sphingomyelin was incorporated as a marker of the liposome membrane the specific radioactivity in the final vesicle preparation was approx. 83.000 dpm/μmol lipid. When [^{3}H]inulin was used as a marker of the aqueous space the specific radioactivity was 60.000 dpm/μmol lipid. Based on phosphorus content and ^{3}H-radioactivity of the concentrated vesicle samples an internal volume of 0.27 ± 0.03 μl/μmol lipid ($n = 5$) was calculated for the liposome preparations used.

Animals. Liposomes (5 μmol of total lipid per 100 g body weight) were injected into the femoral vein of fasted female Wistar rats of 175–200 g body weight under diethyl ether anesthesia. The treatment regimen with chloroquine was adapted from Stein et al. [12]. Details of this procedure are presented in Table II.

Total liver uptake. To determine the uptake of the liposomal radioactive labels the liver was perfused via the portal vein with isotonic saline for 5 min at 37°C at 26 ml/min. Livers were excised and homogenized in a Potter-Elvehjem tube in water.Subsequently the homogenates were subjected at 4°C to six 10-s bursts of 60 W delivered by a Branson B-12 Sonifier® and samples were taken in triplicate for measurement of radioactivity.

Uptake by hepatocytes and non-parenchymal cells. Hepatocytes were isolated by perfusion of the liver for approx. 10 min with 0.03% collagenase followed by low speed centrifugation of the resulting cell suspension to separate hepatocytes from non-parenchymal cells as described before [2]. Non-parenchymal cells were isolated by in situ perfusion of the liver with 0.2% pronase for 3–4 min and subsequent incubation of the liver tissue with the enzyme solution for an additional 60 min at 37°C to digest the parenchymal cells. After removing cell debris and erythrocytes by Metrizamide® gradient centrifugation Kupffer and endothelial cells were separated by elutriation centrifugation. Details of this procedure are also extensively described in Ref. 2.

In some experiments isolation of non-parenchymal cells was performed at 10°C as described by Praaning-Van Dalen and Knook [13] with the aim to slow down intracellular degradation and/or conversion of liposomal phospholipid during the isolation procedure. The liver was perfused in situ with Gey's balanced salt solution (GBSS) at 37°C for 3 min to remove the blood. Pronase perfusion (0.2% pronase-E) was done at 10°C for 10 min (10 ml/min) followed by perfusion with Gey's balanced salt solution containing 1.3% bovine serum albumin at 10°C for 2 min (15 ml/min). The in vitro incubation with pronase was omitted. The Metrizamide® gradient centrifugation was done at 4°C.

Samples (0.5 ml) of liver cell suspensions were mixed with 0.5 ml 30% H_2O_2 and decolorized for 1 h at 60°C. Radioactivity was assayed with Plasmasol [14] as a scintillation mixture.

Aliquots of the cell suspensions were extracted with chloroform/methanol according to Bligh and Dyer [15] and the phospholipids were separated by thin-layer chromatography on 0.5 mm Silica HF Gel from Merck with chloroform/methanol/conc. ammonia/water (90 : 54 : 5.5 : 5.5, v/v) as a solvent system. Relevant spots were scraped from the plates and mixed with 0.5 ml water. Radioactivity

was measured in 5 ml Hydroluma (Lumac BV, The Netherlands) as a scintillation mixture.

Results

The kinetics of hepatic uptake of intravenously injected liposomes labeled with [^{14}C]sphingomyelin or with [^{3}H]inulin is shown in Fig. 1. [^{3}H]inulin was used since it is metabolically inert and, if administered in free form, it is not taken up by liver cells but rapidly excreted via the kidneys. At all times uptake of the vesicles as calculated from the ^{14}C-radioactivity was significantly lower than that calculated from the ^{3}H-radioactivity which was taken to indicate the occurrence of intrahepatic degradation of liposomal sphingomyelin and subsequent release of water-soluble labeled degradation products such as choline or phosphorylcholine.

From experiments with [^{3}H]inulin-containing vesicles we established first that, similar to our previous observations with multilamellar and large unilamellar vesicles [2], also small unilamellar vesicles are not taken up to any appreciable extent by endothelial cells. Virtually all of the radioactivity in the non-parenchymal cells was recovered in the Kupffer cells (Table I). Therefore, in the following experiments we did not bother to further fractionate the non-parenchymal cell fraction but calculated from the radioactivity associated with this fraction the uptake of liposomal radioactivity by the Kupffer cells. The proportion of Kupffer cells in the crude non-parenchymal cell fraction

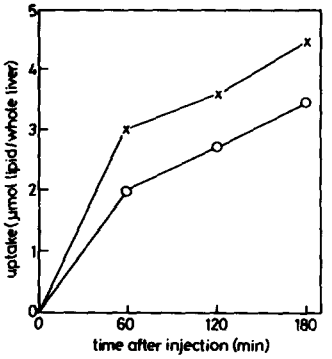

Fig. 1. Liver uptake of small unilamellar vesicles. Rats were injected intravenously with liposomes (5 μmol of total lipid per 100 g body weight; for composition see Methods). The vesicles were labeled with [^{14}C]sphingomyelin or with [^{3}H]inulin. At times indicated the liver was perfused with isotonic saline to remove blood and processed as described in Methods. The results as presented in Figs. 1–3 are from single, representative, experiments. × ——— ×, ^{3}H-radioactivity; ○ ——— ○, ^{14}C-radioactivity.

was determined by cytochemical staining of endogenous peroxidase activity: Kupffer cells are strongly positive while endothelial cells lack peroxidase activity.

Fig. 2 shows the kinetics of the two liposomal labels i.e. [^{3}H]inulin and [^{14}C]sphingomyelin by Kupffer and parenchymal cells. From the [^{3}H]-inulin curve it is clear that, on a per cell base, uptake by Kupffer cells far exceeds uptake by the parenchymal cells. However, uptake by the total amount of parenchymal cells present in the liver is still considerable, since the hepatocytes outnumber

TABLE I

UPTAKE OF [^{3}H]INULIN-LABELED VESICLES BY KUPFFER AND ENDOTHELIAL CELLS IN THE LIVER 2 H AFTER INTRAVENOUS INJECTION

[^{3}H]Inulin-labeled vesicles (5 μmol lipid per 100 g body weight) were injected intravenously. At 2 h after administration non-parenchymal cells were isolated and separated in an endothelial cell fraction and a Kupffer cell fraction as described in Ref. 2. Kupffer cells could be identified cytochemically on basis of endogenous peroxidatic activity, while endothelial cells lack peroxidase activity. Cells were processed for measurement of radioactivity content as described in Methods. Data are shown as the mean of duplicate measurements ± S.D. EC, endothelial cells; KC, Kupffer cells.

Injected dose (μmol/100 g body wt.)	Cell type isolated	Peroxidase-positive cells (%)	dpm per 10^6 cells	dpm per 10^6 cells corrected for contamination	Uptake (nmol lipid/10^6 cells
5	EC	9.5 ± 0.7	57 ± 2.8	−65 ± 11.3	0
	KC	87.0 ± 4.2	1130 ± 162	1305 ± 251	16.7 ± 3.18

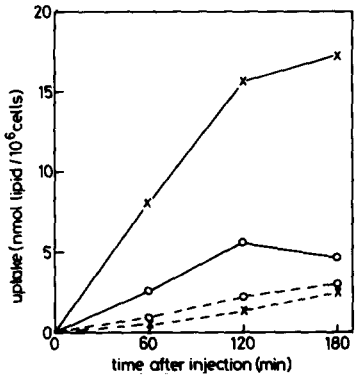

Fig. 2. Uptake of ^{3}H-label or ^{14}C-label by Kupffer and parenchymal cells of rat liver following injection of small unilamellar vesicles labeled with [^{3}H]inulin or with [^{14}C]sphingomyelin. At times indicated livers were perfused with pronase or with collagenase at 37°C to isolate the various cell fractions as described in Methods. For processing of samples of the cell suspensions and for measurements of radioactivity contents see Methods. ×——×, ^{3}H-radioactivity; ○——○, ^{14}C-radioactivity. Uptake by Kupffer cells, solid curves; uptake by parenchymal cells, dashed curves.

the Kupffer cells by a factor of approx. 10.

By comparing the fate of the two labels in both cell types we observed that in the Kupffer cell fraction ^{14}C-radioactivity is substantially lower than ^{3}H-radioactivity, whereas in the hepatocyte

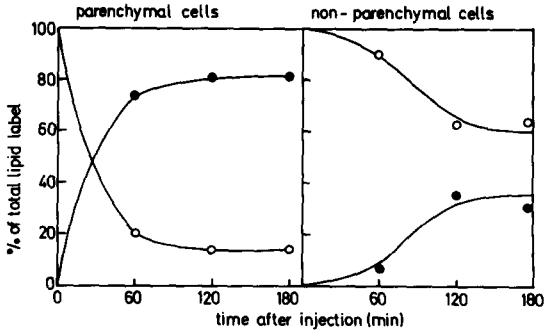

Fig. 3. Conversion of liposomal [*N-Me*-^{14}C]choline-labeled sphingomyelin into phosphatidylcholine in parenchymal and non-parenchymal liver cells. Rats were injected intravenously with [^{14}C]sphingomyelin-labeled small unilamellar vesicles. At times indicated livers were perfused with pronase at 10°C to isolate non-parenchymal cells or with collagenase to isolate parenchymal cells. Lipids were extracted from aliquots of the cell suspensions and separated by thin-layer chromatography. Relevant spots were analyzed for radioactivity contents as described in Methods. ○——○, sphingomyelin; ●——●, phosphatidylcholine.

TABLE II

EFFECT OF IN VIVO CHLOROQUINE TREATMENT OF RATS ON THE CONVERSION OF LIPOSOMAL [^{14}C]SPHINGOMYELIN INTO PHOSPHATIDYLCHOLINE IN PARENCHYMAL AND NON-PARENCHYMAL LIVER CELLS

Rats were injected intravenously with [^{14}C]sphingomyelin-labeled small unilamellar vesicles (5 μmol lipid per 100 g body weight). Pretreatment of the animals with chloroquine consisted of three consecutive intraperitoneal injections of 5, 2.5 and 2.5 mg/100 g body weight at 2 h and 1 h before and 1 h after injection of the liposomes as adapted from Stein et al. [12]. 2 h after liposome injection parenchymal cells were isolated by a 10-min perfusion of the liver with collagenase and subsequent low-speed centrifugation of the liver cell suspension at 4°C. Non-parenchymal cells were isolated by pronase perfusion at 10°C as described in Methods. Aliquots of the cell suspensions were extracted with chloroform/methanol and the lipid extract was fractionated by thin-layer chromatography. The sphingomyelin (Sph) and phosphatidylcholine (PC) spots were analyzed for radioactivity. The amount of radioactivity detected in the various spots was expressed as a percentage of the total amount of radioactivity on the thin-layer plate. The data in Tables II and III are from the same experiment. P, parenchymal cells; NP, non-parenchymal cells.

Cell type	Treatment	Total lipid uptake (nmol/10^6 cells)	% of total lipid label in	
			Sph	PC
P	None	1.03	14.2	82.7
P	Chloroquine	0.94	47.2	47.5
NP[a]	None	0.52	62.3	37.5
NP[a]	Chloroquine	0.96	90.1	7.9

[a] It is to be noted that the uptake values for the non-parenchymal cell fraction are given per 10^6 total non-parenchymal cells. If expressed as uptake per 10^6 Kupffer cells (see Fig. 2) the uptake values would be approx. 4-fold higher because the Kupffer cells make up about 25% of the non-parenchymal cells. See also legend to Table III.

fraction the two labels close coincide. This suggests that especially the Kupffer cells are to be held responsible for the discrepancy in liver uptake between the ^{3}H- and ^{14}C-labels as observed in the experiments presented in Fig. 1.

The close correlation between the ^{3}H- and the ^{14}C-curves in the hepatocyte fraction could be taken to indicate a lack of internalization of the vesicles by these cells; they could have remained merely adsorbed to the cell surface. The experiments presented in Fig. 3 and Table II, however, provide evidence that a major proportion of the

hepatocyte-associated vesicles does become internalized. Not only do these experiments demonstrate that hepatocyte-associated ^{14}C-label is efficiently converted from sphingomyelin into phosphatidylcholine, they also show that chloroquine, a well-known inhibitor of lysosomal enzyme activity, effectively inhibits this conversion. Taken together this indicates that the liposomal lipid label, at least in part, reaches an intracellular site, probably the lysosomal system, where it becomes subject to metabolic alterations.

Metabolic conversion of liposomal phospholipid was not confined to the hepatocytes as is also shown in Fig. 3 and Table II. Although not as extensively as in the hepatocytes a significant proportion of the lipid-soluble radioactivity associated with the non-parenchymal cells was also recovered as phosphatidylcholine. Also in this case, chloroquine had a strongly inhibitory effect on this conversion.

It could be argued that the observed conversions of sphingomyelin into phosphatidylcholine occur, partly or entirely, during the isolation procedure of the cells. Particularly in the non-parenchymal cells, the isolation of which involves a 60-min incubation at 37°C, this may come into effect. To minimize the contribution of such an artifact we adapted our cell isolation procedure according to Ref. 13 by performing the pronase perfusion of the liver for 10 min at 10°C and entirely omitting the subsequent in vitro incubation. Although the yield of the nonparenchymal cells obtained with this method was lower (approx. 27%) as compared with our standard method (recovery approx. 40%), the quality of the cells met our regular standards: trypan blue was excluded in more than 90% of the cells, while approx. 20% of the cells could be identified as Kupffer cells by cytochemical demonstration of peroxidatic activity. The results of this modified procedure, as compiled in Table III, show that the recovery of total ^{14}C-radioactivity in the non-parenchymal cell fraction was not influenced as compared to the 37°C perfusion. The conversion of labeled sphingomyelin into phosphatidylcholine, however, was inhibited to a considerable extent.

In order to establish whether phospholipid conversion was also occurring during the isolation of the hepatocytes we attempted to isolate also these cells at 10°C. Collagenase perfusion of the liver at this temperature resulted, however, in very low yields and viabilities of the cells. Therefore we decided to modify the hepatocyte isolation procedure only in that after the 10-min perfusion at 37°C with collagenase all further cell-handling, including the centrifugations, was carried out at 4°C. The results on parenchymal cells in Fig. 3 and Table II were, in fact, obtained with this method.

TABLE III

EFFECT OF ISOLATION TEMPERATURE ON THE RECOVERY AND CONVERSION OF LIPOSOMAL [^{14}C]SPHINGOMYELIN IN NON-PARENCHYMAL CELLS

[^{14}C]Sphingomyelin-labeled liposomes were injected intravenously and non-parenchymal cells were isolated 2 h after administration. Isolation of the cells was done by perfusion of the liver with pronase at 37°C with subsequent incubation of the liver cell suspension with the enzyme at 37°C for 1 h or, alternatively, at 10°C as described in Methods. Non-parenchymal cell suspensions were processed for measurement of radioactivity contents as described in Methods. Lipids were extracted from aliquots of the cell suspensions, separated by thin-layer chromatography and analyzed for radioactivity. The amounts of radioactivity detected in the sphingomyelin and phosphatidylcholine spots are expressed as a percentage of the total amount of radioactivity on the thinlayer plate. NP cells, non-parenchymal cells.

Perfusion temperature (°C)	Uptake by NP [a] cells (nmol lipid/10^6 cells	$CHCl_3$-soluble radioactivity (% of NP cell radioactivity)	Sphingomyelin label (% of total lipid label)	Phosphatidylcholine label (% of total lipid label)
37	0.64	87.6	26.1	73.7
10	0.52	84.8	62.3	37.5

[a] See legend to Table II.

Discussion

Targeting of liposomes to tissues other than the reticuloendothelial system is frustrated by the inability of liposomes to traverse the capillary walls in most organs as was recently discussed by Poste et al. [6]. The parenchymal cells of the liver, however, seem to be an exception in this respect, since the fenestrated endothelial cells, lining the liver sinusoids, allow direct access of particles with a diameter of approx. 100 nm or less from the bloodstream to these cells as was recently pointed out by Scherphof et al. [7].

Indeed, we and others demonstrated that, after intravenous injection, small unilamellar vesicles became associated not only with non-parenchymal cells but also with parenchymal cells of the liver [5,7]. A considerable further increase in hepatocyte uptake could even be achieved by incorporating lactosylceramide in the vesicle membrane [16,17].

However, thus far solid evidence that the liposomal constituents are really internalized by the cells and by which route is still lacking. Rahman et al. [18] provided indirect evidence that small unilamellar vesicles, containing metal chelating agents, are effective in removing iron stores from liver parenchymal cells. This observation was taken to indicate that the vesicles are really taken up by the hepatocytes probably by way of endocytosis.

By comparing the intrahepatic fate of the inert [^{3}H]inulin label with the [^{14}C]sphingomyelin label (Fig. 1) we concluded that, after uptake by the liver, the liposomal sphingomyelin is subject to degradation with subsequent release of water-soluble degradation products. Sphingomyelin can be degraded by an acid sphingomyelinase [19] a lysosomal enzyme that catalyzes the hydrolytic formation of phosphorylcholine and ceramide. Further degradation of the phosphorylcholine can take place by a lysosomal phosphomonoesterase liberating the choline moiety. Both the choline and the phosphorylcholine moieties, after release from the lysosomal compartment, could serve as building blocks for de novo synthesis of phosphatidylcholine or could be released from the cells.

Of the non-parenchymal cells only Kupffer cells were found to be involved in the uptake of the vesicles (Table I). This result is in line with our previous observations on multilamellar and large unilamellar liposomes: endothelial cells did not take up this type of vesicles [2]. Similar results have been published by Rahman et al. [5].

The results presented in Fig. 2. demonstrate that the Kupffer cells are much more active in liposome uptake than the hepatocytes, particularly as judged from the ^{3}H-label. The ^{14}C-label tends to lag behind the ^{3}H-label, which is particularly evident in the Kupffer cells. Since inulin is a metabolically inert substance, which has a very long half life in cells once internalized, the obvious conclusion emerging from our experiments is that the discrepancy between the two radioactive markers as observed in Figs. 1 and 2 arises from release of ^{14}C-label from the cells subsequent to uptake and intracellular degradation of the liposomes. The results in Fig. 2 suggest that the discrepancy observed in Fig. 1 for whole liver, is mainly accounted for by loss of ^{14}C-label from the Kupffer cells. Release of liposomal lipid label from Kupffer cells in monolayer culture in addition to incorporation into cellular phosphatidylcholine was also observed by Dijkstra et al. [8,20].

The effects of chloroquine treatment of the rats support our opinion that the internalization of liposomes, which, considering the substantial metabolic conversion we observed, must take place also in hepatocytes, is achieved by way of an endocytic process. This would also most obviously account for the degradation of the sphingomyelin molecule as outlined above.

The lack of release of water-soluble degradation products from hepatocytes (Fig. 2) might indicate that in this cell type resynthesis of phosphatidylcholine occurs very efficiently. The results presented in Fig. 3 are compatible with this interpretation: in parenchymal cells the proportional shift of the ^{14}C-label proceeds much more rapidly and more extensively than in the non-parenchymal cells. The relative slow conversion of the label in the non-parenchymal cell fraction is in line with our observations with cultured Kupffer cells [8]. The rapid and extensive conversion in the hepatocytes may be taken to reflect the high synthetic activity of this cell type in relation to lipoprotein and bile production. The close similarity between the two labels, ^{3}H and ^{14}C, in the hepatocyte fraction argues against the possibility that the hepatocytes receive substantial amounts of

degradation products from the Kupffer cells to use these for phosphatidylcholine synthesis, although we cannot entirely rule out this possibility. Preliminary experiments revealed that significant amounts of liposomal sphingomyelin or phosphatidylcholine label (both [*N-Me*-^{14}C]choline) accumulate in the bile, mostly as radioactive phosphatidylcholine, but the rate at which this occurred (1–2% of the injected dose per hour) was insufficient to account for a significant contribution to a loss of label from the parenchymal cells in our present experiments.

The 10-fold excess of parenchymal cells over Kupffer cells in the liver is counterbalanced by a 10-fold higher ^{3}H uptake by Kupffer cells on a per cell basis (Fig. 2), leading to an approximately equal distribution of ^{3}H-radioactivity over the two cell populations, in agreement with results reported by us previously [7]. Since, one hour after injection, we found in the parenchymal cells almost 80% and in the non-parenchymal cells, i.e. the Kupffer cells, only 10% of the [*N-Me*-^{14}C]choline labeled sphingomyelin converted (Fig. 3) we would, by calculation, expect over 40% sphingomyelin conversion in whole liver. We actually found somewhat less than 30% conversion [7]. Calculated and observed results can be satisfactorily reconciled if we assume that some 20% of the total liver-associated radioactivity represents material that is adsorbed to cell surfaces rather than internalized.

As was demonstrated by Praaning-Van Dalen and Knook [13] with colloidal albumin intracellular degradation of ligands endocytosed in vivo can continue during the isolation of the cells. Although we did not observe a decrease in the recovery of ^{14}C-radioactivity from the non-parenchymal cell fraction using our standard isolation method at 37°C (Table III), intracellular degradation of liposomal [^{14}C]sphingomyelin apparently still continues under these conditions evidenced by the relative extents of label incorporation into phosphatidylcholine. For hepatocytes, the problem of intracellular degradation of liposomal lipids during the isolation of the cells is much less pronounced, since the time needed for perfusion of the liver covers a period of maximally 20 min. The following centrifugation procedure to separate parenchymal from nonparenchymal cells was done at 4°C, allowing for minimal metabolic activity.

Finally, the results from the in vivo chloroquine treatment experiments (Table II) provide good evidence that the liposomal sphingomyelin label passes through the lysosomal compartment of the cells. As outlined above, this observation is fully compatible with an endocytic uptake mechanism of the vesicles by both cell types of the liver. On the other hand, as discussed above, it cannot be excluded that part of the vesicles remain adsorbed to the cell surface. However, the use of an EGTA-containing medium during the cell isolations favor the dissociation of any vesicles adsorbed to the cell membrane.

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