Carbon-13 and Proton Nuclear Magnetic Resonance Studies of Gangliosides[1a,b]

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Abstract: Systematic studies of the properties of purified gangliosides are described. These molecules were studied by ^{13}C NMR in THF-d_8–H_2O (5:1), DMSO-d_6, and D_2O buffer (in the potassium salt form in each case). G_{D1a} and mixtures of G_{D1a} with EYL (egg yolk lecithin) were also studied by ^1H NMR and Sepharose chromatography. Chemical shifts revealed a difference in the conformations of the two *N*-acetylneuraminic acid residues in G_{D1a}. Motions determining T_1 for lipid methylenes are independent of head group size. ^1H NMR line-width studies showed that there is restricted chain motion in EYL-G_{D1a} (1:1) aggregates, probably, according to this and other evidence, caused by tighter lipid packing through intercalation of lipid molecules. In D_2O, all three gangliosides have ^{13}C $T_1 \neq T_2$, showing the motions are anisotropic. A reduced NOE for the methylene resonance in ganglioside micelles shows a dependence on the overall correlation time of the micelle. Partially relaxed spectra of G_{D1a} in D_2O showed a motional gradient along the lipid chains, an effective rotational correlation time seven times greater for the sugar ring carbons than for the lipid methylene chain, and no evidence of a motional gradient among the sugar residues.

We report herein the first carbon-13 NMR studies of gangliosides along with related experiments designed to probe their conformations, mobilities, and hydrophobic binding properties. These biologically interesting glycolipids, which contain *N*-acetylneuraminic acid and are therefore negatively charged at physiological pH, are concentrated in nerve endings[2] and are believed to have a role, so far unknown, in neurotransmission.[3,4] When gangliosides are present in biological membranes, their hydrophilic saccharide chains extend into the surrounding regions, where they can be specifically recognized and bound to extracellular proteins, including hormones and toxins.[3] The lipid portion of the molecule remains embedded in the membrane, and it is believed that these molecules can act to transduce the results of saccharide complexation into specific effects within the membrane.[3]

The gangliosides are derivatives of sphingosine, *trans*-D-*erythro*-2-amino-1,3-dihydroxyoctadec-4-ene (the C_{20} homolog is also present in natural sources), N-acylated by a fatty acid, mainly stearic acid (C_{18}) in brain, and glycosidically linked to a saccharide chain, as shown for the most complex one we have studied, G_{D1a}, in Figure 1.[5] Previous work in this laboratory has analyzed the ^{13}C NMR spectra of NeuNAc derivatives,[6] their calcium complexes,[7] and other ganglioside sugar units, including the entire saccharide of G_{M3}.[8] Related NMR studies on NeuNAc derivatives have appeared,[9] but the only references to ganglioside NMR are an unsuccessful attempt to study their ^{13}C NMR[9a] and a ^1H NMR study of mixed brain gangliosides at 60 MHz.[10] Gangliosides are known to exist as micelles in aqueous solution, but as monomers in *N*,*N*-dimethylformamide and tetrahydrofuran,[11] and have been incorporated into lecithin bilayers,[12] including measurements of the choline methyl relaxation times[13] (interpreted in terms of mobility[14]), spin labeling,[15] and fluorescence polarization.[16] Studies of the structurally simpler phospholipids have been fruitful,[17,18] and have led to considerable theoretical understanding of relaxation times.[19-22]

With the background of these studies on the component sugars and analogous phospholipids, we felt that systematic studies on the properties of purified gangliosides would be both feasible and desirable.[23] The "pure" gangliosides, G_{D1a}, G_{M1}, and G_{M3}, which we have studied are somewhat heterogeneous with respect to the chain lengths of both the sphingosine and fatty acid groups, since separation of gangliosides with different chain lengths has never been possible. The G_{D1a} and G_{M1}, both from brain, have almost entirely the saturated stearic acid group, however.[24] Our studies mainly encompass comparison of gangliosides in micellar and monomeric forms, comparison of gangliosides having three different polar head groups,[5] investigation of dynamics by measurements of line widths, T_1 values, partially relaxed spectra, and NOE's, and comparison of mixed egg yolk lecithin–G_{D1a} systems with the two separate components. These results have answered numerous questions and given us a fairly detailed picture of ganglioside properties, mobilities, and conformations, about which very little was heretofore known.

Experimental Section

Materials and Methods. Bovine brains were from a local meatpacker and were transported on ice, then either used immediately or frozen at −20 °C. Solvents and reagents were of reagent grade. Tetrahydrofuran (THF) was distilled immediately prior to use to remove the antioxidant. Spectral grade methanol was used as reference in aqueous ^{13}C NMR spectra. EM silica gel 60 was used in chromatographic columns (70–230 mesh) and as glass-backed TLC plates (without fluorescence indicator). Solvent mixture compositions are specified as volumes before mixing. Egg yolk lecithin (EYL) for NMR experiments was purchased (Sigma, solution in $CHCl_3$–CH_3OH); for Sepharose column studies, crude EYL was purified[25] and then finally chromatographed on silica gel with $CHCl_3$–CH_3OH (1:4).

Sonication was performed with an Ultrasonics W200R sonicator. A Radiometer pH meter 25 or EM 100 Universal Indicator Sticks (±0.5 pH unit) were used for pH measurements. All ^1H and ^{13}C NMR experiments were done on a JEOL PFT-100 spectrometer.

Isolation of Gangliosides.[1b] A crude mixture of gangliosides including G_{M1} and G_{D1a} was isolated from bovine brain by a modification of the method of Gammack.[11e,26] Two TLC systems were needed to examine the purity of ganglioside samples, using silica gel plates. Development with $CHCl_3$–CH_3OH–H_2O (65:25:4)[27] separated neutral and polar lipids from gangliosides (visualized by charring, 40% H_2SO_4, heating at 100 °C, 10 min); crude gangliosides contained at least 15 nonganglioside contaminants. Development with THF–H_2O (5:1) (100 mg KCl/100 mL solvent)[28] separated gangliosides from one another (visualized with Svennerholm reagent,[29] specific for sialic acid containing compounds). The four major and ca. four minor gangliosides present in crude gangliosides were identified by comparison of relative R_f values.[28] A sequence of two column chromatographic procedures purified G_{M1} and G_{D1a} using DEAE-cellulose[30,31] followed by silica gel. G_{M3} was isolated from dog erythrocytes by a modification of the method of Tallman et al.[32]

Sonication Techniques. The following method, adapted from previous reports on lecithin,[33,34] was used to sonicate both lecithin and gangliosides, alone and in mixtures. The buffer was 0.1 M KNO_3, 0.002 M K_2HPO_4; for Sepharose studies, it was in H_2O and titrated to pH 7.8 with dilute H_3PO_4, while for NMR it was in D_2O and the pD, not changed, was ca. 8.6. Single components were suspended in 0.5 to 3.0 mL of buffer. Two-component mixtures were dissolved in $CHCl_3$–CH_3OH; the solvent was removed by rotary evaporation, then

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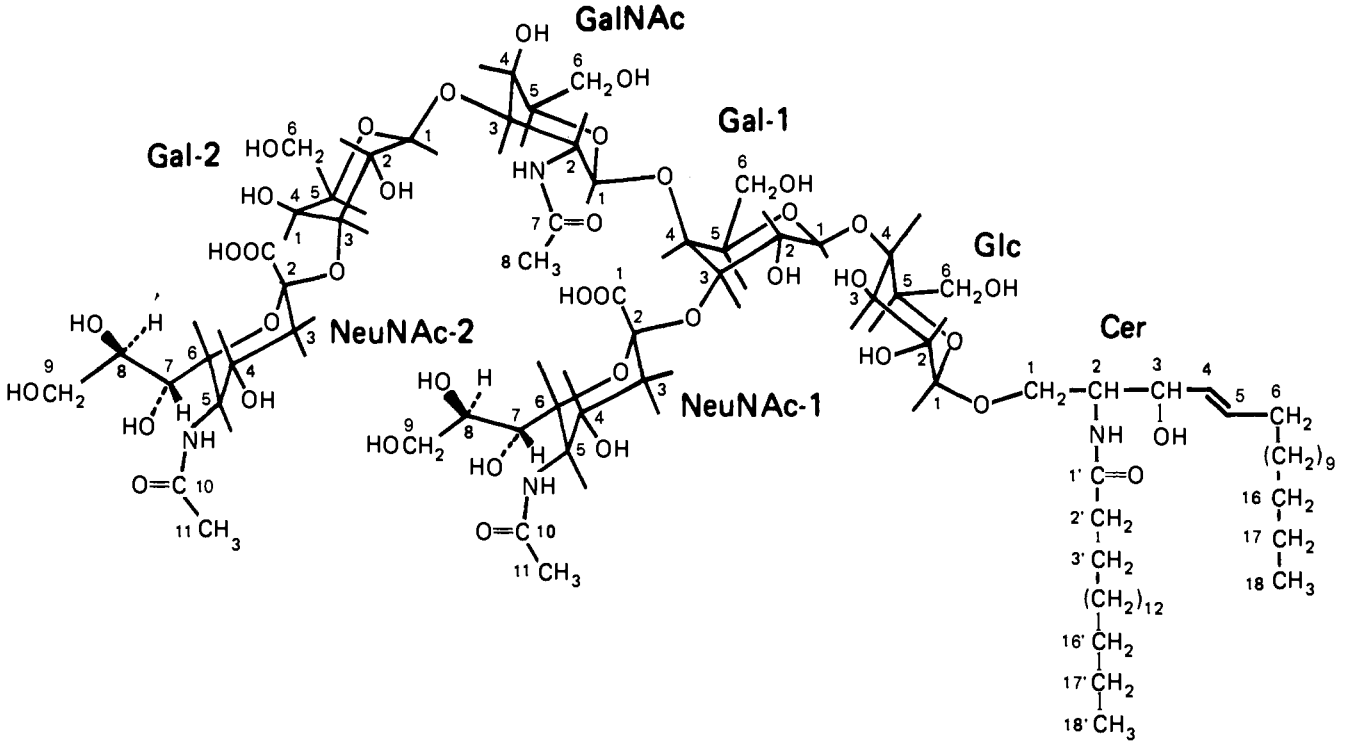

Figure 1. Structural formula of ganglioside G_{D1a}, with numbering system used for all gangliosides studied. Numbers 16, 17, 18 and 16′, 17′, 18′ are used to refer to the antepenultimate, penultimate, and terminal carbons of all homologous chains present in ganglioside samples.

by high-vacuum pumping; buffer was added; and the lipids were suspended by vortexing with two glass beads. Lipid suspensions were sonicated 1 h with a micro tip at power level four, 50% pulse, sample immersed in ice, with N_2 continuously flowing over it. For the last few minutes, the ice bath was removed to allow the sample to warm up. Centrifugation at 40,000g pelleted titanium fragments. Mixed EYL–G_{D1a} samples were sonicated and filtered through a 0.6-μ Millipore filter in a syringe adapter for removal of large, multilamellar structures. TLC of lyophilized and extracted samples of EYL–G_{D1a} showed by comparison with mixtures of known concentration that the ratio remained approximately unchanged through sonication and filtration. This conclusion was confirmed for EYL–G_{D1a} (4:1) by comparison of ^{1}H NMR peak areas of choline methyl vs. *N*-acetyl methyl protons; for (1:4), ^{13}C NMR peak areas of methylene carbons next to the fatty acid carbonyls of the two molecules.

Sepharose Chromatography. The potassium salt of G_{D1a}, EYL, and mixtures of the two were characterized by chromatography on Sepharose 4B (Pharmacia) at 4 °C. The column was calibrated by determination of retention volumes V_r for the proteins concanavalin A, catalase, and glutamate dehydrogenase as well as sonicated EYL vesicles.[33,34] The void volume V_0 was determined using blue dextran, and sizes were interpolated from a graph of V_r/V_0 vs. diameter. Concentrations of lipid in the samples injected were varied, mainly in the range of 40–60 mg/mL. These concentrations are similar to those of NMR samples, which were approximately 20 to 120 mg/mL. The buffer used for samples and column elution was described in the preceding paragraph.

^{1}H and ^{13}C NMR Studies. In all experiments in D_2O solvent, precautions were taken to avoid metal ion contamination.[6,8,35] All D_2O (Stohler, 99.8%) was distilled in glassware previously soaked in alkaline EDTA for 12 h, thoroughly rinsed with deionized H_2O, and dried in a 100 °C oven. D_2O buffer was extracted three times by centrifugation with an equal quantity of 0.05% dithizone solution in CCl_4. All tubes, pipets, plastic caps, vortex plugs, etc., used in sample preparation were soaked in alkaline EDTA solution as above. After the sample was placed in the NMR tube, N_2 was bubbled through it.

Spectra were obtained at 23.5 kG, with a temperature controller accurate to ±2 °C. The 90° pulse was determined and set before each experiment and was 18–25 μs. All spectra were traced on both normal and expanded chemical shift scales, to aid in distinguishing peaks from noise in those cases where question arose. The fast inversion-recovery technique was used for the ^{13}C T_1 studies.[36] NOE values were determined using the gated decoupling technique[37] as modified.[38]

After NMR study, all samples were tested by TLC in the two solvent systems discussed above,[27,28] and little or no evidence of decomposition or contamination was found.

Results

Sepharose 4B Chromatography. Previous studies of aqueous ganglioside micelle molecular weights,[11b,e] giving values in the range 200 000–350 000, were done at acidic pH. These micelles are oblate spheroids.[11b] We have studied the potassium salt in order to have the NeuNAc residues largely ionized as they would be at physiological pH and have added salt (0.1 M KNO_3), since lecithin bilayer vesicles aggregate in low salt media.[34] Because the micelles might differ in size at higher pH, we have carried out Sepharose (size separation) chromatography on G_{D1a}, the most abundant ganglioside of bovine brain, as well as egg yolk lecithin (EYL) and mixtures of the two. The results are shown in Table I.

Sonicated EYL is known to exist as single-bilayer, spherical vesicles with molecular weight 2.1×10^6 and 250 Å average diameter.[33] Therefore, we investigated both sonicated and unsonicated G_{D1a}. It will be noted from Table I that the sizes are similar. We have, however, found a difference in NMR properties and so we will differentiate the true, equilibrium micelles (unsonicated) from those altered by sonication, calling the latter "sonicated micelles". Sonicated and unsonicated G_{D1a} give similar elution profiles consisting of a partially retained peak as shown in Table I and another peak at the excluded volume. The excluded "peak" is believed to be only a minor component representing accumulation of the excluded tail of the broad, retained micelle peak, since larger aggregates scatter light to a much greater extent than smaller ones.[39] This conclusion is supported by the absence of any detectable higher aggregates in sedimentation studies of ganglioside micelles.[11c] Sonicated EYL contains larger aggregates,[33] and it gave a much larger excluded peak relative to the retained peak on our column than did G_{D1a}. The small difference in average size

Table I. Sepharose 4B Chromatography of G_{D1a} Potassium Salt, EYL, and Mixtures[a]

sample	V_r/V_0[b]	diameter, Å[b]
sonicated EYL	1.72	245
sonicated EYL-G_{D1a}		
(83:17)	1.73	240
(1:1)	1.97	190
(1:4)	1.99	185
sonicated G_{D1a}	2.10	160
unsonicated G_{D1a}	2.03	175
EYL-G_{D1a} (1:1), separately sonicated	1.56	280

[a] Buffer 0.1 M KNO_3, 0.002 M K_2HPO_4, pH 7.8; temperature 4 °C; mixed sample composition given in parentheses is mol/mol. [b] Determined from average of at least six injections over a period of several weeks; diameters rounded to multiples of 5 Å, estimated reproducibility and accuracy ±15 Å.

between sonicated and unsonicated G_{D1a} is not enough to account for the distinct differences observed in the ^1H NMR spectra, so it seems probable that sonication alters the size distribution, presumably to favor smaller micelles, even though it does not greatly alter the actual peak position.

From the molecular weight[11b] and partial specific volume[11e] of G_{D1a}, one can calculate a micelle diameter, assuming they are spherical, of 90 Å. The size we have observed is significantly larger. The increased size could arise from the ca. 30-fold higher ganglioside concentrations, 40–60 mg/mL, we have studied, but it may additionally, or instead, arise from the more ionized state of the ganglioside at the higher pH we have studied.

From the data on mixtures, three major conclusions arise. First, ganglioside sonicated micelles incorporate EYL with a small size increase. Second, the near equality of size of EYL-G_{D1a} (83:17) to EYL strongly suggests that EYL vesicles incorporate G_{D1a} without significant change in size. Third, the size of the EYL-G_{D1a} (1:1) aggregates is most consistent with their being mainly enlarged sonicated micelle structures, since a mixture of G_{D1a} sonicated micelles and EYL sonicated vesicles has a very different chromatographic size.

^1H NMR Studies. The proton NMR line width and effective area of the main methylene resonance are useful parameters for characterizing the physical state of lipid chains.[19,34,39-41] We have therefore studied G_{D1a} in this way, as shown in Table II, with the objective of comparing ganglioside micelles and EYL vesicles.

Our data on the magnitude of the line width and the changes with temperature of both line width and effective area for sonicated EYL vesicles are similar to literature data on EYL[39,41] and other lecithins,[34] although some difference in line width was found with lower salt concentration.[41] The change in effective area is known to arise from instrumental delay time before data acquisition, preventing detection of a very broad, fast-relaxing peak subcomponent which increases as temperature is lowered.[34,41] The data demonstrate that the EYL is in the liquid-crystalline state and that there is no phase transition over the temperature range we have studied, as has already been shown.[39,41]

Compared with EYL, the data on G_{D1a} lead to three major points. First, the line widths are in the range characteristic of the liquid-crystalline state for sonicated and unsonicated G_{D1a} as well as EYL-G_{D1a} (1:1), and the temperature dependences of line width and area demonstrate the absence of phase transition phenomena in the temperature range 17–47 °C. This is an important observation, as it allows ganglioside micelles to serve as a useful model for hydrophobic interaction in a membrane-like physical state.

Second, there is a distinct difference between unsonicated and sonicated micelles, especially at lower temperatures. The line widths demonstrate that the sonicated micelles are arranged in a more disordered fashion than unsonicated, which is expected from a smaller radius of curvature, providing more room per lipid molecule, as observed for lecithin bilayer vesicles.[34] This structural difference would permit more off-axis bending of the lipid chains for the sonicated system, decreasing line width, i.e., increasing the spin-spin relaxation time T_2, which is sensitive to such slower motions.[19,34] Sonicated micelles relax toward unsonicated properties on aging, demonstrating that sonication has produced structures with some internal pressure, analogous to the internal pressure in sonicated lecithin vesicles which causes them to aggregate in time.[34]

Table II. Representative Data on Proton NMR Line Width at Half-Height ($\nu_{1/2}$) and Area (A) of the Methylene Peak of G_{D1a}, EYL, and EYL-G_{D1a} (1:1) at Different Temperatures

sample[a]		17 °C	27 °C	37 °C	47 °C
G_{D1a}, unsonicated	$\nu_{1/2}$	65	32	20	15
	A	55	75	90	100
G_{D1a}, sonicated	$\nu_{1/2}$	28	19	14	11
	A	73	86	93	100
G_{D1a}, sonicated, aged 24 h	$\nu_{1/2}$	50	27	20	16
	A	62	82	92	100
EYL-G_{D1a} (1:1), sonicated	$\nu_{1/2}$	65	43	31	26
	A	36	70	91	100
EYL, sonicated	$\nu_{1/2}$	35	28	23	18
	A	70	81	91	100

[a] G_{D1a} present as potassium salt; line width at half-height in Hz; area as % of area at 47 °C; data are averages for two samples in all cases except G_{D1a} aged 24 h and EYL, and are read from graphs constructed from measurements at ca. 5° temperature intervals; buffer 0.1 M KNO_3, 0.002 M K_2HPO_4, in D_2O, pD 8.6; sample sizes were 40–80 mg/mL.

Third, sonicated EYL-G_{D1a} (1:1) has greater line width and greater changes in both line width and effective area with temperature than either sonicated G_{D1a} or sonicated EYL separately. Chemical shift inequivalence cannot account for the greater line width of the mixture than its components, since the separate chemical shifts of the methylene peaks of EYL and G_{D1a} differ by only 0.5 Hz, equal within experimental error, although chemical shift inequivalence is known to account for a great deal of the line broadening of the methylene resonance in lecithin vesicles, as much as 80% at high temperatures.[40] Sonicated sphingomyelin-EYL (1:1) gave line widths between those of the individual components at 20–50 °C,[41] yet sphingomyelin has the same type of ceramide structure as G_{D1a}, contains more unsaturated fatty acids, and would give at least as much structural cause for chemical shift inequivalence as G_{D1a}. Therefore, the extra line broadening in EYL-G_{D1a} (1:1) is very strong evidence of a specific interaction between the two lipids.

^{13}C NMR Chemical Shifts. In view of the possibility of resolving many individual carbon resonances and thereby comparing different gangliosides in some detail, we have made ^{13}C NMR studies of G_{D1a}, G_{M1}, and G_{M3}. The chemical shift data in both D_2O and a largely organic solvent, THF-d_8-H_2O (5:1), are given in Table III as averages (±0.3 ppm) from spectra on at least two samples in each case. Representative spectra for G_{D1a} are shown in Figure 2. Assignments could be made for many peaks based on unambiguous analogies with simpler substances,[6,8,9,13,17] especially NeuNAc-Gal-Glc, which is the complete saccharide group of G_{M3},[8] the individual hexoses,[8] NeuNAc,[6,9] and stearoylcerebroside (Gal-Cer). A good deal of literature analogy exists for the lipid portion.[13,17] Two resonances associated with cis double bonds are found only in G_{M3}, which is the only ganglioside we did not isolate from brain

Table III. ^{13}C NMR Chemical Shift Data for Ganglioside Potassium Salts

assignment[b]	$\delta_C(Me_4Si)$ (external)					
	THF-d_8–H_2O (5:1), 28 °C			D_2O buffer, 35 °C[a]		
	G_{D1a}	G_{M1}	G_{M3}	G_{D1a}	G_{M1}	G_{M3}
GalNAc C-7[c]	174.6	174.5		175.9	176.0	
NeuNAc-1 C-10			174.5			175.9
NeuNAc-2 C-10						
Cer C-1′	174.3	174.2			175.6	
NeuNAc-1 C-1	173.9	173.8	173.8	174.9	175.1	174.9
NeuNAc-2 C-1						
Cer C-4	132.6	132.5	132.6	135.5	135.5	135.5
Cer C-5	130.5	130.6	130.6	130.5	130.5	130.5
-CH=CH—CH_2-			129.5			
GalNAc C-1	104.6	104.8		105.6	105.8	
Gal-1 C-1	102.8	102.9	103.0	103.7	103.8	103.9
Gal-2 C-1						
Glc C-1						
NeuNAc-1 C-2[d]	101.8	101.8		102.6	102.9	
NeuNAc-2 C-2[d]	99.8		99.8	101.0		101.1
	81.0	80.9		81.8	81.6	
	79.1	79.0	79.0	80.0	79.8	79.9
	77.5	77.6		78.7	78.8	
	75.8		75.8	76.7		
		75.0	75.2	75.6	75.7	76.1
CH-OH[e]			74.9			
CH-OC[e]	74.7	74.6	74.6	73.9	73.8	74.1
CH_2-OC[e]	73.2	73.2	73.2	73.0		72.9
	72.4	72.4				
	72.0		71.8		71.9	
	71.4	71.4	71.3		71.3	
	70.1	70.1	69.4		70.6	70.5
	69.2	69.0	69.0	69.4	69.7	69.5
NeuNAc-1 C-9	63.0	63.6	63.2	63.8	64.1	64.0
NeuNAc-2 C-9						
Gal-1 C-6[f]	61.3	61.2	61.2	61.9	62.2	62.1
Gal-2 C-6[f]						
Glc C-6[f]	60.6	60.6	60.4	61.2		61.4
GalNAc C-6						
Cer C-2	53.4	53.3	53.3	54.3	54.6	54.5
NeuNAc-1 C-5	52.2	52.3	52.3	52.9	52.9	53.0
NeuNAc-2 C-5						
GalNAc C-2	51.2	51.2		51.8		
NeuNAc-2 C-3	40.3[g]		40.3	40.7		40.9
NeuNAc-1 C-3	37.0[g]	37.0			38.1	
Cer C-2′	36.0	36.0	36.0	37.6	37.4	37.3
Cer C-16, 16′[h]	32.3	32.3	32.2	33.1	33.2	32.9
Cer C-6[h]	31.7	31.8	31.7			
Cer C-4′-15′	29.6	29.7	29.4	31.2	31.5	31.3
Cer C-7-15						
-CH=CH—CH_2-			26.8			28.3
Cer C-3′	25.8	25.8	25.9	27.3	27.3	
NeuNAc-1 C-11	22.3	22.3	22.4	23.7	23.8	23.7
NeuNAc-2 C-11						
Cer C-17, 17′						
GalNAc C-8						
Cer C-18, 18′	13.4	13.4	13.5	14.8	14.8	14.9

[a] Sonicated, 0.1 M KNO_3, 0.002 M K_2HPO_4, pD 8.6. [b] Not all resonances listed are present in G_{M1} and G_{M3};[5] G_{M1} has only one NeuNAc, while G_{M3} has only one NeuNAc, only one Gal, and no GalNAc. Assignments listed under a single chemical shift value are believed to be in that single resonance peak; brackets indicate cases where complete assignments of sets of more than one peak cannot be made. [c] The *N*-acetylneuraminyllactose and *N*-acetylgalactosamine spectra[8] indicate that NeuNAc C-10 should be downfield from NeuNAc C-1 and that GalNAc C-7 is probably very close to NeuNAc C-10; partially relaxed spectra in D_2O (higher resolution) demonstrate that NeuNAc C-10 is included in the 175.9 (176.0) resonance while NeuNAc C-1 is included in the 174.9 (175.1) resonance; and the spectrum of stearoylcerebroside in THF-d_8–H_2O (sample kindly supplied by P. Tkaczuk, University of Pennsylvania) indicates that Cer C-1′ is included in one of the upfield resonances. [d] Assigned on the basis of long relaxation times as shown by narrow line widths and by partially relaxed spectra of G_{D1a} in D_2O; G_{D1a} assignments based on analogy with similar NeuNAc steric environments in G_{M1} and G_{M3}, respectively. [e] For G_{D1a}, G_{M1}, and G_{M3}, there are 13, 11, and 8 CHOH, 11, 9, and 5 CHOC, respectively, and one CH_2OC in each. [f] The *N*-acetylneuraminyllactose and G_{M3} spectra indicate that at least one Gal C-6 is in the downfield resonance and that the Glc C-6 is in the upfield resonance; the latter may have been obscured by noise in G_{M1} (D_2O). [g] Assignments based on analogy, as in footnote *d*. [h] These assignments may be interchanged.

and which is known to contain cis-unsaturated fatty acid chains.[32] The G_{M3} sugar assignments in THF–H_2O correspond within a few tenths ppm to carbons assigned in NeuNAc-Gal-Glc in D_2O.[8] Although the solvents are different, both substances are monomeric under these conditions.[2,11d] Interestingly, the sugar resonances differ somewhat in D_2O, where G_{M3} is micellar rather than monomeric.

Further assignments for G_{D1a} and G_{M1} could be made by

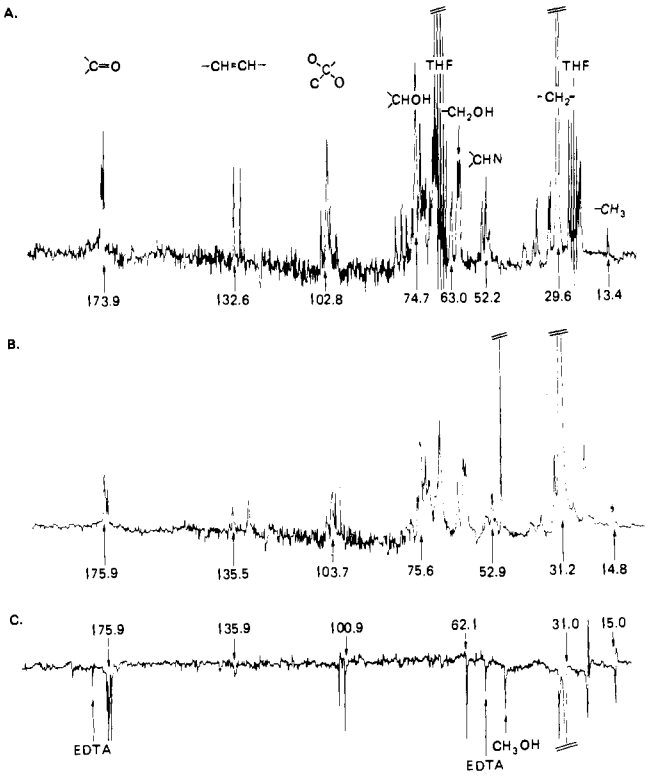

Figure 2. ^{13}C NMR spectra of G_{D1a} potassium salt. (A) THF-d_8–H_2O (5:1), 28 °C. (B) Sonicated in D_2O buffer (0.1 M KNO_3, 0.002 M K_2HPO_4, pD 8.6), 35 °C, CH_3OH internal reference. (C) Same as B, but partially relaxed, τ = 40 ms. All spectra were also traced with expanded chemical shift scale.

comparing with G_{M3} and with NeuNAc,[6] GalNAc and Gal[8] spectra, noting the presence of new or shifted resonances not present in G_{M3}. A particularly interesting pattern was observed in the anomeric carbon region, ca. 100 ppm, where the two NeuNAc C-2 resonances were resolved in G_{D1a} and had chemical shifts virtually identical with the NeuNAc C-2 resonances of G_{M1} and G_{M3}, respectively. The narrower line widths in D_2O of these carbons relative to other, protonated carbons are characteristic of the slower spin–spin relaxation expected for quaternary carbons and demonstrate that the assignments as NeuNAc C-2 are correct. These assignments were confirmed by the partially relaxed G_{D1a} spectrum shown in Figure 2, which demonstrates that the two resonances have long T_1 values relative to the other sugar anomeric carbon resonances.

Comparison of the three gangliosides provides very strong evidence that the downfield one of this pair of resonances belongs to the interior NeuNAc-1 of G_{D1a}, which is in a structural position very similar to that of the NeuNAc of G_{M1}; likewise, the upfield resonance of G_{D1a} belongs to the chain-end NeuNAc-2, which is structurally very similar to the NeuNAc of G_{M3}. The GalNAc C-1 assignment is then confirmed by its presence in G_{D1a} and G_{M1} but not in G_{M3}.

Two NeuNAc C-3 resonances are also resolved in the spectra of G_{D1a} in THF–H_2O; they correspond to resonances in G_{M3} and G_{M1} and so can be assigned to NeuNAc-2 C-3 (downfield) and NeuNAc-1 C-3 (upfield). A similar situation exists in D_2O; although the upfield resonance was not detected for G_{D1a} in D_2O, it was very likely masked by noise.

Since C-2 and C-3 of NeuNAc-1 differ significantly from C-2 and C-3 of NeuNAc-2 in both monomeric and micellar states, since both G_{D1a} NeuNAc moieties are bound to Gal C-3, and since normal structural effects on ^{13}C chemical shifts do not extend beyond the γ position, it follows that the two types of NeuNAc in G_{D1a} must differ by some intramolecular steric or conformational effect. The fact that, relative to NeuNAc-2, C-2 of NeuNAc-1 is shifted downfield while C-3 of NeuNAc-1 is shifted upfield is very strong evidence that this effect on chemical shifts is specific and structurally related.

Comparison of absolute values of chemical shifts in different solvents is of necessity qualitative, but observed large changes in the *differences* between chemical shifts of structurally similar carbons are significant. Such an effect is seen in the difference between the Cer C-4 and C-5 sphingosine double bond resonances, which is 5.0 ppm in D_2O, 2.1 ± 0.1 ppm in THF–H_2O, and zero in Me_2SO (Me_2SO spectra not shown[1b]) for all three of the gangliosides studied. Incorporation of EYL into ganglioside micelles does not change these chemical shifts, strongly suggesting that simple steric environmental effects are not the main factor (chemical shift data not shown[1b]). It will be noted that the major solvent effect is on C-4, which is adjacent to the free hydroxyl group at C-3. However, solvation of this group is still farther removed than the γ position relative to C-4. Therefore, we believe the observed strong solvent effect is evidence of a solvation effect on the conformation of the sphingosine chain in the region of C-3 and C-4. Although we do not have a detailed explanation of this effect, it is unusually large and therefore of considerable interest.

Table IV. ^{13}C NMR T_1 and NOE Data for Main Methylene Peak of Ganglioside Potassium Salts and EYL–G_{D1a} Mixtures, 28 °C

solvent	sample	T_1, s[a]	NOE (η + 1)[a]
THF-d_8–H_2O (5:1)	G_{M3}	0.68 ± 0.02	2.88 ± 0.18
	G_{M1}	0.74 ± 0.09	3.01 ± 0.16
	G_{D1a}	0.66 ± 0.04	2.92 ± 0.22
D_2O, unsonicated[b]	G_{M3}	0.24 ± 0.03	2.12 ± 0.19
	G_{M1}	0.18 ± 0.01	2.23 ± 0.32
	G_{D1a}	0.21 ± 0.02	2.03 ± 0.18
D_2O, sonicated[b]	G_{D1a}	0.20 ± 0.02	2.05 ± 0.15
	EYL–G_{D1a} (1:4)	0.25 ± 0.02	2.28 ± 0.17
	EYL–G_{D1a} (4:1)	0.32 ± 0.03 (0.38 ± 0.03)[c]	2.90 ± 0.25
	EYL	0.32 ± 0.02 (0.44 ± 0.03)[c]	3.08 ± 0.35

[a] Standard deviations given. [b] Buffer, 0.1 M KNO_3, 0.002 M K_2HPO_4, pD 8.6. [c] Value for the EYL choline methyl carbons.

^{13}C NMR Relaxation Times. Because of the enormous machine time requirements for molecules as large as gangliosides, T_1 values were determined only for the main methylene peak of the gangliosides, as shown in Table IV. T_1 decreases with the mobility of the vectors between a given carbon and neighboring protons (dominated by directly bonded protons, if any) if these mobilities are greater than ω_C, the carbon resonance frequency, but goes through a minimum at ω_C.[14,21] The T_1 values determined for the ganglioside methylenes in D_2O are ca. 0.2 s, but Cer C-2 has a much shorter T_1 (cf. Table V, discussed further below). Cer C-2, being part of the backbone in the head group region, must have less mobility than the methylenes, and it also has a lower value of NT_1, N being the number of directly attached protons, demonstrating that the T_1's are on the fast side of the minimum, i.e., are influenced by motions (believed to be rapid kink migration along the lipid chains) that are faster than ω_C.[19] Therefore, decreased mobility leads to decreased T_1 values in our experiments.

The first significant observation from Table IV is the lack of dependence of the methylene T_1 on head group size. Since T_1 is determined by kink migration (conformational changes around the lipid chain axis),[19,20] the data demonstrate that this motion is unaffected by head group changes in spite of the fact that the T_1 values are considerably altered in micelles compared with monomers (in THF–H_2O). Furthermore, G_{D1a} has

the same T_1 in sonicated and unsonicated micelles, in contrast with the line width differences noted previously in the proton NMR. Therefore, differences in packing or micelle size for the different gangliosides do not affect the kink-migration rate.

Although the micelles, being smaller, have a lower radius of curvature than EYL bilayer vesicles, the T_1 values (under identical conditions) show that the carbons contributing to the main methylene peak are more mobile in the case of EYL vesicles. The contrast is not greatly altered by adding 20% EYL to ganglioside sonicated micelles or 20% ganglioside to EYL vesicles. This result is unexpected, since the larger radius of curvature of the vesicles should permit efficient packing of lipid chains and, hence, lower kink mobility.

The main methylene peak width may be affected by small chemical shift differences in the case of carbon NMR, but the methyl peak is clearly resolved and gives an approximate T_2 = 0.03 s in sonicated micelles of all three gangliosides. Since T_2 increases monotonically with internal motion, the main methylene peak must have $T_2 < 0.03$ s, yet $T_1 \cong 0.2$ s. If T_1 is on the fast side of the minimum, as we have shown it is in this case, and the motions leading to relaxation are isotropic, T_2 must equal T_1. The observed very significant inequality demonstrates that the fatty acid chain motions are anisotropic.[19]

It was not feasible to do accurate T_1 studies on ganglioside resonances other than the main methylene peak, because their ca. 20-fold lower intensities would require 400 times as many pulses to achieve the same signal/noise ratio.[14a] However, it was possible to do partially relaxed spectra of G_{D1a} in D_2O at delay times τ of 40 and 150 ms. With the repetition rate of 3 s used, T_1 values < ca. 0.6 s will be reflected reasonably accurately by the null condition for the peak, as shown in Table V. We recognize that the null condition cannot be determined precisely, but we believe the relaxation times calculated for peaks which appear to be nulled are accurate to ±25%.

Several interesting conclusions can be derived from the data. First, the sugar ring carbons all have nearly the same NT_1 values, which is very strong evidence that they have the same mobility. There is no significant mobility gradient in the saccharide chain, which accords with our prediction from analysis of NeuNAc-Gal-Glc relaxation times.[8] The NeuNAc C-2's are exceptional because they are quaternary, as discussed above in connection with their chemical shifts, and therefore cannot be directly compared with the protonated sugar carbons. Second, the sugar groups are not entirely frozen conformationally by intermolecular interactions in the sonicated micelles, since the exocyclic C-6 of at least one of the hexoses and C-9 of both NeuNAc groups have added mobility (greater NT_1) relative to the ring carbons; however, the NeuNAc C-9's are less mobile than at least one of the hexose C-6's.

Third, the main methylene peak shows considerably more mobility than the sugar carbons, reflecting the extra mobility resulting from rapid kink migrations.[19] Fourth, at least part of the sphingosine chain near the polar head group region (Cer C-2) has the same mobility as the sugars. This sameness provides strong evidence that the motion leading to relaxation is the same for both the sugars and the head group region. The best explanation is that this motion is the rotation of the entire ganglioside molecule around its long axis. The first methylene group of the fatty acid (Cer C-2′) shows the beginning of the mobility gradient resulting from kink migration, as seen in phospholipid vesicles.[19] Although Cer C-5 is nulled, C-4 remains slightly inverted; however, both carbons have mobilities close to those of head-group carbons. The long relaxation time of the methyl groups demonstrates that in the sonicated micelles, as in phospholipid vesicles, molecular packing is such that the methyl groups retain considerable rotational freedom.

^{13}C NOE. The nuclear Overhauser enhancement for the main methylene peak of sonicated EYL vesicles is maximal, 3.0.[42] Our data (Table IV) show that the NOE is also maximal for EYL–G_{D1a} (4:1) vesicles and for all three gangliosides as monomers in THF–H_2O, but is considerably reduced for both sonicated and unsonicated ganglioside micelles as well as in EYL–G_{D1a} (1:4). This observation is consistent with the size of ganglioside micelles and provides evidence that their overall rotational rates approach ω_C, which is 1.6×10^8 rads/s in our experiments.[43,44] Reductions in NOE have been observed in high polymers.[45]

The similar NOE values for micelles of all three gangliosides demonstrate that all have comparable magnitudes of overall rotational rate in spite of the differences in polar head group size and charge. Therefore, even though a great deal of hydrogen bonding of the saccharide chains with surrounding water must be present, the similar micelle sizes[11b] and not the structures of the saccharide groups must be dominant in determining overall micelle rotation rate.

Table V. ^{13}C NMR T_1 Data for G_{D1a} Potassium Salt Sonicated in D_2O Buffer, 35 °C[a]

carbon atoms	NT_1, s
sugar CH (all)	0.06
NeuNAc C-9	0.12
Cer C-2	0.06
Cer C-2′	0.12
Cer C-5	0.06
Cer C-4	>0.06; <0.22
sugar C-6 (one or more)	>0.12
NeuNAc-1 C-2	>0.22[b]
NeuNAc-2 C-2	>0.22[b]
CH_3 (C-18, C-18′)	>0.66
$(CH_2)_n$	>0.12; <0.44

[a] Buffer 0.1 M KNO_3, 0.002 M K_2HPO_4, pD 8.6; T_1 values calculated from $\tau_{null} = 0.69T_1$, giving T_1 = 0.06 and 0.22 s for peaks nulled in the two partially relaxed spectra at τ = 40 and 150 ms, respectively; N = number of protons directly attached to the carbon; results expressed as > or < are derived from peaks remaining inverted or having recovered above null, respectively. [b] T_1 given for quaternary carbons (N = 0).

Discussion

The experiments reported here have provided a specific picture of the binding properties of gangliosides.

Ganglioside Micelles. We have shown that ganglioside micelles have properties which make them useful for investigation of hydrophobic binding of these interesting molecules. Unsonicated and sonicated micelles have similar sizes, but average lipid chain packing is altered, as shown by the differences in proton NMR line widths and effective areas. We interpret the narrower methylene line widths of sonicated G_{D1a} as indicating that smaller micelles have a more disordered packing, since sonication appears to alter the size distribution in favor of smaller micelles, although it is possible that the small size difference causes the sonicated micelles to rotate sufficiently faster to affect line width.[19,34] A minimum correlation time for rotation of these micelles can be calculated by the method used for lecithin vesicles[46] to be 5×10^{-8} s, which is fast enough to contribute to line width, in contrast with the 10^{-6} s for lecithin vesicles.[19,46] Relaxation of sonicated micelles to nearly the properties of unsonicated ones shows that the latter are more stable, but if they are larger and more ordered, then the stability cannot be an entropy phenomenon and must have to do with packing enthalpy. The segmental motions determining T_1 for ^{13}C in the methylene chain are the same in sonicated and unsonicated G_{D1a} micelles, however, so the difference in order involves only the slower, off-axis motions which influence the line widths.[19]

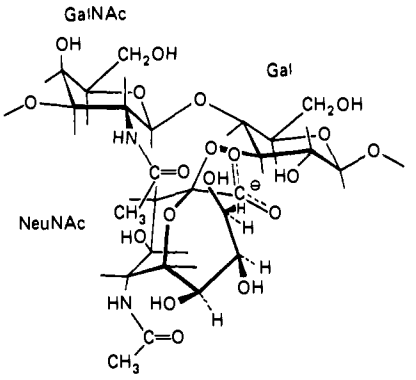

Figure 3. Schematic diagram of GalNAc, NeuNAc-1, and Gal-1 residues of G_{D1a} and G_{M1} potassium salts, showing how GalNAc carbonyl would influence NeuNAc C-2 and C-3 chemical shifts. The possible oxygen cage is also illustrated.

This picture of the properties of the lipid chains is similar to that of the lipid chains in lecithin vesicles.[19] The similarity is further seen in the anisotropic motion which leads to $T_1 \neq T_2$, even though T_1 is on the fast-motion side of its minimum, and in the motional gradient with greater mobility of segmental motion toward the interior region of the chains than in the head group region.

There is a difference in mobility of the chains under our conditions between EYL vesicles and ganglioside micelles, however. Those of the gangliosides are significantly less mobile as indicated by lower ^{13}C T_1 values. Also, there is more mobility for monomeric gangliosides in THF–H_2O than for either EYL vesicles or ganglioside micelles. T_1 values near 0.5 s and nearly equal for sonicated EYL vesicles and EYL dissolved in methanol have been found at an unspecified temperature.[47]

From our partially relaxed spectra of G_{D1a} sonicated micelles, we showed that the saccharide chain has little motional gradient among the saccharide residues and that, in fact, their carbons relax with similar NT_1 to Cer C-2, part of the sphingosine polar region. These results give a picture of relatively "rigid" saccharide chains whose fastest motion is the rotation of the entire lipid molecule about its long axis. This view is supported by the effective correlation time for $NT_1 = 0.06$ s, which is ca. 10^{-9} s,[14] over ten times faster than the calculated minimum correlation time of 5×10^{-8} s for overall micelle rotation (see above). Since NT_1 for the main methylene peak of G_{D1a} sonicated micelles is 0.40 s (Table IV), the effective correlation time for the methylene motions is about seven times shorter than that for the sugar rings.[14]

The fact that C-2 of the interior NeuNAc-1 residue in G_{D1a} is shifted significantly downfield while C-3 is shifted upfield, relative to the corresponding resonances of NeuNAc-2, suggests a neighbor anisotropy effect. Since the adjacent GalNAc *N*-acetyl carbonyl group is in the vicinity of the shifted atoms (cf. Figure 1), space-filling models were constructed which showed steric congestion, making it very likely that the average plane of the NeuNAc ring must be perpendicular to that of the GalNAc ring. The neighbor anisotropy effect of the GalNAc carbonyl group should be deshielding in the carbonyl plane and shielding above and below,[48] and the conformation for this region shown in Figure 3, derived from the models, places NeuNAc C-2 in the deshielding region and C-3 in the shielding region. This interpretation is supported by the fact that no differences in chemical shift of C-5, C-9, or C-11 between NeuNAc residues are observed. These are the other NeuNAc carbons which can be separately identified in the ^{13}C NMR, and their distance from the GalNAc carbonyl would preclude significant neighbor anisotropy shifts.

The lack of binding of Ca^{2+} to NeuNAc-Gal-Glc[49] in spite of the strong binding of Ca^{2+} by gangliosides[9a] (G_{M3} has, however, not been tested) suggests that GalNAc may be involved. The conformation in Figure 3 has a cage-like cavity, lined with oxygen atoms, including the GalNAc carbonyl oxygen, which we tentatively predict to be a binding site for Ca^{2+}.

EYL–G_{D1a} Mixtures. A cosonicated (1:1) mixture of EYL and G_{D1a} was found to have unique properties. Sepharose chromatography showed that this mixture is different from either EYL vesicles or a (1:1) mixture of separately presonicated EYL and G_{D1a}. It is close in size to the EYL–G_{D1a} (1:4) sonicated mixture and not far larger in average size than G_{D1a} micelles. The only reasonable interpretation of this information seems to be that EYL is largely intercalated into ganglioside micelles on sonication. The Sepharose column resolution is not sufficient to resolve a mixture of vesicles and micelles, if present, but significant numbers of vesicles seem extremely unlikely.

Intercalation of EYL molecules, with their smaller head groups, between ganglioside molecules in such mixed micelles seems quite possible. This interpretation is supported by the observed ^1H NMR line width and the temperature dependence of line width and effective area for the main methylene peak, where it was found that (1:1) EYL–G_{D1a} was uniquely different from pure EYL, G_{D1a}, or (1:4) or (4:1) mixtures. The extra line-broadening would be caused by the more restricted off-axis bending of lipid chains resulting from intercalation of the lipid molecules.[19,34] An interaction between the negatively charged NeuNAc residues of G_{D1a} and the positively charged choline groups of EYL could occur and could lead to further tightening of the lipid chain region.

Thus, sonicating G_{D1a} gives a short-term disordering of lipid chains, but, in contrast, cosonicating G_{D1a} and EYL (1:1) creates lipid aggregates which are more ordered than the ones observed for either lipid by itself. This latter effect may be important in ganglioside-containing biomembranes, which contain significant quantities of lecithin.

Because of the very large time requirements and the resolution problems associated with the many different carbons present in a (1:1) EYL–G_{D1a} mixture, ^{13}C NMR studies of this system have not yet been feasible. However, it was possible to study EYL–G_{D1a} (1:4) and (4:1) and show that the main methylene carbon mobility, as measured by T_1, resembles G_{D1a} and EYL, respectively. The latter effect shows that G_{D1a} has little effect on the kink migration rates of EYL even though it is a sphingolipid. Also, the data (Table IV) show a trend toward lessened mobility of the choline methyl carbons when G_{D1a} is present in EYL vesicles, which may reflect a choline–NeuNAc electrostatic interaction as suggested in the second paragraph above. A very similar effect was found for choline methyl carbons in the presence of G_{M3}.[13]

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References and Notes

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Nuclear Magnetic Resonance Studies of Catecholamines. Temperature and Solvent Effects on the Association with Adenosine Triphosphate and Its Divalent Metal Ion Chelates

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Abstract: The effects of temperature and organic solvents on the association between catecholamines and ATP or divalent metal ion–ATP chelates have been investigated by means of ^{1}H NMR chemical-shift and line-width measurements. The average thermodynamic parameters for the association of catecholamines with ATP, either in the absence or in the presence of divalent metal ions, are found to be -5.0 ± 0.5 kcal/mol, -11.1 ± 1.5 eu, and -1.7 kcal/mol for $\Delta H°$, $\Delta S°$, and $\Delta G°$ (300 K), respectively. These data, coupled with the observations that organic solvents which are less polar than water or possess large electric-dipole moments disrupt the catecholamine–ATP association, are interpreted in terms of possible mechanisms for the complexation between catecholamines and ATP. It is suggested that van der Waals–London interactions provide the major stabilizing force for the ring stacking involved in the complex formation. Hydrophobic and charge-transfer interactions, as well as hydrogen bond formation and an electrostatic interaction, are also considered to contribute to the stability of the association. An upper limit of 3×10^{-6} s is derived for the mean residence time in the complexed state of catecholamines.

Recent NMR studies[1-4] have established that at the region of neutral pH catecholamines bind to ATP, either in binary complexes or in ternary complexes with divalent metal ions, mainly through stacking between the catechol and the purine rings. This interaction was found to be augmented by hydrogen bond formation involving the catecholamine hydroxyls and by an electrostatic interaction between the protonated ammonium group and the negatively charged phosphate moiety. The specific interactions between aromatic moieties are of great importance in biological systems. Numerous experiments have provided evidence that parallel base stacking is the major stabilizing force for the self-association of purine and pyrimidine nucleosides and nucleotides, the folded structure of oligo- and dinucleotides, and the secondary

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