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Ceramide excluded from cell-free vesicular lipid transfer from endoplasmic reticulum to Golgi apparatus. Evidence for lipid sorting

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The distribution and cell-free transfer of ceramide and other lipids were compared using highly purified fractions of endoplasmic reticulum, transitional endoplasmic reticulum, transition vesicles and Golgi apparatus from rat liver. Ceramides were present in both endoplasmic reticulum and Golgi apparatus where they represented between 0.3 and 1% of the total lipids. Ceramides, however, were much reduced or absent (< 0.05%) from transition vesicles. Transition vesicles were induced to form from transitional endoplasmic reticulum by incubation with ATP and a cytosol fraction. When transfer of [^{14}C]choline-labeled phosphatidylcholine from transitional endoplasmic reticulum to Golgi apparatus was followed, transition vesicles were more efficient in transfer than the transitional endoplasmic reticulum from which they were derived. This transfer was temperature- and ATP-dependent and inhibited by *N*-ethylmaleimide. When transfer of [^{3}H]ceramide was followed, there was little or no transfer via transition vesicles and that transfer which occurred was temperature-, ATP- and *N*-ethylmaleimide independent. Transfer of ceramide in the cell-free system did occur from endoplasmic reticulum to Golgi apparatus but via a non-vesicular mechanism that was temperature-dependent but not dependent on ATP or cytosol, alone, or in combination, nor was it inhibited by *N*-ethylmaleimide. A component of phosphatidylcholine transfer exhibited similar characteristics. The results provide evidence for two distinct mechanisms for cell-free transfer of lipids from endoplasmic reticulum to Golgi apparatus. The first is via 50 to 70 nm transition vesicles which is temperature- and ATP-dependent, inhibited by *N*-ethylmaleimide and from which ceramides are excluded. The second is non-vesicular, temperature-dependent, and neither ATP- nor cytosol-dependent. It accounts for the bulk of the ceramide transfer. As a result during cell-free lipid transfer from endoplasmic reticulum to Golgi apparatus, lipid sorting occurs such that ceramides are largely absent from the transition vesicles and, apparently are delivered to the Golgi apparatus by another mechanism.

Introduction

Cell-free systems for the study of transfer and sorting of membrane constituents [1–3] have been extended only recently to the investigation of membrane lipids [4–7]. Moreau et al. [4] and Moreau and Morré [5] demonstrated two components of lipid transfer from endoplasmic reticulum to Golgi apparatus in a cell-free system from rat liver. The first was vesicular and ATP-dependent. The second was non-vesicular and ATP-independent. Both were temperature-dependent and neither was highly cytosol dependent. These observations were extended to plant fractions [6] where preliminary indications suggested transfer of phospholipids by both mechanisms, but exclusion of triacylglycerols from the ATP-dependent vesicular pathway. These results were taken as evidence of lipid sorting during the formation of the 50–70 nm transition vesicles that mediate the cell-free transfer between endoplasmic reticulum and Golgi apparatus [8].

Ceramides, precursors of the glycosphingolipids of the plasma membrane [9–11], also are thought to be synthesized in endoplasmic reticulum (Ref. 9 and references therein) and transported via bulk flow to the Golgi apparatus [12], where the glycosyltransferases leading to lactosyl ceramide [13,14] and ganglioside [15]

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Abbreviations: GA, Golgi apparatus; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TER, transitional endoplasmic reticulum; TV, transition vesicles.

formation primarily are located. In this report, the cell-free transport of ceramide is compared with that of phosphatidylcholine and other lipids. In the cell-free system, ceramide is transported exclusively via the non-vesicular pathway providing a second example, in addition to triacylglycerols, where lipids are excluded from transition vesicles during their formation.

Materials and Methods

Isolation of the transition element fraction

Livers were from male Holtzman rats, 150–180 g, provided with rat chow and drinking water ad libitum. Homogenates were prepared in isotonic, buffered sucrose as described [16]. Briefly, livers were homogenized in two volumes of 37.5 mM Tris-maleate (pH 6.5), 0.5 M sucrose, 5 mM $MgCl_2$ and 1% dextran (average molecular mass 225 kDa) for 45 s with a Polytron 20 ST operated at 6000 rpm. The homogenates were centrifuged for 15 min at $6000 \times g$ and the pellets used as a source of Golgi apparatus as described [17]. The supernatant was diluted 1:5 with the homogenization medium and, following a second centrifugation at $10000 \times g$ to remove mitochondria, was layered onto a discontinuous sucrose gradient consisting of 2.0, 1.5 and 1.3 M sucrose layers. After centrifugation at $85000 \times g$ for 90 min, membranes collecting at the 1.3 M sucrose/sample interface were removed using a Pasteur pipette and pelleted by centrifugation for 20 min at $70000 \times g$ as the starting material for transition vesicle formation.

Initiation of transition vesicle formation

Transition vesicles were induced to form by incubating the part-rough, part-smooth elements of the endoplasmic reticulum collected from the 1.3 M sucrose/sample interface of the sucrose gradients described above by incubation in a total volume of 6.0 ml with 200 μl of a cytosol fraction (5 mg protein/ml), 2 ml of an ATP/ATP-regenerating system and 3.8 ml of resuspended membranes. The cytosol fraction consisted of a microsome-free supernatant cleared by centrifugation at $90000 \times g$ for 60 min and then fractionated by filtration through a Centricon YM 10 filter (Amicon) to yield a > 10 kDa-enriched fraction. The ATP-regenerating system [18,19] contained 30 mM Hepes-KOH, 30 mM KCl, 2.5 mM magnesium acetate, 50 mM ATP (rabbit muscle), 300 mM UTP, 2 mM creatine phosphate and 10 IU/ml creatine phosphokinase (rabbit muscle) (pH 7.0).

Preparation of the Golgi apparatus (acceptor) fraction

Highly purified Golgi apparatus from the same livers as the donor membranes, were isolated as described [20]. The purified Golgi apparatus membranes next were adsorbed on nitrocellulose strips following resuspension at a final concentration of 1–2 mg of protein per ml in 33 mM Hepes (pH 7.0) containing 2.5 mM magnesium acetate and 33 mM KCl (Hepes/Mg $(OAc)_2$/KCl). Incubation with the 1 cm^2 nitrocellulose strips (10 strips per ml) was at 4°C for 1 h with continuous shaking. The strips then were transferred to Hepes/$Mg(OAc)_2$/KCl containing 5% bovine serum albumin and incubated for 1 h at 4°C with shaking to block unoccupied sites. The strips, each loaded with about 100 μg of Golgi apparatus protein, then were rinsed through four changes of Hepes/$Mg(OAc)_2$/KCl, blotted, and added to the reconstituted system as described below.

Reconstituted membrane transfer

Incubations were in 8 ml glass shell vials with three acceptor strips per vial. The same medium was used as for initiation of transition vesicles. The strips were arranged vertically in the vial in the form of a triangle around the circumference. All solutions were maintained at 4°C until initiation of the reaction by transfer to 37°C. At the end of the incubation, the strips were rinsed through four changes of Hepes/$Mg(OAc)_2$/KCl, edge blotted on Whatman No. 1 filter paper and dried. The individual dried strips were placed in scintillation vials and, after addition of 10 ml of aqueous counting scintillant (ACS, Amersham), radioactivity was determined.

Isolation of transition vesicles

For isolation of transition vesicles, the donor fractions were incubated for 1 h in the absence of acceptor strips at 37°C to induce the formation of transition vesicles. Transition vesicle-enriched fractions were obtained by preparative free-flow electrophoresis [8]. Identical conditions as described previously for Golgi apparatus subfractionation [21] were used with a VAP-22 continuous free-flow electrophoresis unit (Bender and Hobein, Munich, Germany). Absorbance was measured at 280 nm and appropriate peak fractions were pooled and concentrated by centrifugation at $85000 \times g$ for 30 min. Proteins were estimated by the BCA [22] procedure.

Alternatively, transition vesicles were enriched from a $15000 \times g$ (10 min) transitional endoplasmic reticulum supernatant after incubation with the complete ATP + ATP regenerating system with cytosol using microultracentrifugation for 10 min at $225000 \times g$ (Hitachi Himac CS100, Rotor RP80AT). To insure complete release of transition vesicles, at the end of the incubation the mixture was sonicated for 10 s (Ultrasons Annemasse) and mixed by vortexing for 20 s at minimal speed (Jenke and Kunkel IKA Labortechnik VF2). Transition vesicles were resuspended in an appropriate volume of Hepes buffer, an aliquot being fixed for morphology analysis.

Labeling of radioactive donor

In a glass tube, [^{14}C]phosphatidylcholine and [^{3}H]ceramide, prepared from liver sphingomyelin [23] by hydrolysis with placental sphingomyelinase (Sigma) according to Iwamori et al. [24], 0.5 μCi of each, were evaporated to dryness. Hepes buffer (50 μl) was added and the mixture was sonicated for 30 s (Ultrason Annemasse) to resuspend the lipids. Then, the transitional endoplasmic reticulum membranes were added in 1 ml Hepes buffer and the mixture was homogenized by vortexing at minimal speed for 15 s, incubated 5 min at 37°C to ensure lipid incorporation and finally vortexed again for 15 s. The radiolabeled membranes were collected by centrifugation for 10 min at 15000 × g (Hitachi Himac CS 100, Rotor RP80AT), resuspended in 1 ml of Hepes buffer and centrifuged again at 15000 × g for 10 min to pellet the radiolabeled membranes and eliminate the bulk of nonincorporated lipids.

Analysis of lipids

Lipids from the various membranes were extracted by $CHCl_3/CH_3OH$ (1:1) at 60–70°C for 20 min. The lipid extracts were washed and then analyzed by thin layer chromatography according to Heape et al. [25]. Neutral lipids were analyzed as described by Juguelin et al. [26]. Radiolabeled ceramides were separated on silica gel HPTLC plates (Merck 60F-254) eluted by $CHCl_3$/acetic acid (90:10). Ceramides were identified by comparing with the migration of standards, scraped off the plates and their radioactivity determined by liquid scintillation counting.

Electron microscopy

Material was prepared for electron microscopy by fixation in 2.5% glutaraldehyde in 0.1 M sodium phosphate (pH 7.2), followed by postfixation in osmium tetroxide in the same buffer. Dehydration was through an acetone series with embedment in Epon. Thin sections were observed and photographed using a Philips EM 200 electron microscope.

Results

Lipid analysis of different membrane fractions revealed the same major lipids in endoplasmic reticulum and Golgi apparatus of rat liver (Tables I and II). Major phospholipids included phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Phosphatidylserine (PS) and sphingomyelin were less abundant. For neutral lipids (Table II), mono- and diglycerides were not quantitated. Only sterols, triacylglycerols and ceramides were determined. Among the neutral lipids, the ceramides were the least abundant and consisted chiefly of non-hydroxy ceramides.

TABLE I

Phospholipid composition comparing transitional endoplasmic reticulum (TER) transition vesicles (TV) and Golgi apparatus (GA) from rat liver

Lipids were analyzed by the densitometric method of Heape et al. [25] following analysis by thin layer chromatography. Values are average of 3 to 5 determinations ± S.D.

Phospholipid	% of total		
	TER	TV	GA
Sphingomyelin	4.6±1.4	–	8.8±0.5
Phosphatidylcholine	58.7±1.3	71.4±2.9	56.1±2.1
Phosphatidylethanolamine	19.0±1.6	16.0±3.0	21.8±0.8
Phosphatidylinositol	10.4±0.2	5.4±0.1	5.4±0.4
Phosphatidylserine	2.7±0.8	7.2±1.6	4.8±0.6
Other	4.6±1.2	–	3.1±0.6

When transition vesicles derived from transitional endoplasmic reticulum were analyzed, major compositional differences were noted. Whereas transition vesicles contained about the same amounts of the major phospholipids and sterols as the endoplasmic reticulum from which they were derived (Tables I and II), there was an increase of PS in the transition vesicles, in agreement with the vesicular transfer pathway of PS to the plasma membrane as proposed [9]. Moreover, triacylglycerols and especially ceramides (Table II) were low in the transition vesicles. These results are suggestive of lipid sorting between the endoplasmic reticulum and the transition vesicles to select, for example, PS and exclude triacylglycerols and ceramides.

TABLE II

Ceramide, sterol and triacylglycerol content (μg/mg protein) of rat liver fractions comparing two fractions of rough endoplasmic reticulum (RER) of differing density (ER_{II}·ER_I) and transitional endoplasmic reticulum (TER) with transition vesicles (TV), Golgi apparatus and plasma membrane

Values are means ± S.D. among determinations. The number of determinations is given in parentheses. Data shown in brackets for ceramides are expressed as μg/μmol phospholipid.

Fraction	Ceramides		Sterols	Triacylglycerols
Homogenate (5)	2.8±0.4	[13.5±2.9]		
RER_{II} (3)	2.1±0.6	[5.2±0.6]		
RER_I (3)	3.9±0.7	[11.1±1.6]		
TER (4)	7.3±0.2	[9.6±1.1]	30.8±4.8	28.8±3.3
TV (2)	0.5±0.02	[0.7±0.03]	10.5±3.1	4.3±0.8 [a]
Golgi apparatus (3)	4.1±0.7	[7.5±2.0]	31.7±5.6	51.7±8.9
Plasma membrane (1)	5.9	[11.6]		

[a] For triacylglycerols, the small amount found in the transition vesicles may include a contribution from secretory lipoproteins which may contaminate the fraction.

To examine the transfer of ceramides from endoplasmic reticulum to Golgi apparatus, a comparison was made between transitional endoplasmic reticulum and the transition vesicles derived from transitional endoplasmic reticulum (Fig. 1) in the transfer of both PC and ceramide. The transition vesicle fractions utilized in the study were highly enriched in the characteristic 50 to 70 nm vesicles (Fig. 1B). Ribosomes were

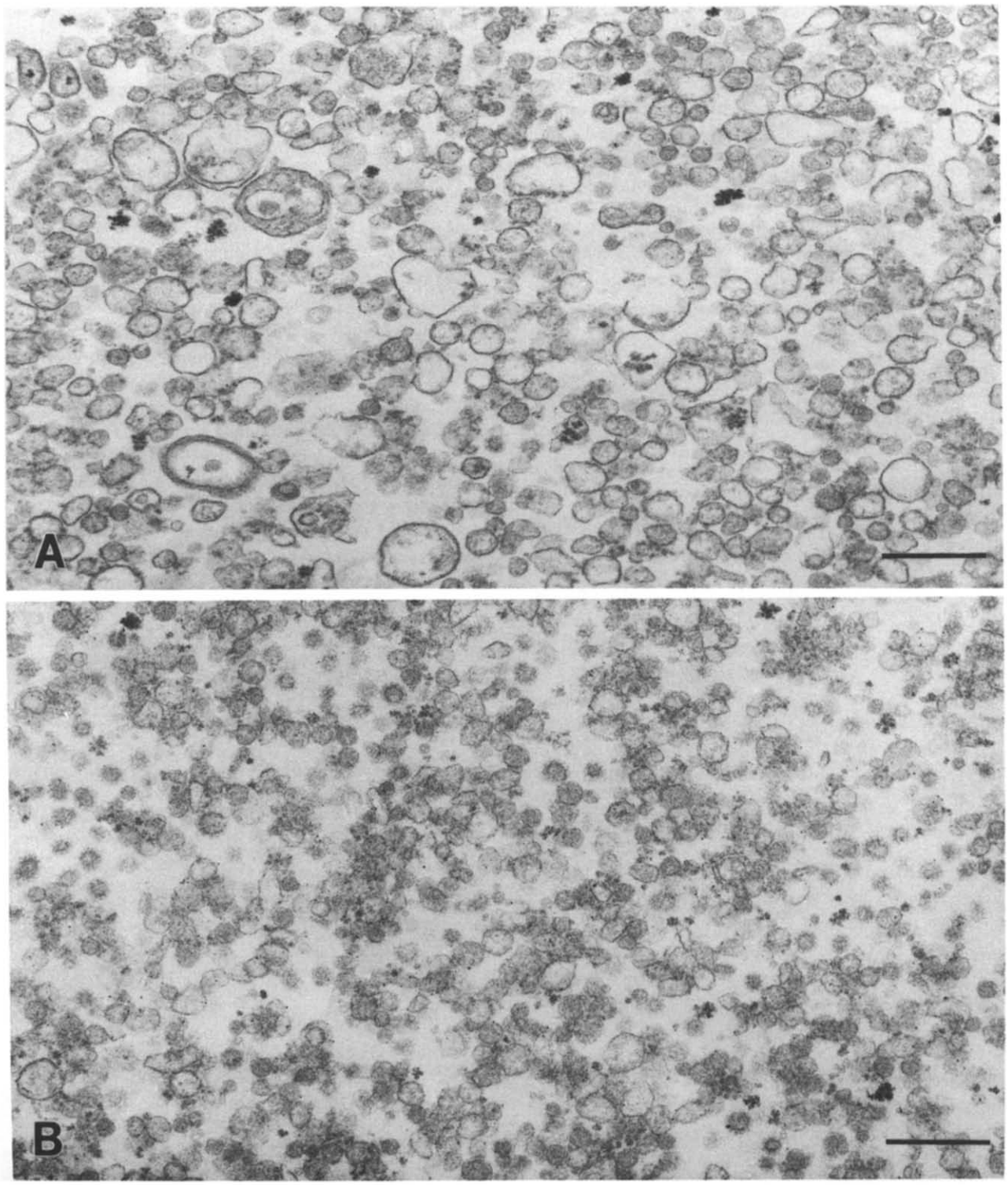

Fig. 1. Electron micrographs of isolated transitional endoplasmic reticulum from liver (A) and the purified transition vesicles derived from the transitional endoplasmic reticulum by incubation with ATP plus a > 10 kDa cytosol fraction (B). The transition vesicle fraction is enriched in 50–70 nm vesicles whereas the levels of the transitional endoplasmic reticulum fraction contains larger vesicles of part-rough, part-smooth membrane vesicles. Scale bar = 0.5 μm.

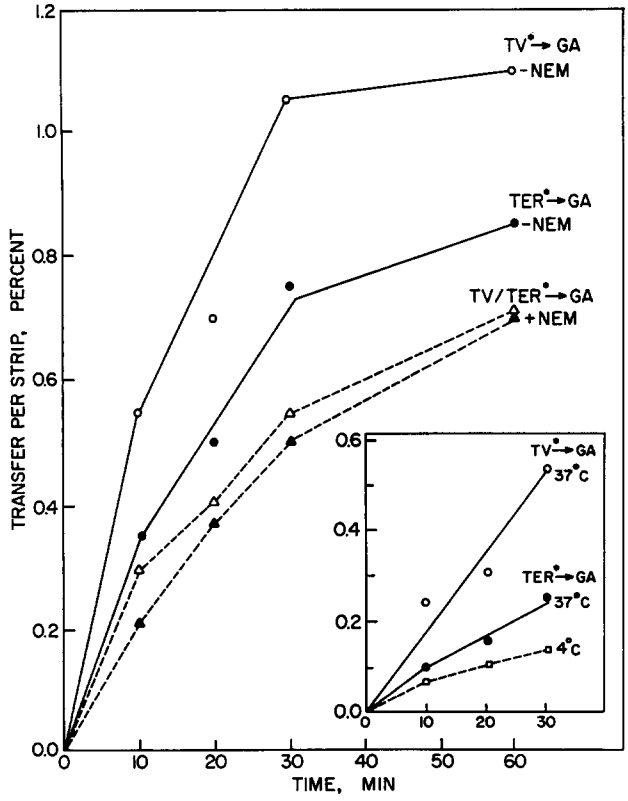

Fig. 2. Transfer of [^{14}C]phosphatidylcholine from radiolabeled donor transitional endoplasmic reticulum (TER*) or transition vesicles (TV*) to unlabeled Golgi apparatus (GA) acceptor of rat liver. The inset shows the transfer sensitive to inhibition by 1 mM *N*-ethylmaleimide (NEM) at 37°C compared to the transfer from TER at 4°C in the absence of NEM. Transfer sensitive to NEM is transfer in the absence of NEM at 37°C minus transfer in the presence of NEM at 37°C.

largely absent. In contrast, the bulk of the transitional endoplasmic reticulum fraction from which the vesicles were derived contained much larger vesicles representative of the part-rough (with ribosomes), part-smooth endoplasmic reticulum (Fig. 1A) normally capable of producing transition vesicles within the intact cell.

For PC (Fig. 2), transfer from radiolabeled donor to unlabeled Golgi apparatus acceptor was greater for purified transition vesicles as donor than for transitional endoplasmic reticulum as donor. For transitional endoplasmic reticulum as donor, transfer was ATP-dependent (Table III) whereas for both donors, transfer was sensitive to *N*-ethylmaleimide (Fig. 2). For transition vesicles, the *N*-ethylmaleimide-sensitive (ATP-dependent) transfer was approximately twice that of transitional endoplasmic reticulum (Fig. 2, inset).

With ceramide, the transfer was largely *N*-ethylmaleimide-insensitive (Fig. 3) and ATP-independent (Table III, Fig. 4). In contrast to results with PC (Fig. 2), the percent transfer from transitional endoplasmic reticulum labeled with ceramide was more efficient than from transition vesicles. *N*-ethylmaleimide-inhibited transfer from ceramide-labeled transition vesicles to unlabeled Golgi apparatus was not time-dependent and less than 0.1 that for PC (Fig. 3, inset).

TABLE III

Transfer after 60 min of [^{14}C]phosphatidylcholine (PC) or [^{3}H]ceramide as donor from transitional endoplasmic reticulum to unlabeled Golgi apparatus as acceptor

Radiolabeled donor lipid	Additions	Temperature	Transfer per strip (%) [b]
PC	none	37°C	0.5 ± 0.03
		4°C	0.13 ± 0.01
	complete [a]	37°C	0.8 ± 0.08
		4°C	0.2 ± 0.02
Ceramide	none	37°C	2.3 ± 0.10
		4°C	0.6 ± 0.02
	complete [a]	37°C	2.3 ± 0.12
		4°C	0.7 ± 0.02

[a] ATP + ATP regenerating system plus > 10 kDa cytosol fraction from rat liver.

[b] Mean of two determinations ± mean average deviations.

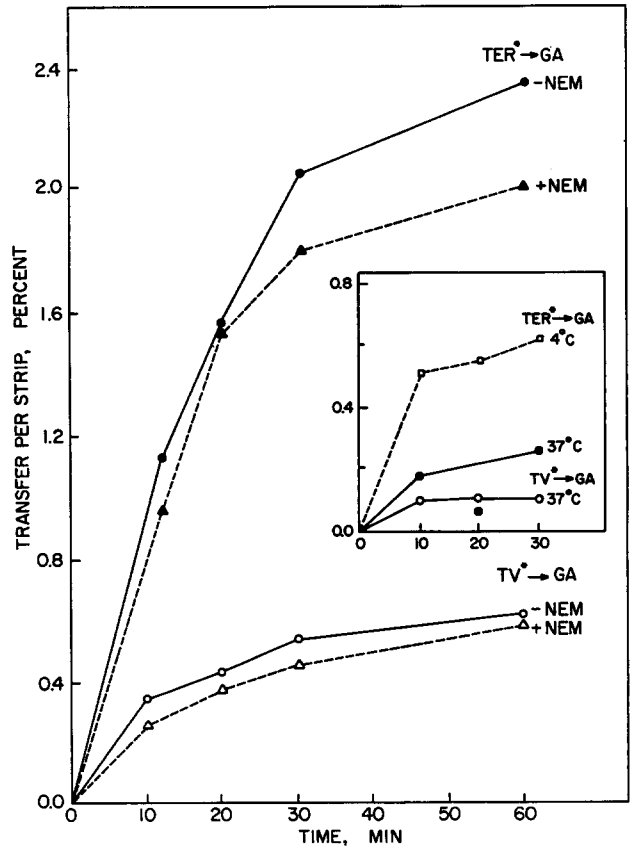

Fig. 3. Transfer of [^{3}H]ceramide from radiolabeled donor transitional endoplasmic reticulum (TER*) or transition vesicles (TV*) to unlabeled Golgi apparatus (GA) acceptor of rat liver. The inset shows the transfer-sensitive to inhibition by 1 mM *N*-ethylmaleimide (NEM) at 37°C compared to the transfer from TER at 4°C in the absence of NEM.

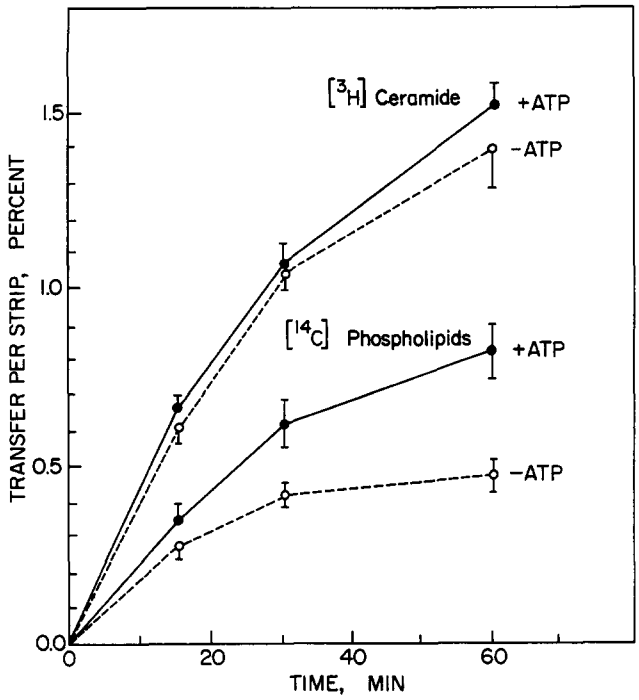

Fig. 4. Transfer of [^{3}H]ceramide from radiolabeled donor transitional endoplasmic reticulum (TER) to unlabeled Golgi apparatus in the presence or absence of ATP plus ATP regenerating system and comparison to transfer of ^{14}C-labeled phospholipids. For the latter, the TER donor was isolated from liver slices labeled for 1 h with [^{14}C]acetate as described [4].

Despite the lack of ATP-dependence and *N*-ethylmaleimide-sensitivity, the transfer of ceramide was temperature-dependent as was the ATP-independent component of PC transfer (Table III). With PC transfer, the Q_{10} was approx. 2. For ceramide, the Q_{10} was approx. 1.7.

To demonstrate that endoplasmic reticulum and/or transitional endoplasmic reticulum of liver were, in fact, sites of ceramide synthesis, liver slices were incubated for 2 h with [^{14}C]acetate. Ceramides when isolated by thin layer chromatography from transitional endoplasmic reticulum and the bulk of the rough endoplasmic reticulum, were labeled to about the same specific activity (Table IV). The ceramide of a more dense endoplasmic reticulum fraction, considered to be derived from the stacked arrays of rough endoplasmic reticulum and designated rough ER-II, was unlabeled. This endoplasmic reticulum fraction contained vesicles uniformly covered with attached ribosomes. Finally, the transition vesicles were also unlabeled, the radioactivity found being easily accounted for by the low levels of transitional endoplasmic reticulum contamination confirm the absence of ceramides in these vesicles (Table II).

TABLE IV

Labeling of non-hydroxy ceramides within different endoplasmic reticulum (ER) fractions and transition vesicles of rat liver

Liver slices were labeled for 2 h with [^{14}C]acetate. Lipids were extracted and analyzed for labeled ceramides. Hydroxy and non-hydroxy ceramides were separated from other lipids by HPTLC using $CHCl_2$/acetic acid (90:10) as eluent. Standards were used to determine the mobility of ceramides and to determine recovery during separation. No radioactivity was found associated with hydroxyceramides.

Fraction	dpm/mg protein	% of total lipids
Transitional ER	370	0.3
Rough ER-I	330	0.7
Rough ER-II	50	< 0.05
Transition vesicles	26	< 0.05

Discussion

Moreau et al. [4] previously demonstrated cell-free transfer of acetate labeled phospholipids from transitional endoplasmic reticulum as donor and to unlabeled Golgi apparatus as acceptor. One component of transfer, approx. 50%, was temperature and ATP-dependent and mediated via transition vesicles. A second component comprising the remainder of the transfer was ATP-independent, not mediated by transition vesicles, but was temperature dependent. Cytosol was beneficial for vesicle transfer but was not required for the ATP-independent transfer [4].

Similar findings have been demonstrated in the present study for transfer of PC. In addition, data are presented to show that the ATP-dependent transfer of PC from transitional endoplasmic reticulum to Golgi apparatus and both the ATP-dependent and ATP-independent transfer of PC from transition vesicles to Golgi apparatus are sensitive to 1 mM *N*-ethylmaleimide. *N*-ethylmaleimide is a well established inhibitor of vesicle formation/fusion events in cell-free systems [2,27–29].

In contrast to PC and other phospholipids, ceramides as well as triacylglycerols (see also Ref. 6) are low or absent from transition vesicles, suggestive of lipid sorting to exclude these lipids from the vesicle membrane as the transition vesicles are formed. As has been reported previously (Ref. 9 and references therein), newly synthesized ceramides are present in endoplasmic reticulum of rat liver and do not appear to be preferentially excluded from the transitional endoplasmic reticulum.

It is possible that the method of radiolabeling resulted in non-uniform incorporation of ceramides. However, radiolabeled sterols, incorporated in a similar manner do reach transition vesicles and are transferred via an ATP-dependent mechanism from endoplasmic reticulum to Golgi apparatus in the cell-free system.

Despite lipid sorting to restrict entry of ceramide into the transition vesicles, ceramide from the endoplasmic reticulum does reach the Golgi apparatus [9,10,30]. In the cell-free system, transfer appears to occur but by a temperature-dependent, non-vesicular mechanism that requires neither ATP nor cytosol and is insensitive to *N*-ethylmaleimide.

The nature of the mechanism that accounts for nearly half of the cell-free transfer of phospholipids and nearly all of the ceramide from transitional endoplasmic reticulum to Golgi apparatus is not known. Only the strict temperature dependency ($Q_{10} \equiv 2$) distinguishes it from a physical process. Cytosolic transfer proteins seem not to be obligatorily involved and ceramide is transferred several times more efficiently than are phospholipids. The transfer is proportional to time and donor amount.

A major role for ceramide is as an essential precursor for both sphingomyelin [30] and the glycosphingolipids, including gangliosides [9], which are synthesized at the Golgi apparatus and eventually concentrate in the plasma membrane. It appears that membrane proteins are delivered from endoplasmic reticulum to Golgi apparatus predominantly, if not exclusively, by a vesicular mechanism [1–3,12]. However, for lipids and especially biosynthetic precursors such as diacylglycerides and ceramide, the alternative pathway suggested by the cell-free observation is probable. Transfer might be micellar or via a completely soluble intermediate, or might even involve direct membrane contacts, although the latter seem less likely. Most biochemical assays for synthesis of sphingomyelin, glucosylceramide and lactocylceramide, the immediate Golgi apparatus products from ceramide, remain problematic and endogenous precursors frequently appear to be utilized differently than those added exogenously.

The Golgi apparatus has been identified as a unique site of sphingolipid synthesis using ceramide analogs [31,32]. In contrast, when labeled ceramide or phosphatidylcholine was used, the plasma membrane appeared also to be an important site of sphingomyelin synthesis [33]. Even if their plasma membrane fraction was contaminated by Golgi membranes, it is difficult to totally rule out any plasma membrane biosynthetic activity. Alternatively, the high amount of ceramides in the plasma membrane (Table II) could be a consequence of active neutral sphingomyelinases [34]. Ceramides also can be formed in lysosomes by an acidic sphingomyelinase [34]. Quinn and Allan [35] have shown that two pools of sphingomyelin (one in the plasma membrane and another in the intracellular membranes, probably the endoplasmic reticulum) occur in BHK cells. These results lead to the question as to how these different pools are supplied. What we have shown is that ceramides present in the endoplasmic reticulum can reach the Golgi apparatus and presumably other membrane locations for further sphingolipid synthesis. This transfer occurs independently from the endoplasmic reticulum to Golgi apparatus vesicular pathway but could represent an important route for ceramide supply to the various intracellular pools where ceramides accumulate or are utilized as biosynthethic precursors.

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