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GALACTOSE OXIDASE ACTION ON G_{M1} GANGLIOSIDE IN MICELLAR AND VESICULAR DISPERSIONS

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G_{M1} ganglioside was dispersed in different membrane-mimicking systems and the effect of dispersion on G_{M1} oxidation by galactose oxidase was studied. The following membrane-mimicking systems were used: homogeneous micelles of G_{M1}; mixed micelles (at different proportions of constituents) of G_{M1} with either G_{D1a} ganglioside (which is resistant to the enzyme), or the non-ionic detergent Triton X-100, or bovine serum albumin; small unilamellar vesicles of egg phosphatidylcholine (PC), containing various proportions of G_{M1}. As a reference substrate not involved in membranous systems and freely interacting with the enzyme, the oligosaccharide portion of G_{M1} ($DesG_{M1}$) was employed.

The apparent V_{max} of the enzyme was dramatically dependent on the type of G_{M1} dispersion. The lowest value was recorded on homogeneous micelles of G_{M1} and on mixed G_{M1}-G_{D1a} micelles. From this value, the V_{max} increased 2-fold with G_{M1}-bovine serum albumin lipoprotein micelles, up to 1400-fold with mixed G_{M1}-Triton X-100 (optimal molar ratio, 1:13.8) micelles, and up to 14000-fold on PC vesicles containing 8 mol% G_{M1} (this proportion was optimal for enzyme activity on vesicles). The activity developed on these latter vesicles turned out to be still greater (2-fold) than that displayed on $DesG_{M1}$. The apparent K_m had very similar values in all different membrane systems; in contrast, it was markedly greater on $DesG_{M1}$. Both Triton X-100 micelles and PC vesicles did not appreciably alter the kinetics of galactose oxidase action on pure galactose, indicating that the above effects are dependent on the intrinsic characteristics of the membrane-like systems containing gangliosides.

Introduction

Gangliosides are sialic acid containing glycosphingolipids present in the plasma membranes of most vertebrate cells and particularly abundant in the neuronal membranes. They appear to be involved in a variety of cell surface events based on recognition phenomena [1–5]. These events, especially after the detailed studies on cholera toxin binding to G_{M1} ganglioside [6–8], are viewed as due to specific interactions between the ganglioside carbohydrate moiety, protruding from the membrane surface, and the external ligand.

It is known that the availability to external enzymes of membrane-bound phospholipids and cholesterol depends on a number of physical and chemical conditions of the membrane: surface pressure, spacing, association, transition temperatures of the same lipids, etc. [9–13].

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine.

This paper follows the ganglioside nomenclature of Svennerholm [34] and the IUPAC-IUB recommendations [35] $G_{M1} = II^3NeuAc\text{-}GgOse_4Cer$; $G_{D1a} = IV^3NeuAc,II^3NeuAc\text{-}GgOse_4Cer$.

On this basis we thought it worth studying whether and how the physicochemical features of the membrane do also influence the bound ganglioside-external ligand interactions. Due to the paucity of information in this specific field [14,15] our first, more general, approach was to investigate how a peculiar recognition phenomenon, that is the action of an enzyme affecting gangliosides, was influenced by different dispersing systems of gangliosides. The ganglioside-containing systems used, providing surfaces and actually mimicking membranes, were: micelles of pure ganglioside or of ganglioside plus a detergent, and small unilamellar vesicles of egg phosphatidylcholine (PC). The ganglioside used was G_{M1} and the enzyme galactose oxidase, which catalyzes the oxidation (at C-6) of the galactose residue terminally located in G_{M1} [16]. As a reference substance, not involved in surface and freely interacting with the enzyme, the oligosaccharide portion of G_{M1} was employed; galactose was used when testing the effect of pure detergent micelles and PC vesicles on the enzyme.

Materials and Methods

Chemicals and other products

Commercial chemicals were of analytical grade or of the highest purity available. Solvents were distilled before use. The water routinely used was freshly distilled using a glass apparatus. Egg phosphatidylcholine and egg phosphatidylethanolamine were purchased from B.D.H. (Milan, Italy) and used without further purification provided that both of them showed a single spot when assayed by high-performance thin-layer chromatography on silica gel thin-layer plates (HPTLC 60 Merck, Darmstadt, F.R.G.). The solvent system was chloroform/methanol/water (60:35:4, v/v, 1 h run at 20°C), and spots were revealed by exposure to iodine.

Gangliosides G_{M1} and G_{D1a} were extracted and purified from beef brain according to Tettamanti et al. [17]. Their identification, structure analysis and purity were assayed as described by Sonnino et al. [18]. The final purity was over 99% for both gangliosides. The composition of the ganglioside lipid portions, analyzed according to Sonnino et al. [18], is reported in Table I.

The oligosaccharide portion of G_{M1} ganglioside ($DesG_{M1}$) was obtained from the original ganglioside (50 mg) after ozonolysis and alkaline treatment as described by Wiegandt [19]. The oligosaccharide was purified from the reaction mixture by chromatography on a 2 × 30 cm Biogel P2 column previously equilibrated and eluted with distilled water. The elution profile was monitored by TLC on HPTLC precoated plates (Merck, Darmstadt, F.R.G.) using *n*-propanol/water (7:3, v/v) as the developing solvent; the spots were detected by treatment with a *p*-dimethylaminobenzaldehyde spray reagent and heating at 130°C for 10 min. Triton X-100 (isooctylphenylpolyethoxyethanol) of gas-liquid chromatography purity grade and *o*-dianisidine were purchased from Merck (Darmstadt, F.R.G.). Delipidized bovine serum albumin was purchased from Sigma (St. Louis, MO, U.S.A.); its monomeric form was prepared according to Tomasi et al. [20]. Galactose oxidase (EC 1.1.3.9) (from *Polyporus circinatus*, 98 IU/mg protein) was from Kabi (Stockholm, Sweden); horseradish peroxidase (EC 1.11.1.7) from Boehringer (Mannheim, F.R.G.); 2,4,6-trinitrobenzenesulphonic acid (TNBS) from B.D.H. (Milan, Italy); Sepharose 4B from Pharmacia (Uppsala, Sweden).

TABLE I

LIPID COMPOSITION OF GANGLIOSIDES G_{M1} AND G_{D1a}

Compositions are expressed as mol%.

Ganglioside	Long-chain bases				Fatty acids					
	$C_{18:0}$	$C_{18:1}$	$C_{20:0}$	$C_{20:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$
G_{M1}	6.3	59.6	2.7	31.4	1.0	0.0	97.0	0.0	2.0	0.0
G_{D1a}	8.1	53.4	1.0	37.5	2.6	0.8	87.9	1.3	5.2	2.2

Preparation of micelle solutions of G_{M1} ganglioside

G_{M1} was dissolved in chloroform/methanol (2:1, v/v). Known aliquots of the mixture were transferred into glass tubes and the solvent was completely removed by a gentle flow of nitrogen. The residue was dissolved with a proper volume of 25 mM sodium phosphate buffer, pH 7.0, at 37°C and briefly vortexed. Mixed micelles or G_{M1} with either G_{D1a} or Triton X-100 were prepared starting from separate mixture of each component in chloroform/methanol (2:1, v/v). Known aliquots of each mixture were mixed in glass tubes and processed as described above.

Preparation of G_{M1}-bovine serum albumin complex (micellar lipoproteic complex)

G_{M1}-bovine serum albumin complex, was prepared as described by Tomasi et al. [20]. The low-molecular-weight complex, which consists of one molecule of bovine serum albumin and one micelle of G_{M1}, as described by the same authors, was employed.

Preparation of small unilamellar vesicles

PC or PC-G_{M1} small unilamellar vesicles were prepared following the procedure of Barenholz et al. [21], already applied to ganglioside-containing systems [14]. Further purification was achieved by chromatography of the high-speed supernatant on Sepharose 4B column (1.5 × 30 cm) previously equilibrated and eluted with 25 mM sodium phosphate buffer, pH 7.0. The elution was monitored by turbidity measurements at 450 nm. The integrity of small unilamellar vesicles was assessed according to Barenholz et al. [21] by: (a) turbidity measurements (reading at 450 nm); (b) assaying the percentage of amino groups (carried by PE inserted as a marker in the vesicle – about 5 mol%) available to TNBS (reading at 410 nm). The percentage of outer-sided PE amino groups was reported to be about 65% in the case of small unilamellar vesicles [21]; we confirmed this value also in G_{M1}-containing vesicles. All mixtures were prepared in 25 mM sodium phosphate buffer, pH 7.0. The distribution of vesicle-bound G_{M1} in the inner and outer side of the vesicles was determined as follows. A sample of vesicle preparation (carrying about 20 μg G_{M1}, as bound sialic acid) was incubated under the conditions specified below with an excess of galactose oxidase (generally 5 IU) till no more oxidation occurred and the extinction value was recorded. This corresponded to the G_{M1} exposed on the outer vesicle side and directly available to galactose oxidase. An equal sample, after addition of 1% Triton X-100 (final concentration) was vortexed till the mixture became perfectly clear (a few seconds) (indicating complete destruction of the vesicle structure) and treated with galactose oxidase as above. The recorded extinction value corresponded to the total G_{M1} present. As a control, at the end of the oxidation oxidized G_{M1} was separated from G_{M1} by TLC performed as described by Masserini et al. [22] and densitometrically quantitated. The results of a typical experiment establishing the sidedness of G_{M1} in the vesicle are given in Fig. 1. It is shown that in small unilamellar vesicles carrying

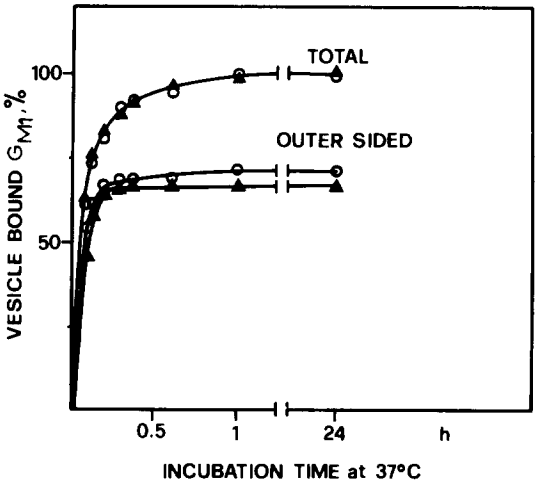

Fig. 1. Assessment of sidedness of G_{M1} in small unilamellar vesicles of PC containing from 3 (▲——▲) to 20 (○——○) mol% G_{M1}. Small unilamellar vesicles carrying 20 μg G_{M1} as bound sialic acid were incubated with an excess of galactose oxidase of (5 IU) till no more oxidation occurred. Details on small unilamellar vesicle preparation and incubation with galactose oxidase are given under Materials and Methods. The amount of G_{M1} oxidized under these conditions (expressed as percentage of total small unilamellar vesicle-bound G_{M1}) corresponds to the portion of small unilamellar vesicle-bound G_{M1} available to galactose oxidase, that is, of outer-sided G_{M1} (lower curves). The amount of total small unilamellar vesicle-bound G_{M1} (upper curve) was established by exhaustive galactose oxidase oxidation after vesicle treatment with 1% Triton X-100 (final concentration) and vortexing. Mean values of four sets of experiments.

3–20% (as moles) of G_{M1}, the proportion of gangliosides sided in the outer layer was almost constantly levelled at the value of 63–67% of the total.

Galactose oxidase assay

This assay was carried out at 37°C according to the coupled peroxidase-*o*-dianisidine method described in a previous paper [22] and using an incubation time never exceeding 20 min. Preliminary experiments established, for each type of substrate or substrate-carrying particle, the saturating amounts of substrate, the range of linearity between reaction rate and incubation time, and the range of linear response between reaction rate and amount of enzyme protein present. The buffer used was 25 mM sodium phosphate/5 mM disodium EDTA. The pH optimum was confirmed to be 7.0 for galactose, $DesG_{M1}$ and micellar G_{M1} and it was assumed to be 7.0 for vesicular-bound G_{M1} too (the pH optimum in this latter state was not established, since pH variations could markedly alter the vesicle structure). The amount of enzyme used in each assay ranged from a minimum of 0.25 IU (in the case of small unilamellar vesicles) to a maximum of 20 IU (in the case of homogeneous micelles of G_{M1}). The reaction rates were expressed as μmol oxidized substrate (as galactose) per min per mg protein. Under the experimental conditions used, an absorbance variation of 0.100 corresponded to 5.6 nmol oxidized galactose. The apparent V_{max} and K_m values of the enzyme were determined by the double-reciprocal plot method of Lineweaver and Burk [23].

Other methods

Protein content was determined according to Lowry et al. [24], bovine serum albumin being used as the reference standard. Ganglioside-bound sialic acid (NeuAc) was determined according to Svennerholm [25].

Results

Galactose oxidase activity on pure G_{M1} became measurable, by the assay procedure used, only by employing very high enzyme amounts (20 IU) and G_{M1} concentrations exceeding 0.4 mM.

Galactose oxidase action on micellar dispersion of G_{M1}

Homogeneous micelles. As shown in Fig. 2, galactose oxidase featured regular hyperbolic kinetics on homogeneous micelles of G_{M1} and on $DesG_{M1}$ (the oligosaccharide portion of G_{M1}) but expressed much higher (about 7000-fold) velocity on the second substrate. This can be taken as an indication that the aggregation of G_{M1} in homogeneous micelles strongly prevented the enzyme from acting.

Mixed micelles. Mixed micelles of G_{M1} were prepared, as specified under Materials and Methods, by addition of ganglioside G_{D1a} (a ganglioside which is resistant to the action of galactose oxidase), or of Triton X-100, a non-ionic surfactant, or of bovine serum albumin. G_{D1a}, at different concentrations, did not influence the activity of galactose oxidase on G_{M1} (see Fig. 3); conversely, Triton X-100 caused a huge enhancement (even more than 1000-fold) of the enzyme activity. This effect was dependent on the amount of Triton X-100, and, by consequence, on the G_{M1}/Triton X-100 molar ratio in the micelle. At high ratios the enzyme activity increased very slowly: when a molar ratio value of about 1:4–1:5 was reached, the enzyme started working at a much higher rate, a break in the course of the reaction being visible (Fig. 3). The activity reached its maximum at a ratio 1:13.8–1:20, then it began decreasing. Using two fixed G_{M1}/Triton X-100 molar ratios, 1:13.8 and 1:88, the V/S relationships followed hyperbolic kinetics (see Fig. 4a) and displayed an early inhibition by excess substrate. The V/S rela-

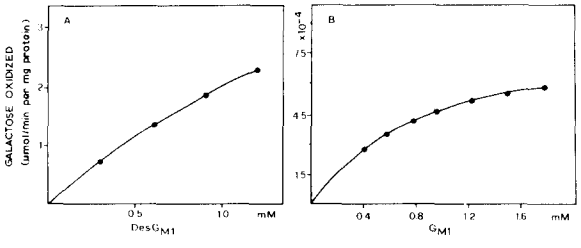

Fig. 2. Kinetics of galactose oxidase action on the oligosaccharide portion of G_{M1} ($DesG_{M1}$) (A) and on homogeneous micelles of G_{M1} (B). The incubation were carried out as described under Materials and Methods with 0.25 IU (A) and 20 IU (B) enzyme. Incubation time, 20 min. Mean values of four sets of experiments.

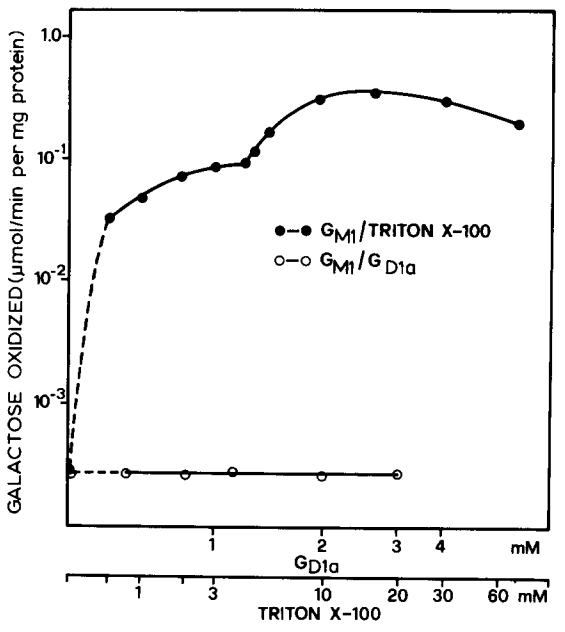

Fig. 3. Galactose oxidase action on mixed micelles of constant G_{M1} (0.8 mM), containing increasing proportions of Triton X-100 or of G_{D1a}. The incubations were carried out as described under Materials and Methods, with the following amounts of enzyme: 20 IU for G_{M1}/G_{D1a} mixed micelles (○———○) at all proportions; 2.5 IU for G_{M1}/Triton X-100 mixed micelles (●———●) till a molar ratio of 1:5; 0.25 IU with G_{M1}/Triton X-100 mixed micelles of molar ratio lower than 1:5. Incubation time: 20 min. Mean values of four sets of experiments.

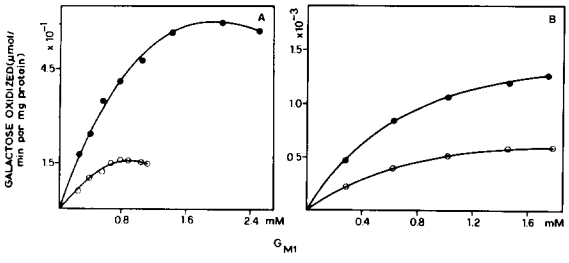

Fig. 4. Kinetics of galactose oxidase action on mixed micelles of G_{M1} at fixed ratios between components. A: G_{M1}/Triton X-100 mixed micelles at 1:13.8 (●———●) and 1:88 (○——○) molar ratios; B: G_{M1}-bovine serum albumin mixed lipoproteic micelles (●———●) and G_{M1}/G_{D1a} mixed micelles (1:1 molar ratio) (○———○). The incubations were carried out as described under Methods with 0.25 IU (A) and 20 IU (B) of enzyme. Incubation time: 20 min. Mean values of four sets of experiments.

tionship obtained with G_{M1}-G_{D1a} (1:1, molar ratio) mixed micelles exactly overlapped that of homogeneous G_{M1} micelles; the same was observed with lipoproteic mixed micelles (bovine serum albumin-G_{M1}), the only difference being that the value of apparent V_{max} doubled that measured with pure G_{M1}.

Galactose oxidase action on vesicular dispersions of G_{M1}

G_{M1} dispersion in small unilamellar vesicles was followed by a striking facilitation of galactose oxidase action. The V/S relationships at different mol% values of G_{M1} in the vesicles (3–20%) have been exposed in fig. 5. In all cases, the curves were hyperbolically shaped and the maximum value of apparent V_{max} was reached at 8% G_{M1} in the vesicle. This value was about 14000-fold greater than that on pure G_{M1}. With increasing G_{M1} concentration in the vesicle the V_{max} rapidly diminished. The variations of V_{max} and K_m values with increasing G_{M1} concentration in the vesicle are graphically shown in Fig. 6. V_{max} changes were

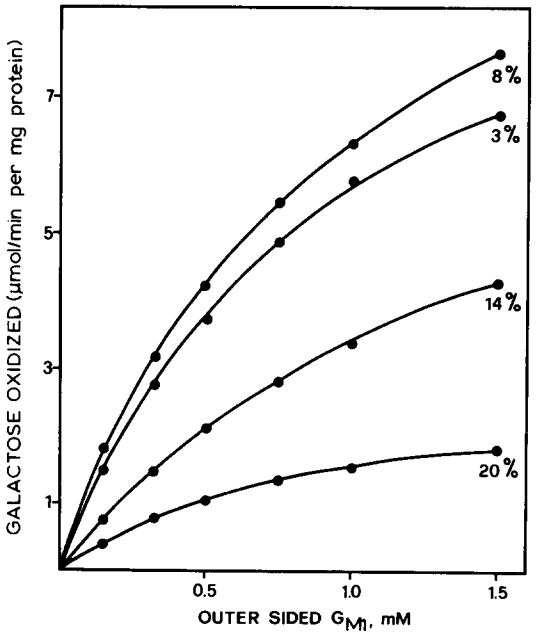

Fig. 5. Kinetics of galactose oxidase action on PC small unilamellar vesicles containing various proportions of G_{M1}. The incubations were carried out as described under Materials and Methods using 0.24 IU of enzyme. Mean values of four sets of experiments.

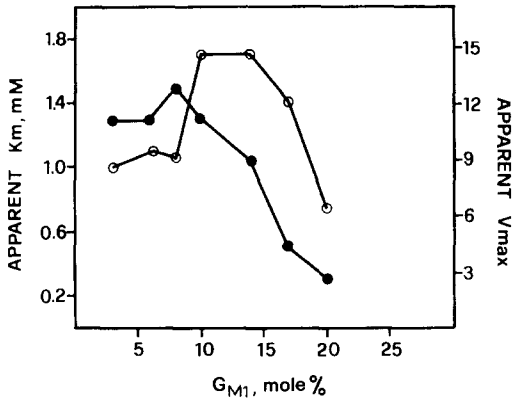

Fig. 6. Galactose oxidase action on small unilamellar vesicles of PC containing various proportions (from 3 to 20 mol%) of G_{M1}. Variations of apparent K_m (○——○) and V_{max} (●—●) values with increasing proportions of G_{M1} in the vesicles. The incubations were carried out as described under Materials and Methods using 0.25 IU of enzyme. Mean values of four sets of experiments.

greater than those in K_m and, generally, higher V_{max} values corresponded to lower K_m values.

Table II contains the values of apparent V_{max} and K_m obtained in the different systems. It appears that K_m values were higher with free substrates (like $DesG_{M1}$ and galactose) than with substrates linked to supramolecular structures. In all membrane-mimicking systems the variations of K_m were of the order of less than 2-fold, while V_{max} changes ranged from 1 (G_{M1} homogeneous micelles) to 14000-fold (vesicular dispersion of G_{M1}, 8 mol%). It is noteworthy that with 8% G_{M1} in vesicular systems the V_{max} value more than doubled that found for the free substrate $DesG_{M1}$.

Effect of pure Triton X-100 micelles and of PC vesicles on galactose oxidase activity

This effect was studied using galactose as substrate, and concentrations of Triton X-100, in micellar form (0.5–90 mM) and of PC, as vesicles (0.5–80 mM), corresponding to the range employed in ganglioside-containing systems. As shown in Table II, the apparent V_{max} and K_m values for galactose were not appreciably modified by the presence of Triton X-100 micelles and PC vesicles.

TABLE II

VALUES OF APPARENT K_m AND V_{max} OF GALACTOSE OXIDASE WORKING ON DIFFERENT FREE (GALACTOSE AND $DesG_{M1}$) OR SURFACE-LINKED SUBSTRATES (G_{M1} IN MICELLAR OR VESICULAR DISPERSIONS)

For details see Materials and Methods. Mean values of four different sets of experiments. TX, Triton X-100; BSA, bovine serum albumin.

Substrate	K_m (mM)	V_{max} (μmol/min per mg protein)
Free molecules		
Galactose	250	180
+0.5 mM TX	238	178
+90 mM TX	242	185
+0.5 mM PC	243	172
+80 mM PC	249	176
$DesG_{M1}$	7.20	6.50
Micellar dispersions [a]		
G_{M1}	1.05	$0.91 \cdot 10^{3}$
G_{M1}-G_{D1a}, 1:1	1.02	$0.91 \cdot 10^{3}$
G_{M1}-TX, 1:88	0.97	0.33
G_{M1}-TX, 1:14	1.35	1.24
G_{M1}-BSA [b]	1.01	$2.01 \cdot 10^{3}$
Vesicular dispersions (PC s.u.v.) [c]		
G_{M1}, 3 mol%	1.00	11.30
G_{M1}, 8 mol%	1.07	13.10
G_{M1}, 14 mol%	1.70	8.68
G_{M1}, 20 mol%	0.75	2.60

[a] Ratios are molar ratios.
[b] Lipoproteic complex.
[c] s.u.v., small unilamellar vesicles.

Discussion

Galactose oxidase catalyses the oxidation to aldehyde of the primary alcoholic group of galactose. It works on free galactose as well as on galactosyl derivatives, such as G_{M1} ganglioside, carrying a galactose residue in the terminal position [16]. The present paper shows that the action of galactose oxidase on G_{M1} ganglioside is greatly influenced by: (a) the kind of physical dispersion – micelle, vesicle – of the ganglioside; (b) the molar ratio between the components in the case of Triton X-100-G_{M1} mixed micelles and of egg phosphatidylcholine-G_{M1} small unilamellar vesicles. The enzyme works with the lowest rate on homogeneous G_{M1} micelles; the apparent V_{max}

increases more than 1000-fold with mixed G_{M1}-Triton X-100 micelles (optimal molar ratio: 1 : 13.8), and more than 10000-fold with PC vesicles containing an optimal molar proportion of 8% G_{M1}. Since micelles of pure Triton X-100 and vesicles of PC alone do not appreciably modify the kinetics of galactose oxidase action on galactose, the above effects do not appear to depend on direct enzyme stimulation, but on the supramolecular organization of the particles embedding the gangliosidic substrate. In order to define the role played by these particles in the enzyme-substrate interactions it would have been interesting to know how the enzyme worked on free ganglioside molecules, that is, on monomeric gangliosides. This point could not be explored. In fact, no measurable enzyme activity was recorded for G_{M1} concentrations lower than $1 \cdot 10^{-4}$ M. On the other hand, G_{M1} is known to micellise at about $1 \cdot 10^{-6}$ M [15] or lower [26–28] concentrations. In the lack of monomeric gangliosides, the oligosaccharide portion of G_{M1}, $DesG_{M1}$, served as an analogous substance freely interacting with the enzyme. The K_m value for $DesG_{M1}$, 7-fold higher than that for micellar or vesicular G_{M1}, and the V_{max} value for $DesG_{M1}$, which exceeded about 7000-fold that of homogeneous G_{M1} micelles but was only a half of that provided by PC-G_{M1} vesicles, clearly focused the importance of being part of a surface for G_{M1} recognition by galactose oxidase.

The micelle and vesicle features which are responsible for the huge variation of galactose oxidase action on bound G_{M1} are not easy to define. In mixed micelles of ganglioside and detergent (Triton X-100, bile salts) the presence of ganglioside surely affects the size [15,29] of the particle and the consolution properties of the system [29]. These changes, together with possibly concurrent modifications in the curvature and shape of the particle, were already demonstrated to be recognized by enzymes [15,29]. In artificial lipid bilayers gangliosides induce a decrease of membrane fluidity [30,31]. The strong side-side headgroup interactions on the bilayer surface – between ganglioside oligosaccharide chains and between ganglioside and phospholipid polar headgroups – lead the lipid chains to assembly in a more rigid fashion. Moreover, the formation of ganglioside-rich phases (ganglioside clusters), by lateral separation, was described to occur in both artificial and natural membranes [31–33]. Of course, these events are expected to affect some basic properties of the bilayer, such as transition temperature of the components, surface pressure, surface charge density which, on the other hand, are known to greatly influence the action of phospholipase on vesicle- or membrane-bound phospholipids [9–12]. Particular attention should also be paid to intermolecular attraction between neighbouring ganglioside oligosaccharides, which occurs in homogeneous ganglioside micelles and results in a packing of ganglioside molecules not suitable for interacting with enzymes. This applies to bacterial sialidases, as shown previously by Gatt and associates [14,15], and to galactose oxidase, as demonstrated here. In mixed Triton X-100 micelles and in PC vesicles the detergent, or phospholipid, acts as 'spacer', separating the ganglioside molecules and decreasing interaction between them, thereby facilitating galactose oxidase binding and action. On the other hand when G_{D1a}, a non-substrate ganglioside, was used as the spacer, the intercarbohydrate attraction being thus maintained, the enzyme action remained at the low level expressed on homogeneous G_{M1} micelles.

In conclusion, the interaction of galactose oxidase with a gangliosidic substrate appears to be dependent on the intrinsic characteristics of the membrane-like system in which ganglioside is embedded. It would be interesting to know whether the same phenomenon occurs for other enzymes, physiologically involved in ganglioside metabolism, for the various ligands known to specifically interact with gangliosides, and for gangliosides bound to natural membranes.

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