Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5490–5493, September 1982
Biochemistry

Stability of carbohydrate-modified vesicles *in vivo*: Comparative effects of ceramide and cholesterol glycoconjugates

(liposome/perturbed angular correlation spectroscopy/NMR/drug delivery system)

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Contributed by John D. Baldeschwieler, June 7, 1982

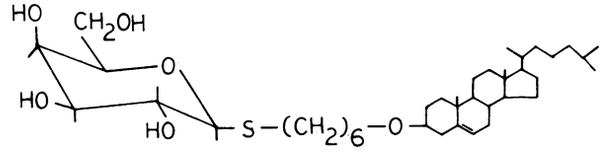
ABSTRACT **The stability and tissue distribution of lipid vesicles modified at the surface by the incorporation of either a galactosyl ceramide (GalCer) or a galactosyl cholesterol (GalChol) glycoconjugate have been studied in mice by measuring the release of vesicle-entrapped ^{111}In. Although the tissue distributions of both vesicle types were similar, the GalCer-containing vesicles were markedly less stable than those prepared with GalChol, whether administered orally or by intraperitoneal injection. Physical characterization of the vesicles *in vitro* suggests that the increased disruption rate for GalCer vesicles *in vivo* is related to structural instabilities induced by the cerebroside, which can then result in either an increased rate of vesicle uptake by tissues or a greater susceptibility to lysis. These studies demonstrate the importance of the nonpolar anchoring groups in determining the fate of surface-modified vesicles *in vivo*.**

The targeting of encapsulated agents, such as drugs or enzymes, to specific tissues is one of the goals in the development of liposomes as exogenous delivery systems. To achieve this result, a number of strategies have been attempted, including the addition of charged lipids to neutral vesicles (1), the covalent binding of antibodies to vesicle surfaces (2), and the use of localized hyperthermia to induce phase transitions in synthetic liposomes (3). The finding that mammalian hepatocyte surfaces contain a galactose-specific glycoprotein binding site (4) has led to studies on the usefulness of carbohydrate-modified vesicles in targeting encapsulated materials (5–7). Initial work has shown great promise (7), leading to further investigation of variables that can affect the system.

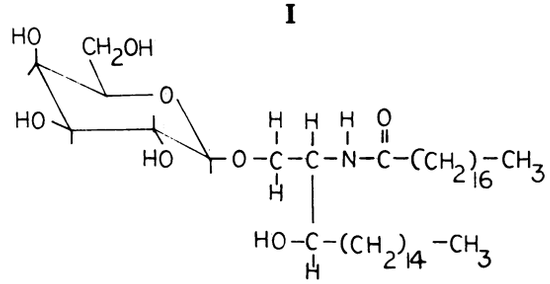
Due to the amphiphilic nature of vesicle bilayers, it is convenient to incorporate polar carbohydrate molecules by attaching them to nonpolar lipid groups. In view of the importance of lipid shape to vesicle structure (8), it is likely that the choice of lipid conjugate or anchoring group will, in addition to the type of carbohydrate used, affect the fate of modified vesicles *in vivo*. In the present communication, we report studies of carbohydrate-modified vesicles containing glycolipids differing in the structure of their nonpolar group. The results, comparing the effects of *N*-stearoyl-DL-dihydrogalactocerebroside (galactosyl ceramide, GalCer, II) with those of 6-(5-cholesten-3β-yloxy)hexyl-1-thio-β-D-galactopyranoside (galactosyl cholesterol, GalChol, I) demonstrate the importance of the carbohydrate anchoring group in maintaining stable vesicles *in vivo*.

MATERIALS AND METHODS

Materials. L-α-Distearoylphosphatidylcholine from Calbiochem and cholesterol from Sigma were used without further purification. GalChol was synthesized at Merck. GalCer was purchased from Miles. GalCer and GalChol were labeled at the C-6 position of galactose, with tritium or deuterium, by the method of Suzuki and Suzuki (9) using NaB^3H_4 (specific activity, 350 Ci/mol; 1 Ci = 3.7×10^{10} becquerels) from ICN and NaB^2H_4 (98 atom % ^2H) from Merck. The trisodium salt of nitrilotriacetic acid was purchased from Aldrich and ultrapure $InCl_3$ was obtained from Ventron (Danvers, MA). $^{111}InCl_3$ was purchased from Medi+Physics (Glendale, CA) and purified according to the method of Hwang and Mauk (10). The ionophore A23187 was a gift from Eli Lilly.



I



II

Preparation of Vesicles. Small unilamellar vesicles, prepared according to the method of Mauk and co-workers (6, 7, 10–12), were composed of L-α-distearoylphosphatidylcholine, cholesterol, glycoconjugate (GalChol or GalCer), and A23187 at 2:0.5:0.5:0.004 (mol/mol).

Electron Microscopy. Vesicle size distributions were determined by electron microscopy of fresh preparations, negatively stained with 2% phosphotungstic acid as described by Sheetz and Chan (13).

Vesicle Stability *in Vivo*. Vesicles containing $^{111}In^{3+}$ were administered to Swiss–Webster mice (18–22 g) orally or intraperitoneally (i.p.). $^{111}In^{3+}$ permeability was monitored by the γ-ray perturbed angular correlation technique (6, 7, 10–12). The tissue distribution of radioactivity was determined by assaying samples in a well-type γ-ray spectrometer (7).

Studies *in Vitro*. Vesicle thermolability in serum was measured by the γ-ray perturbed angular correlation technique,

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Abbreviations: i.p., intraperitoneal; GalChol, galactosyl cholesterol, 6-(5-cholesten-3β-yloxy)hexyl-1-thio-β-D-galactopyranoside; GalCer, galactosyl ceramide, *N*-stearoyl-DL-dihydrogalactocerebroside.

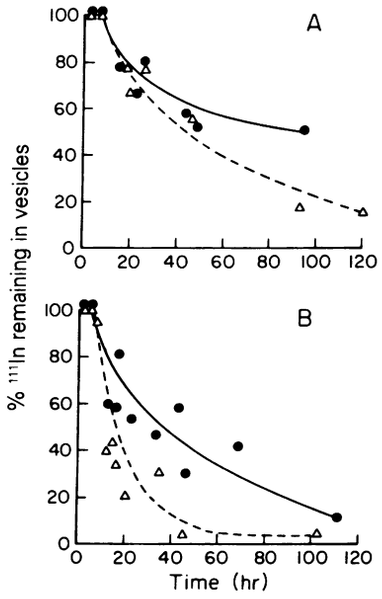

FIG. 1. Stability of vesicle preparations in live mice administered by i.p. injection (*A*) or orally (*B*). ●, GalChol-containing vesicles; △, GalCer-containing vesicles.

as described (14). Vesicle aggregation and fusion in buffered solution was monitored by light scattering (15). ^{2}H NMR spectra were obtained with a Bruker WM 500 spectrometer equipped with a BVT 1000 variable-temperature controller.

RESULTS

***In Vivo* Studies.** Thirty hours after oral administration, approximately half of the GalChol-containing vesicles are intact, while less than 20% of the vesicles prepared with GalCer have retained their contents (Fig. 1*B*). Similarly, when vesicles are injected i.p., the breakdown rate of GalCer vesicles is more rapid than that of GalChol vesicles, which change very slowly after 40 hr (Fig. 1*A*). This differential breakdown rate is also reflected in the time course for urinary excretion of ^{3}H-labeled galactose. As shown in Fig. 2*B*, after oral administration of GalCer vesicles, the bulk of the labeled material appears in the urine in approximately 5 hr while, for the GalChol vesicles, the peak in excretion of labeled material is reached almost 3 hr later. A markedly different pattern emerges when vesicles are administered by i.p. injection. In this case, the peak in excretion of labeled material for GalCer-injected mice is not reached until 30 hr while, with GalChol vesicles, little or no labeled material is ever present, even after 80 hr.

Table 1. Tissue distribution of ^{111}In-labeled vesicles

Oral administration*			i.p. administration		
	GalChol	GalCer		GalChol	GalCer
Stomach	46.1%	58.9%	Liver	31.4	31.3
Large intestine	34.1	12.7	Skin	12.5	9.4
Small intestine	5.6	4.5	Pelvic tissue	11.2	9.4
Pelvic tissue	5.6	3.3	Abdomen	10.6	7.2
Legs	2.3	3.0	Small intestine	7.3	9.2
Skin	1.7	3.6	Kidney	8.4	7.5
Liver	1.6	5.7	Legs	5.4	4.6
Kidney	1.4	5.9	Blood	4.1	11.5
Abdomen	0.9	1.7	Large intestine	3.0	2.4
Blood	0.5	0.3	Spleen	2.5	2.4
Heart	0.2	0.2	Stomach	1.5	2.6
Spleen	0.06	0.2	Lung	1.5	1.9
			Heart	1.0	0.9

* Lung tissue was not included.

In agreement with previous studies (7, 11), it was found that the method of administration also has a significant effect on the tissue distribution (Table 1). Most of the ^{111}In was found in the stomach and intestines, 24 hr after oral administration, while i.p. injection resulted in a wider distribution with about a third of the vesicle material located in the liver. In comparing the results for the carbohydrate-modified vesicles, it was found that, when administered by the same route, there is a remarkable similarity in the tissue distribution, despite the marked difference in stability. The major difference after oral administration appears in the distribution between stomach and large intestine, with more material from the unstable GalCer vesicles being retained in the stomach and less found in the large intestine. i.p. injection showed only a slight difference in the amounts of material found in the blood, the difference for all other tissues being insignificant.

***In Vitro* Studies.** To gain some insight into the mechanism responsible for the variability of vesicle stability *in vivo*, a number of experiments were carried out *in vitro*. The thermolability of carbohydrate-modified vesicles in serum is shown in Fig. 3. As with the studies carried out in mice, the GalCer vesicles are much less stable than the GalChol vesicles. In related work, the binding of serum proteins to vesicles was measured and found to be identical for the two types of vesicles. The amount bound

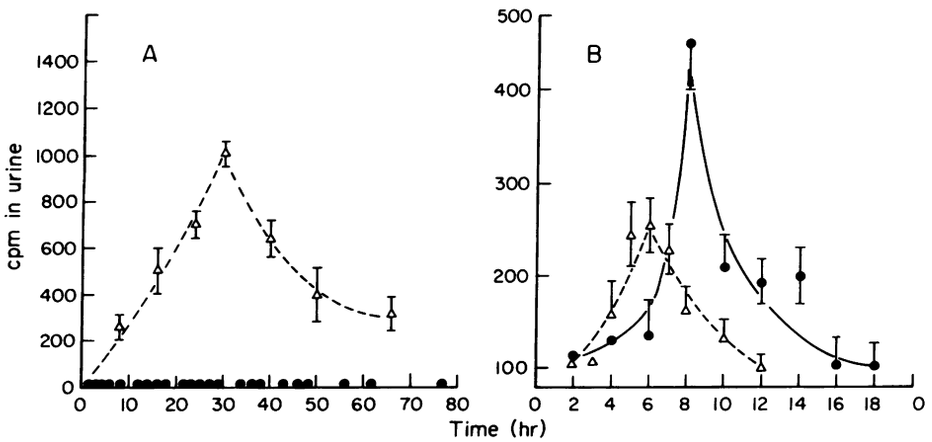

FIG. 2. Time course of urinary excretion of [^{3}H]galactose after i.p. injection (*A*) or oral administration (*B*) of vesicles containing [^{3}H]GalChol (●) or [^{3}H]GalCer (△).

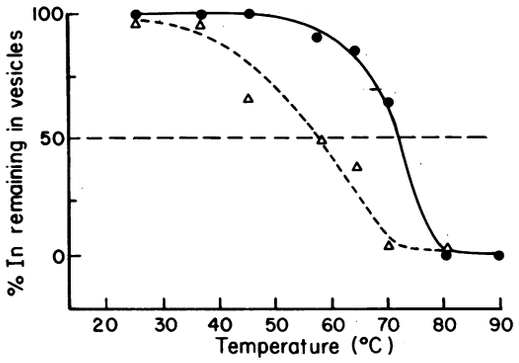

FIG. 3. Thermolability of galactose-modified vesicles in serum. ●, GalChol-containing vesicles; △, GalCer-containing vesicles.

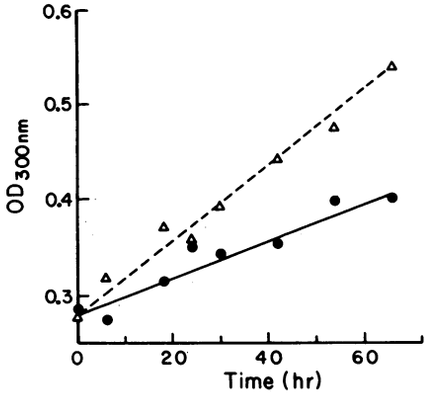

FIG. 4. Change in turbidity of galactose-modified vesicle preparations stored at 37°C. ●, GalChol-containing vesicles; △, GalCer-containing vesicles.

was less than 1/10th the value found for control vesicles without carbohydrates (unpublished data). These results suggest that the observed instability of GalCer vesicles is an inherent property. This possibility is further supported by the results from light-scattering experiments shown in Fig. 4. The increased scatter seen after sonication of the GalCer vesicles in buffer indicates that they are either aggregating or undergoing fusion to form larger vesicles at a faster rate than are the GalChol vesicles (15, 16).

DISCUSSION

The use of lipid bilayer vesicles to deliver exogenous agents to specific organs and tissues is one of the more recent efforts in the development of therapeutically targeted agents (17). However, the vesicle approach has several promising advantages, not the least of which is that, once the method has been perfected, practically anything can be delivered to a specific site. Of more current usefulness, in the development of this technique, is the variety of ways in which the vesicles can be modified. Since the objective is to bring vesicles together with the membrane surfaces of specific types of cells, most of the initial approaches have involved alterations in the outer surfaces of the vesicles (1, 2, 5–7). It is also, of course, important that the modification not significantly decrease the stability of the vesicle before it reaches the target.

Figs. 1 and 2 and Table 1 present evidence showing that there is a marked difference in the relative stabilities *in vivo* of galactose-modified vesicles that differ in the structures of their hydrophobic lipid groups. In general, such differences can be caused by a variation in either the rate of vesicle uptake by tissues or the rate of vesicle lysis, a process that could also be occurring extracellularly. The latter possibility is supported by the similar results found when vesicles are administered by two different routes, i.p. and oral, that expose them to different biochemical environments. Further evidence that the instability of GalCer vesicles, relative to that of GalChol vesicles, is an inherent property, is shown in Fig. 4, where marked differences

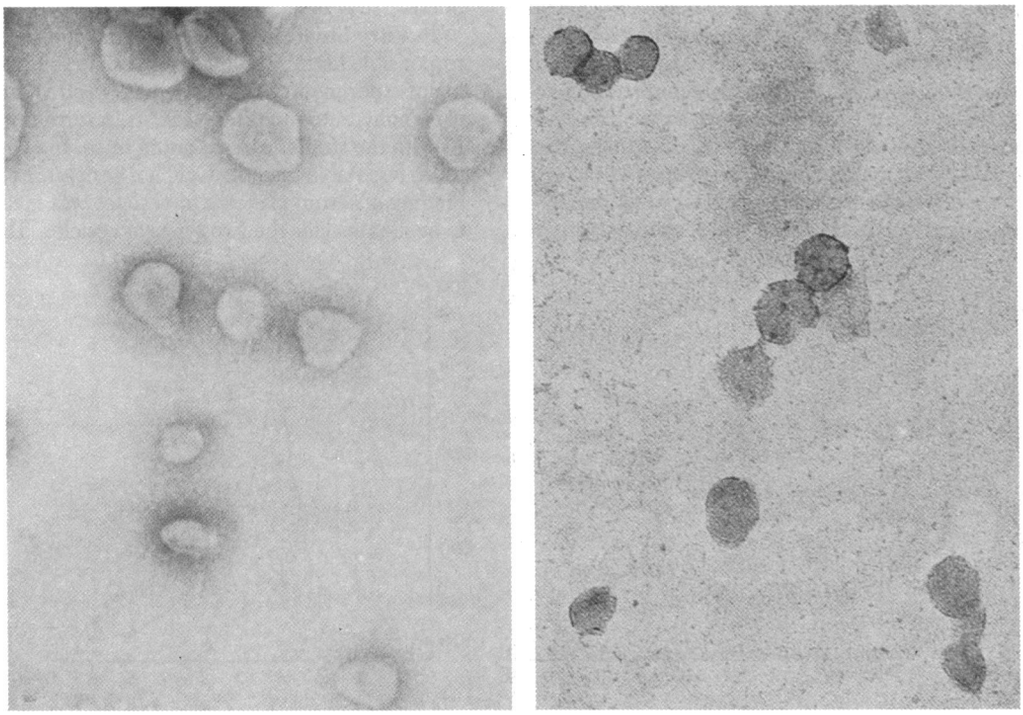

FIG. 5. Electron micrographs of negatively stained vesicle preparations. (*Left*) GalChol-containing vesicles (average diameter, 662 ± 60 Å). (*Right*) GalCer-containing vesicles (average diameter, 639 ± 60 Å). (×13,900.)

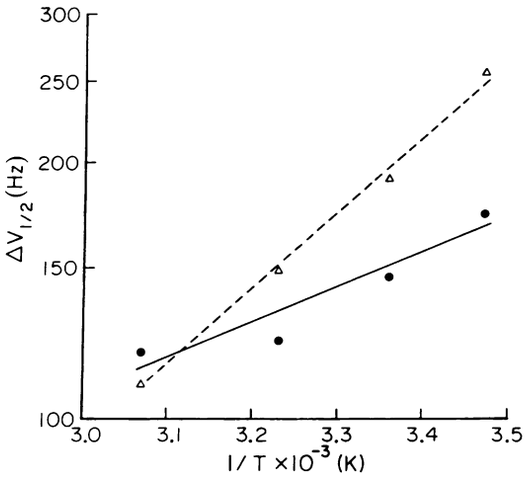

FIG. 6. Temperature dependence on the deuterium linewidth arising from ^{2}H-labeled galactose conjugates in chloroform/methanol (1.5:1.0). ●, GalChol; △, GalCer.

are observed for the rates of spontaneous aggregation and fusion. Significantly, these data also show that the decreased stability of GalCer-modified vesicles in buffer at 37°C occurs within the same time period found for the instability *in vivo* (see Fig. 1).

The spontaneous aggregation and fusion of neutral phospholipid vesicles has been studied in some detail and is believed to be caused by structural instabilities brought on by the packing requirements for bilayer lipids to form small unilamellar vesicles (15, 16). Because the initial sizes of the two types of vesicles are similar (Figs. 4 and 5), the shapes of the individual lipid molecules present must be compatible with the packing requirements for these structures (8). Thus, we can assume that, immediately after sonication, the free energies of the two types of vesicles are similar (18) and that the increase in the relative rate of aggregation or fusion seen for GalCer vesicles is due to a reduction in the small energy barrier stabilizing them. This reduction in stability is likely to be caused by the formation of more packing defects in the GalCer vesicles than in the GalChol vesicles. However, because of the initial size similarity, the lipid packing should also be similar. One mechanism for introducing additional packing instabilities after sonication is the formation by the cerebrosides of stable complexes that result in a net reduction in the bilayer volume.

Indirect evidence for the formation of hydrogen-bonded cerebroside complexes has been reported in unsonicated phospholipid bilayers (19). We have obtained direct evidence for the formation of GalCer aggregates, which are more stable than GalChol aggregates, from ^{2}H NMR studies of deuterium-labeled glycolipids in nonaqueous solvents. The aggregation of amphiphiles in such a system depends strongly on their headgroup structure (20) and serves as a convenient model for comparing the polar interactions of lipids dissolved in a bilayer. For relatively rapid motions, the linewidth of the deuterium signal is determined by the molecular tumbling rate (21), which in turn depends on the aggregate size. Thus, from measurement of the linewidth, one can monitor changes in the average size of the lipid aggregates. These studies, in which both solvent polarity and temperature were varied, show that a marked difference exists in the aggregation states of the two glycolipids. The formation of a relatively stable aggregate by the cerebroside in chloroform/methanol (1.5:1.0) is shown in Fig. 6. The Arrhenius plots indicate an energy barrier of 4.1 kcal/mol (1 cal = 4.18 J) for the GalCer micelles and one of 1.9 kcal/mol for GalChol. For comparison, the activation energy for phosphatidycholine micelles in chloroform/water is only 0.46 kcal/mol (22). In pure chloroform, GalCer was practically insoluble while the GalChol aggregates became much larger. The observation that GalCer forms complexes in phospholipid bilayers (19) correlates with the relatively high activation energy reported here for GalCer aggregates. The lower activation energy found for GalChol aggregates suggests that formation of GalChol complexes in the bilayer plane is less likely. These data further support, but do not confirm, the possibility that lipid–lipid interactions are responsible for the reduced stability of GalCer-containing vesicles.

The studies presented here provide evidence that galactose-modified cerebrosides reduce the stability of phospholipid/cholesterol vesicles, both *in vivo* and *in vitro*. Similarly modified vesicles, prepared with a galactose-cholesterol conjugate, show much greater stability. The effects of the cerebrosides may result from complex formation within the plane of the bilayer. This process could be useful for the timed release of labile molecules from vesicles. However, and perhaps more significantly, this work also shows the importance of the lipid-anchoring group in maintaining vesicle stability and further indicates that cholesterol conjugates are preferable for the formation of stable carbohydrate-modified vesicles.

The NMR spectra were obtained at the Southern California Regional NMR Facility, California Institute of Technology, Pasadena, CA. This research was supported by grants from the National Science Foundation (CHE79-19401) and the National Institutes of Health (GM21111-09) and a grant from Merck and Co., Inc.

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