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GLYCOLIPID GLYCOSYL TRANSFERASES OF A HAMSTER CELL LINE IN CULTURE

II. SUBCELLULAR DISTRIBUTION AND THE EFFECT OF CULTURE AGE AND DENSITY

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Summary

The activities of two galactosyl transferases catalysing the formation of di- and tri-glycosyl ceramides in NIL-2 hamster cells have been studied with respect to culture age and density, subcellular distribution, and transformation of cells by virus. The activity of the transferases was found to increase considerably as culture density increased, although maximal activities were found before appreciable cell contact occurred. The highest transferase activities were found in the endoplasmic reticulum. Virus transformation reduces the activity of the transferase catalysing triglycosyl ceramide synthesis, while the transferase catalysing diglycosyl ceramide synthesis is slightly increased. There is no evidence that the transformed cells produce a dialysable soluble inhibitor of transferase activities.

Introduction

The properties of enzyme systems that catalyze the formation of di- and triglycosyl ceramides have been described in the accompanying paper [1]. Kijimoto and Hakomori [2] reported on the enzyme activities of growing and confluent cell populations, and in cells transformed by polyoma and hamster sarcoma viruses. We have confirmed some of their observations, and in addition studied the full cycle of cell growth in culture from trypsinization and the initiation of a sparse culture, through the exponential growth phase to dense confluent cultures and finally to the stage when the cells detach from their supporting substrate. In addition the subcellular distribution of enzyme activities are reported.

Materials and Methods

Membrane fractionation

NIL-2 cells (approx. $5 \cdot 10^8$) were suspended in 0.25 M sucrose, 5 mM Tris · HCl (pH 7.4), 0.2 mM Mg^{2+} to a volume of 30 ml and ruptured by nitrogen cavitation after equilibration with oxygen-free nitrogen at 750 lb · in^{-2} for 15 min. Nuclei were removed from the homogenate by centrifugation at 1000 × *g* for 5 min and the supernatant which was made 1 mM with respect to EDTA, was diluted to 40 ml with 0.25 M sucrose, 5 mM Tris · HCl (pH 7.4). An MSE BXIV zonal rotor was loaded with the following material: 50 ml 5% * sucrose, 40 ml sample, 90 ml 15% sucrose, 40 ml 19% sucrose, 50 ml 22.5% dextran (M_r 40 000), 200 ml 35% sucrose and approx. 140 ml 60% sucrose, and centrifuged at 105 000 × *g* for 90 min. All gradient solutions contained 1 mM EDTA and 5 mM Tris · HCl (pH 7.4). The fractionation of plasma membrane, endoplasmic reticulum and mitochondria attainable in such a gradient has been discussed previously [3]. Fractions from the zonal gradient were diluted with an equal volume of 5 mM Tris · HCl (pH 7.4); centrifuged at 100 000 × *g* for 30 min and resuspended in 1 ml 0.25 M sucrose, 5 mM Tris · HCl (pH 7.4) prior to enzymic analysis.

Subfractionation of material isolated from the zonal gradient was achieved in isopycnic sucrose gradients. Sedimented membrane fractions were washed twice in 5 mM Tris · HCl (pH 7.4), to release trapped soluble proteins; suspended in 7 ml of 50% sucrose and incorporated into the following gradient: 6 ml 50%, 6 ml 40%, 5 ml 30% and 5 ml 20% sucrose. All these solutions contained 1 mM EDTA and 5 mM Tris · HCl (pH 7.4). After centrifugation at 80 000 × *g* for 16 h the gradient was unloaded by upward displacement with 61% sucrose, and collected in 1 ml fractions.

Enzyme assays

The following enzymes were determined in the isolated membrane fractions: ($Na^+ + K^+$)-stimulated Mg^{2+} · ATPase (EC 3.6.1.3) [4], NADH diaphorase (EC 1.6.4.3) [4] and succinate cytochrome *c* reductase (EC 1.3.99.1) [5]: and protein was assayed by the method of Lowry et al. [6]. The broad specificity galactosyl transferase activity, using *N*-acetylglucosamine as substrate was assayed by the method of Fleischer et al. [7].

The methods used for the determination of glycolipid glycosyl transferases are described in the accompanying paper [1]. The conditions described were found to be optimal not only for enzyme particles from dense cells but also for particles from sparse cells except that substrate inhibition became apparent at much lower glycolipid concentrations. Because of this, initial velocities were determined at six different substrate concentrations ranging from $5 \cdot 10^{-4}$ to 10^{-2} μmol of glycolipid substrate in 0.2 ml of incubation mixture as described, when enzyme activities were determined from sparse cells.

Preparation of labelled ceramide trihexoside

NIL-2 cells were grown in medium containing [^{14}C]palmitate (10 μC/ml)

* Concentration of gradient solutions expressed as % (w/w).

for 48 h. Total lipids were extracted with chloroform/methanol (2 : 1), and the lipid extract was saponified with 1 M methanolic NaOH in chloroform/methanol (1 : 1) for 1 h at room temperature. After neutralisation with aqueous HCl the mixture was partitioned and glycolipids isolated by the two dimensional chromatography described previously. Ceramide trihexoside was further purified by column chromatography using a silicic acid column (0.5 g silicic acid, 0.6 × 6 cm column) and eluting successively with 95 : 5 : 1, 80 : 20 : 2, and 70 : 30 : 3 chloroform/methanol/water mixtures. The counts eluted with 70 : 30 : 3 gave a single radioactive spot on autoradiography which corresponded with standard ceramide trihexoside. Its specific activity was then determined. An aliquot of the radioactive ceramide trihexoside prepared (the yield from approximately 10^8 cells) was methanolysed, and the sugars were estimated by gas-liquid chromatography using mannitol as internal standard as described by Esselman et al. using an OV_1 column and a temperature programme of 150—200°C (1°C/min) [8]. An average specific activity of $1.2 \cdot 10^6$ dpm · μmol^{-1} was obtained from three determinations. The yield from 10^9 cells was approximately 100 μg of pure ceramide trihexoside.

Results

Subcellular distribution of enzymes

The distribution of material within the zonal gradient is described in Fig. 1. Table I gives the enzyme content of certain fractions isolated from this gradient and of the nuclei and homogenate. Using $(Na^+ + K^+)$-stimulated Mg^{2+} · ATPase as a plasma membrane marker, NADH diaphorase as an endoplasmic reticulum marker and succinate-cytochrome *c* reductase as a mitochondrial marker it is clear that these three membranes are concentrated in fractions 15—18, 21—25 and 35—40, respectively. The "light" membrane fraction (9—14) contains the "broad-spectrum" galactosyl transferase in high concentration but no $(Na^+ + K^+)$-stimulated Mg^{2+} · ATPase. The glycolipid galactosyl transferase activities are present in both the endoplasmic reticulum and plasma membrane, whilst the nuclei contain no activity. The levels in the mitochondria are probably due

TABLE I

ENZYME CONTENT OF ISOLATED SUBCELLULAR FRACTIONS FROM NIL-2 CELLS

ND, not determined.

Fraction	Specific activity (μmol substrate converted per h/mg protein)				Total activity (μmol substrate converted per h/mg protein)				
	$(Na^+ + K^+)$-Mg^{2+} · ATPase	NADH diaphorase	Succinate cytochrome *c* reductase	Galactosyl transferase	Protein	$(Na^+ + K^+)$-Mg^{2+} · ATPase	NADH diaphorase	Succinate cytochrome *c* reductase	Galactosyl transferase
9—14	<0.02	85	<0.1	0.41	1.1	< 0.1	93	< 3.0	0.44
15—18	2.54	95	<0.1	0.26	3.8	9.7	360	< 3.0	1.0
21—25	0.31	340	0.43	0.40	11.0	3.4	3720	4.8	4.4
35—40	0.10	ND	2.10	0.05	8.9	0.9	ND	18.7	0.45
Nuclei	0.05	ND	<0.1	<0.01	38.0	1.8	ND	< 3.0	<0.3
Homogenate	0.11	ND	0.21	0.03	157	17.3	ND	33	4.7

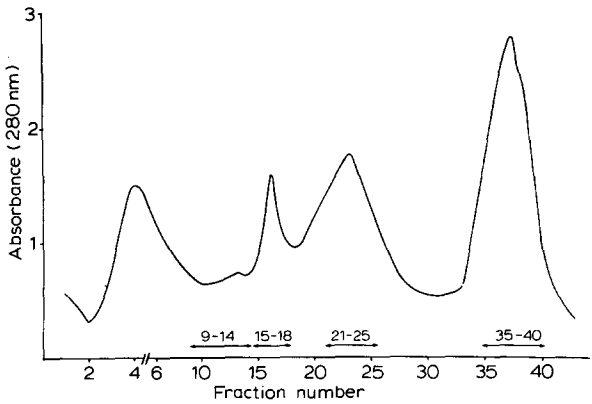

Fig. 1. The 280 nm absorbance profile of a sedimentation rate zonal gradient. Experimental details are given in the Materials and Methods section.

to contamination from the endoplasmic reticulum, since if mitochondria are prepared from NIL-2 cells using a standard Dounce homogenisation and differential centrifugation procedure [9], virtually no activities can be detected.

The presence of the galactosyl transferase enzyme and absence of (Na^+ + K^+)-ATPase in fractions 9—14 suggests that this region may contain membranes derived from the Golgi. Nitrogen cavitation unfortunately vesiculates all the plasma membrane and endoplasmic reticulum, including Golgi membranes. Vesiculation destroys the possible morphological identification of these membranes and no enzyme markers for the Golgi, other than sugar transferase have been conclusively shown to exist. Since, however, the Golgi membranes from animal tissues [10] contain a higher proportion of lipid to protein they therefore possess a lower density than other membranes. It is therefore probable that the galactosyl transferase activity in the low density part of the gradient is due to the Golgi membrane. It is difficult to discount, however, the possibility that the galactosyl transferase activity in the plasma membrane and endoplasmic reticulum is not due to contamination from Golgi membranes. We attempted to resolve this problem by isopycnic sucrose gradient centrifugation of the plasma membrane and endoplasmic reticulum fractions.

TABLE II

SYNTHESIS OF CERAMIDE DIHEXOSIDE AND CERAMIDE TRIHEXOSIDE BY THE SUB-CELLULAR FRACTIONS OF NIL-2 CELLS

Each of the subcellular fractions assayed for transferase activities was characterized as given in Table I. The results given are the mean of four different determinations. Endogenous activities obtained without added glycolipid substrate have been subtracted in the data tabulated. ND, not detected.

Sub-cellular fractions	[^{14}C] Galactose incorporated into glycolipid substrate in pmol · mg protein^{-1} · h^{-1}	
	Transferase I	Transferase II
Homogenate	508	436
Nucleus	ND	ND
Mitochondria	128	205
Plasma membrane	810	672
Endoplasmic reticulum	1580	1610

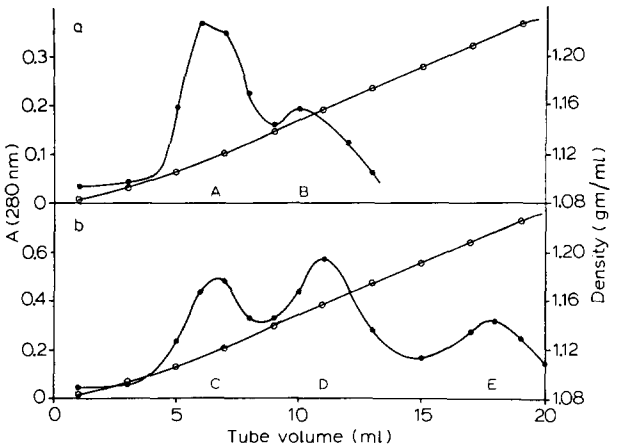

Fig. 2. Protein profiles of plasma membrane (a) and endoplasmic reticulum (b) material fractionated in isopycnic sucrose gradients ●———●; density profiles of these gradients ○———○.

Fig. 2 demonstrates the disposition of plasma membrane (a) and endoplasmic reticulum (b) material within these gradients. The two major bands from the plasma membrane fraction both contain (Na^+ + K^+)-stimulated Mg^{2+} · ATPase (Table III), although the specific activity in the upper band (A) is approx. 4 times higher than in the lower (B). Both of the glycolipid galactosyl transferases however, are concentrated almost entirely in the less dense band (A). On the other hand the lighter subfraction (C) derived from the zonal endoplasmic reticulum contained exclusively transferase I whilst the heavier subfraction (D) contained exclusively transferase II. Both of these subfractions contained NADH diaphorase, while (Na^+ + K^+)-stimulated Mg^{2+} · ATPase was only detectable in subfraction C. Subfraction E contained NADH diaphorase, but no significant quantities of the other enzymes.

Effect of growth conditions and viral transformation on enzyme activities

One of the most interesting observations in glycolipid metabolism of NIL-2

TABLE III

RECOVERY OF ENZYMES IN ISOPYCNIC SUCROSE GRADIENT FRACTIONS FROM PLASMA MEMBRANES AND ENDOPLASMIC RETICULUM

Fraction	(Na^+ + K^+)-Mg^{2+} · ATPase		NADH diaphorase		Transferase I (% recovery)	Transferase II (% recovery)	Protein (μg total)
	Spec. act.*	Total**	Spec. act.*	Total**			
A	2.9	1.25	1.3	0.57	90	90	430
B	0.7	0.12	4.8	0.83	3	3	170
C	0.3	0.18	15.2	9.0	98	0	600
D	0	0	32.8	36.0	0	96	1126
E	0	0	36.0	17.0	0	2	900

* Specific activity: μmol substrate converted · h^{-1} · mg $protein^{-1}$.

** Total activity: μmol substrate converted · h^{-1}. Fractions were identified as in Fig. 2.

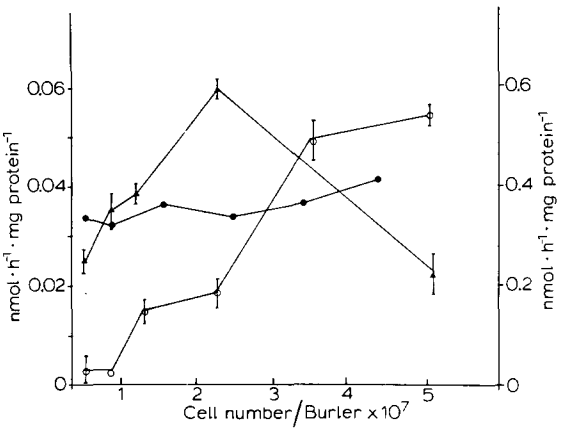

Fig. 3. Changes in enzyme activities during exponential growth of NIL-2 cells: ▲———▲, ceramide monohexoside to ceramide dihexoside; ○———○, ceramide dihexoside to ceramide trihexoside; ●———●, sugar hydrolase activity. Assay condition for both transferases were as given in the accompanying paper. Sugar hydrolase activities were determined as previously described [11], using a crude homogenate as enzyme source. Each point represents the arithmetic mean of five separate determinations.

cells is the increased rate of synthesis of ceramide tri-, tetra- and pentahexosidases a logarithmically growing cell population approaches confluence in growth [11,12]. Critchley et al. [13], gave the levels of relevant enzymes in growing and confluent cell populations. We have confirmed and extended this observation to cover the entire period of cell growth from the time of trypsinization and seeding to a time considerably after the cells had reached their saturation densities. The sugar hydrolase activities for ceramide trihexoside were also measured during these experiments. The results are shown in Fig. 3. Beyond the last point shown ($5 \cdot 10^7$ cells per 2 l roller bottle) no increase in activity was obtained. Continued measurements after this time show that activities of both enzymes decline gradually if the culture is kept longer. The decline in transferase I is more abrupt than that of transferase II, an observation also supported by the data in vivo with respect to ceramide dihexoside synthesis by metabolic labelling [12]. The glycolipid sugar hydrolase activities

TABLE IV

SPECIFIC ACTIVITIES OF UDP-GAL: GLYCOLIPID GALACTOSYL TRANSFERASES OF NORMAL NAD VIRALLY TRANSFORMED NIL-2 HAMSTERD CELLS

The conditions of assay were the same as given in Table I. A crude homogenate was used as the enzyme source. The homogenates were mixed 1 : 1 by vol. when they were used in testing for inhibition of transferase by transformed cells. The data shown is hte mean of six separate determinations.

Cells	[^{14}C] Galactose incorporated in nmol · mg protein^{-1} · h^{-1}	
	Transferase I	Transferase II
NIL	650 ± 58	512 ± 46
NIL-HSV	780 ± 72	22 ± 12
NIL + NIL-HSV	680 ± 71	265 ± 30

using ceramide trihexoside as substrate show little variation in these experiments throughout the cell densities tested. These activities are very much smaller when compared to the rate of synthesis of glycolipids.

Our previous studies [12] showed that NIL-2 cells incorporate considerably more precursor label to ceramide trihexoside compared to ceramide dihexoside, when sparse dividing cells are made quiescent by placing them in medium containing only 0.25% serum. When such cells were assayed for transferase II they gave activities in the order of 800—1000 units which are approximately twice as high as the activities obtainable with 10% serum when cells are dense.

Viral transformation is shown to result in complete loss of the ability of cells to synthesize ceramide trihexoside and other higher glycolipids, while the synthesis of ceramide dihexoside continues [12]. Comparative studies of the two transferases were therefore conducted in normal and transformed cells. Table IV shows the result.

The observed level of activity of transferase I is slightly higher in transformed cells, an observation consistent with those of Kijimoto and Hakomori [1]. If a soluble inhibitor was responsible for the observed lack of activity in transformed cells, a reduction in activity much greater than that observed in the mixed homogenate experiment might have been expected.

Sonication of the particles followed by detergent extraction overnight and centrifugation at $100 \cdot 10^3 \times g$ for 1 h released approximately 30% of the activity of the original particle preparation enzymes. However, the activity thus obtained is rapidly lost by standing over 24 h at 0—4°C, or removal of detergent by dialysis or by freeze drying.

The following attempts were made to get an in vitro synthesis of ceramide trihexoside by transformed cells. (1) Solubilization by detergent; (2) sonication of particles followed by extraction with detergent or buffers; (3) repeated dialysis of washed particles. All gave negative results. Transferase I was detectable throughout these procedures, indicating that protein denaturing conditions were not responsible for the lack of activity.

Discussion

(a) Cell cycle dependence of galactosyl transferases

It is generally believed that the glycosyl transferases specific for a given acceptor molecule and specific for a given nucleotide sugar are the control points in regulating the amount and variety of complex polysaccharides on the surface of animal cells. It has also been suggested that in addition to the above role glycosyl transferases function as cell surface receptors in cellular adhesion [14]. Suggestive evidence has accumulated in support of the above hypothesis [15—18]. The report by Roth and White [14] is of a particular interest, because they implicate cell-to-cell contact resulting in inter-cellular glycosylation between an acceptor molecule on one cell and an enzyme molecule on another. Normal Balb/c 3T3 cells were shown to catalyze such a reaction when incubated with UDP-galactose, while a malignant and relatively "non-contact inhibited" 3T12 cell line carried out the same reaction without any requirement for cell contact.

This hypothesis fits the observations on glycolipid metabolism of NIL cells if

one accepts that ceramide trihexoside, ceramide tetra- and ceramide pentasaccharides are made by transglycosylation, as Roth et al. [17] suggest. However, our data on the rate of increase of the activity of the key enzyme, transferase II is insufficient to clarify this point. Additional evidence would be required to establish whether or not the observed increase in activity is confined to the external surface of the plasma membrane. Cellular contact triggering of the induction of this enzyme appears unlikely, because the enzyme is fully induced before cell contacts are made as a result of increased culture density ($2.5 \cdot 10^7$ in a 2.5 l roller bottle, total surface area 690 cm^2). Culturing the cells further to their usual saturation densities ($8 \cdot 10^7$) results in very little increase in the activity of this enzyme, and indeed it falls off if the cultures are kept after they are confluent. This observed increase in activity (approx. 5-fold) is quite sufficient to explain the observed enhancement of synthesis of ceramide trihexoside, and consequently of the other higher glycolipids in the series, assuming that the essential precursor limiting the synthesis was ceramide trihexoside.

Our data differs from report in ref. 2 in the following: (a) the activities reported here are considerably higher for transferase I, than transferase II; (b) we have not been able to demonstrate any significant variation in the level of hydrolase activities throughout the cell densities tested. The latter observation might arise from the fact that the incubation times we employed were shorter. In longer incubations the reaction rate was not linear with respect to time.

The first point in this experiment was taken after 24 h when the cells were seeded from a dense to a sparse culture. The activity of transferase II is not detectable at this time. Since trypsinization does not affect the enzyme activities, this would mean that the enzyme is either degraded within 24 h, or is in an inactive form. Even after the cell number has doubled the transferase II activity is barely detectable (an observation which is supported by incorporation of [^{14}C]palmitate) [12]. While there is appreciable activity of transferase I at both these points, the possibility must be considered that transferase II is being synthesized only in certain phases of the cell cycle, and since the first two divisions are relatively synchronous, it is conceivable the points were taken on a synchronous population at a time when the enzyme was absent. We have subsequently obtained evidence that ceramide trihexoside synthesis is indeed limited to the G_1 part of the cell cycle [12]. If this is the correct explanation then the enzyme molecule must have a relatively short half life.

The activity of transferase II closely reflects increased ceramide trihexoside synthesis in all reported situations, namely synthesis in serum-block, cells of low density as opposed to high, and its absence in transformed cells. This makes it very unlikely that alternative pathways of ceramide trihexoside synthesis exist other than that catalyzed by transferase II.

(b) Subcellular distribution of galactosyl transferases

We have been unable to eliminate entirely the possibility that the galactosyl transferase activities detected in the plasma membrane and endoplasmic reticulum from NIL-2 cells, are actually due to contamination by Golgi membranes. However, it is significant that galactosyl transferase activity can be detected in slowly-sedimenting material (fractions 9—14) from the zonal gradient which contains no ($Na^+ + K^+$)-stimulated Mg^{2+} · ATPase and which is lighter than the

plasma membrane. Golgi membranes identified either morphologically or by galactosyl transferase activity, isolated from rat liver [19—21] and rat submaxillary-sublingual glands [22] have a density or sedimentation rate lower than the other membranes of the cell. It is feasible, therefore, that the light material in the zonal gradient is derived from Golgi membranes. In the absence of any other enzymatic or morphological evidence it is impossible to be unequivocal on this point.

The results (Table I) indicate that these galactosyl transferase enzymes occur in a number of membrane systems in the NIL-2 cell. This is in accordance with previous observations on the distribution of galactosyl transferases using either *N*-acetylglucosamine or a protein as a receptor. They have been detected in the plasma membrane of rat kidney [23] neural retina cells [17] and bovine submaxillary glands [24]; in the smooth internal membranes of HeLa cells [24] and rat brain (in this case the enzyme was ceramide glucosyl transferase) [25]; in the Golgi membranes from rat submaxillary-sublingual glands [23], rat liver [26] and Ehrlich Ascites cells [27]. Furthermore, although one membrane fraction may contain the highest level of transferase activity, the latter is frequently detected at significant levels in other membrane fractions [22,24,28].

Whether the galactosyl transferase observed in the plasma membrane fraction is a true component of this membrane or whether it is due to contamination from either the "light" membranes or the endoplasmic reticulum, or both, is not clear from the results. Only 10% of the total NADH diaphorase activity in the endoplasmic reticulum (Table I) is in the plasma membrane; on the other hand the total galactosyl transferase activity in the plasma membrane is 25% of that in the endoplasmic reticulum. Unless the endoplasmic reticulum which contaminates the plasma membrane is specifically enriched in galactosyl transferase and impoverished in NADH diaphorase, these figures indicate that the degree of contamination of the plasma membrane by the endoplasmic reticulum cannot account on its own, for all the galactosyl transferase in this fraction.

A comparison of the galactosyl transferase in the plasma membrane and "light" membrane fractions shows that although the specific activity of this enzyme is higher in the latter, it contains only half of the total activity of the plasma membrane. It is interesting that the transferase activity cannot be resolved from the $(Na^+ + K^+)$-Mg^{2+} · ATPase either in a sedimentation rate gradient (Table I) or in an isopycnic gradient (Table III). It is tempting to speculate that the transferase is a true component of the surface membrane but in the absence of any data regarding the density and sedimenting properties of Golgi membranes from these cells it is impossible to be unequivocal about this point.

From the enzyme data (Table II) it appears that the endoplasmic reticulum from the zonal gradient can be fractionated isopycnically into two major components (C and D) both of which contain NADH diaphorase activity while only C contains some contaminating plasma membrane ($Na^+ + K^+$-stimulated Mg^{2+} · ATPase). The glycolipid galactosyl transferase data on these fractions suggest that the two enzymes I and II are segregated within the endoplasmic reticulum, such that nitrogen cavitation produces vesicles containing one or other of the enzymes. These two activities would thus be associated with two different

particles rather than an oligomeric complex with two different sub-units catalysing each reaction. Whilst in the plasma membrane the two activities are associated with the same particle. It seems unlikely that the transferase I activity within subfraction C of the endoplasmic reticulum is contributed entirely by the contaminating plasma membrane, in view of the restriction of both enzymes I and II to a single population of vesicles derived from the zonal plasma membrane fraction; unless the plasma membrane which contaminates the endoplasmic reticulum is a specific heavy fraction containing only enzyme I.

However this data is interpreted in detail, it is clear that specialisation with respect to these transferases and other enzymes exists within both plasma membrane and endoplasmic reticulum. The production of membrane subfractions, each of a specific density and each containing some specific determinants has been frequently observed [29—31], and its implications for the structure of the membrane are important. For, if the membrane is a fluid structure in which proteins are allowed to move laterally and freely within the lipid bilayer then some proteins must be restricted in their movement and/or some protein molecules must be preferentially associated with others in an organised structure.

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