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Interaction of Choleragen with the Oligosaccharide of Ganglioside G_{M1}: Evidence for Multiple Oligosaccharide Binding Sites†

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ABSTRACT: The oligosaccharide moiety of the monosialoganglioside galactosyl-*N*-acetylgalactosaminyl[*N*-acetylneuraminyl]galactosylglucosylceramide (G_{M1}) induced a blue shift in the fluorescence spectra of choleragen and its B protomer similar to that observed previously with G_{M1} [Mullin, B. R., et al. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1679-1683; Moss, J., et al. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74,* 74-78]. The decrease in λ_{max} was maximal when the molar ratio of oligosaccharide to choleragen was 5.6. The circular dichroic spectra of choleragen and its A and B protomers indicated that the secondary structures were composed predominantly of β-pleated sheets. In the presence of oligosaccharide, the mean residue ellipticity of choleragen and its B protomer decreased between 220 and 240 nm; a maximal effect occurred when the molar ratio of oligosaccharide to choleragen was 5.7. Multiple oligosaccharide binding sites on choleragen were confirmed by equilibrium dialysis and gel permeation chromatography. With either procedure, each toxin molecule was observed to bind between 5 and 6 molecules of oligosaccharide. In addition, the apparent weight average molecular weight of choleragen and its B protomer, obtained from sedimentation equilibrium measurements, was increased by approximately 5500 in the presence of oligosaccharide; these results are consistent with 5-6 binding sites for the oligosaccharide (M_w = 1021). The determinants for the binding of choleragen to its ganglioside receptor reside in the B protomer of the toxin and the carbohydrate chain of the ganglioside; presumably, there is one binding site on each of the polypeptide chains that comprise the B protomer. The multivalent nature of choleragen and the observed perturbations of the toxin molecule induced by the oligosaccharide portion of its cell membrane receptor may be involved in the mechanism by which choleragen eventually activates adenylate cyclase within the cell.

Choleragen, an exotoxin of *Vibrio cholerae,* is responsible for the clinical manifestations of cholera and appears to exert its effects on vertebrate cells through activation of adenylate cyclase (Finkelstein, 1973). The toxin is a protein (M_w = 84 000) composed of two protomeric species, A and B (Finkelstein, 1973; Lönnroth & Holmgren, 1973; Cuatrecasas et al., 1973; van Heyningen, 1974; Sattler et al., 1975). The A protomer contains two nonidentical polypeptide chains, A_1 and A_2, linked by a single disulfide bridge. A_1 can activate adenylate cyclase in cell-free systems independent of the B protomer (Gill & King, 1975; Bitensky et al., 1975; Sahyoun & Cuatrecasas, 1975) or the toxin receptor (Moss et al., 1976a).

The B protomer is believed to contain five identical polypeptide chains (Gill, 1976; Kurosky et al., 1977; Lai et al., 1977) and binds the toxin to its cell membrane receptor, the monosialoganglioside G_{M1}[1] (Cuatrecasas, 1973a-c; Holmgren et al., 1973; King & van Heyningen, 1973; van Heyningen, 1974; Staerk et al., 1974). Although the B protomer is polymeric, choleragen can be precipitated and inactivated by equimolar amounts of G_{M1} (Holmgren et al., 1973, 1974).[2] The ability of choleragen to induce redistribution of ganglioside receptors on lymphocytes indicated that choleragen is functionally multivalent (Révész & Greaves, 1975; Craig & Cuatrecasas, 1975; Sedlacek et al., 1976).

Determining the stoichiometry of the choleragen-ganglioside interaction is complicated by the self-association of gangliosides in solution (Gammack, 1963; Yohe & Rosenberg, 1972; King et al., 1976). Since the oligosaccharide chains of gangliosides would not be expected to form micelles and the specificity of G_{M1} in the choleragen-ganglioside interaction resides in the carbohydrate moiety (Holmgren et al., 1974; Staerk et al., 1974), we examined the interaction of oligosaccharides derived from gangliosides with choleragen. Our results indicate that choleragen and its B protomer contain multiple binding sites for the oligosaccharide portion of G_{M1}.[3]

Experimental Procedure

Materials. G_{M1} and G_{D1a} were purified from bovine brain by silicic acid column chromatography (Penick et al., 1966).

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[1] Abbreviations used are: oligo-G_{M1}, Galβ1→3GalNAcβ1→4-[AcNeuα2→3]Galβ1→4Glc; gangliosides are designated by the nomenclature of Svennerholm (1963a).

[2] At low concentrations of choleragen, equimolar concentrations of G_{M1} precipitated the toxin whereas at higher concentrations, molar ratios of 2:1 or more were required (Holmgren et al., 1974; Staerk et al., 1974). Thus the precipitation and inactivation of choleragen by G_{M1} appear to be complex and may be influenced by the self-association of gangliosides in solution.

[3] A preliminary report of this work was presented at the annual meeting of the American Society of Biological Chemists, April 3-7, 1977 (Fishman et al., 1977b).

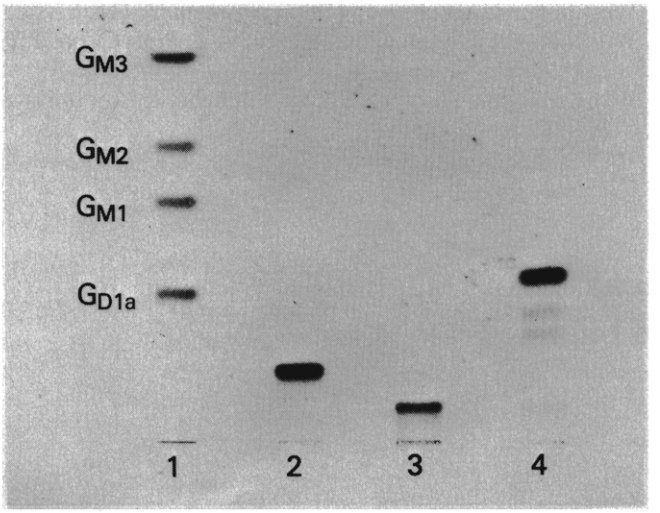

FIGURE 1: Thin-layer chromatogram of oligosaccharides. Oligosaccharides were prepared from G_{M1} and G_{D1a} as described under Experimental Procedure; neuramin lactose (*N*-acetylneuraminylgalactosylglucose) was a commercial preparation and corresponds to the oligosaccharide chain of G_{M3}. The silica gel 60 coated glass plate (E. Merck, Darmstadt, West Germany) was developed in 1-propanol–0.2% $CaCl_2$ (8:2, v/v) and sprayed with resorcinol reagent (Svennerholm, 1963b). (Lane 1) Ganglioside standards; (lane 2) oligo-G_{M1}; (lane 3) oligosaccharide from G_{D1a}; (lane 4) neuramin lactose.

Neuramin lactose was purchased from Bohringer-Mannheim, choleragen from Schwarz/Mann and NaB^3H_4 from Amersham/Searle. [^{125}I]Choleragen was prepared by the method of McFarlane (1958). Dowex resins and Bio-Gel were obtained from Bio-Rad Laboratories.

Preparation of Oligosaccharides. The oligosaccharide moieties of G_{M1} and G_{D1a} were prepared by ozonolysis and alkaline fragmentation (Wiegandt & Bücking, 1970). A solution of 5 μmol of ganglioside in 1 mL of methanol was saturated with O_3 at 25 °C and then evaporated under reduced pressure. The residue was dissolved in 1 mL of 0.1 M $NaHCO_3$. After 16 h at 25 °C, the solution was diluted with 4 mL of H_2O and applied to a Dowex 50W-X8 (200–400 mesh in the hydrogen form) column (0.7 × 7 cm), which was eluted with 5 mL of H_2O. The eluate was lyophilized and the residue, dissolved in 4 mL of H_2O, was applied to a Dowex 1-X8 (200–400 mesh in the acetate form) column (0.9 × 15 cm). The column was eluted with a linear gradient of 200 mL of pyridine–acetate, pH 4.8 (0–300 mM); fractions containing the oligosaccharide were pooled and lyophilized. The residue, dissolved in 0.5 mL of double-distilled H_2O, was chromatographed on a Bio-Gel P-2 (100–200 mesh) column (0.9 × 60 cm) in the same solvent. The oligosaccharide was recovered in the void volume; overall yields were between 50 and 55%.

Other Methods. Fluorescence measurements were made with a Perkin-Elmer Model MPF-4 spectrofluorometer as described previously (Moss et al., 1977a). Circular dichroic spectra were obtained from 270 to 205 nm with a Cary Model 61 spectrophotometer. The temperature was maintained at 24 °C. Mean residue ellipticities were obtained by using the following equation:

$$[\theta]_\lambda = [\theta]_{obsd}(112/10lc)$$

where $[\theta]_\lambda$ is the mean ellipticity at wavelength λ; $[\theta]_{obsd}$, the observed ellipticity; 112, the mean residue molecular weight; l, the pathlength in cm; and c, the concentration in g/mL.

The concentrations of choleragen and its B protomer were determined from published values for extinction coefficients and molecular weights (Finkelstein, 1973). The $E_{cm}^{1\%}$ (280 nm) for choleragen was 11.41 and for the B protomer, 9.56. The molecular weights employed were 84 000 and 56 000, respectively. Oligosaccharide concentrations were determined by a modification of the resorcinol method (scaled down by a factor of 8) using a standard solution of *N*-acetylneuraminic acid (99% pure from Serva, Heidelberg, West Germany). Carbohydrate composition of oligo-G_{M1} was determined by gas–liquid chromatography (Esselman et al., 1972) after methanolysis of the oligosaccharide (16 h at 80 °C in 1 M HCl in methanol). [^{3}H]Oligo-G_{M1} was prepared by reducing the oligosaccharide with NaB^3H_4 (Takasaki & Kobata, 1974). The A and B protomers of choleragen were prepared by the method of Finkelstein et al. (1974) and were homogeneous as assessed by electrophoresis on polyacrylamide gel in sodium dodecyl sulfate (Moss et al., 1977a).

Sedimentation equilibrium experiments were performed in a Spinco Model E ultracentrifuge equipped with a photoelectric ultraviolet scanner. Double sector cells with charcoal-filled centerpieces and quartz windows were centrifuged in an ANG-Ti rotor at 21.5 °C. Each protein solution was dialyzed against a buffer containing 50 mM Tris(Cl^-), pH 7.4, 200 mM NaCl, 3 mM NaN_3, and 1 mM EDTA prior to use. Oligo-G_{M1} (67 μM final concentration) was added to the protein and corresponding reference solutions just prior to centrifugation. The layering fluid was omitted and each cell contained 100 μL of sample and 110 μL of buffer. Initial protein concentrations and rotor speeds are given in Table I. After equilibrium had been reached, monitored by comparing concentration profiles (λ = 280 nm) at 4-h intervals starting at 20 h, the rotor speed was increased to 44 000 rpm for baseline determinations. Apparent weight average molecular weights were obtained in the usual manner. The partial specific volumes of choleragen and the B protomer, 0.728 and 0.73 mL/mg respectively, were calculated from the corresponding amino acid compositions of the proteins (Finkelstein, 1973; Sattler et al., 1975).

Results

Analysis of Oligosaccharides. The oligosaccharides from G_{M1} and G_{D1a}, prepared by ozonolysis and alkaline fragmentation of the gangliosides, were homogeneous by thin-layer chromatography (Figure 1). The commercial neuramin lactose, which corresponds to the oligosaccharide moiety of G_{M3}, contained some minor contaminants. In each case, the oligosaccharide was less mobile than the corresponding ganglioside. Following methanolysis and reacetylation, oligo-G_{M1} was analyzed by gas–liquid chromatography and contained glucose, galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid in the molar ratio of 1.0:1.9:1.0:0.8 which is, within experimental error, the same as the carbohydrate composition of G_{M1}. When [^{3}H]oligo-G_{M1} was hydrolyzed (2 h at 100 °C in 2 M HCl) and the hydrolysate was analyzed by high voltage paper electrophoresis in 0.06 M $NaBO_4$, pH 9.1 (Takasaki & Kobata, 1974), all of the radioactivity migrated as sorbitol.

Effect of Oligosaccharides on the Fluorescence Spectra of Choleragen and Its B Protomer. Oligo-G_{M1} induced a "blue shift" in the fluorescence spectra of choleragen and its B protomer similar to that previously reported for G_{M1} (Mullin et al., 1976; Moss et al., 1977a). No shift was detected with neuramin lactose, the oligosaccharide from G_{D1a}, galactose, or sialic acid. With increasing concentrations of oligo-G_{M1}, the λ_{max} of the fluorescence spectrum of choleragen decreased almost linearly until a plateau at 328 nm was reached when the molar ratio of oligo-G_{M1} to toxin was 5.6 (Figure 2A).

As the λ_{max} of tryptophan in a nonpolar environment is ~328 nm, the oligosaccharide appears to induce a maximum

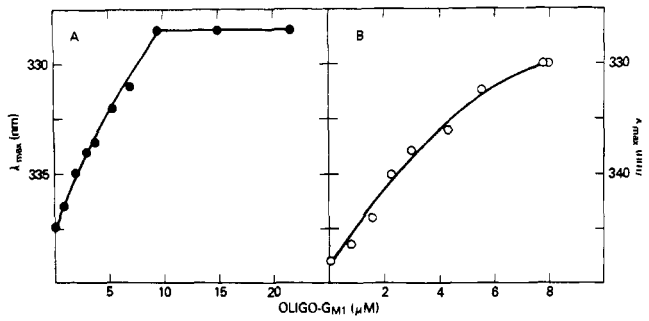

FIGURE 2: Effect of oligo-G_{M1} on the fluorescence spectra of choleragen and its B protomer. The fluorescence spectra of choleragen and the B protomer were scanned in the presence of increasing amounts of oligo-G_{M1} as described in Experimental Procedure and the λ_{max} of each spectrum was plotted against the concentration of oligo-G_{M1}. (A) Shift in λ_{max} of choleragen; the concentration was 174 μg/mL with no oligo-G_{M1} and 141 μg/mL with 9.48 μM oligo-G_{M1}. (B) Shift in λ_{max} of the B protomer; the concentration was 175 μg/mL without oligo-G_{M1} and 141 μg/mL with 8 μM oligo-G_{M1}. The proteins were dissolved in a buffer containing 50 mM Tris(Cl^-), pH 7.4, 200 mM NaCl, 1 mM EDTA, and 3 mM NaN_3.

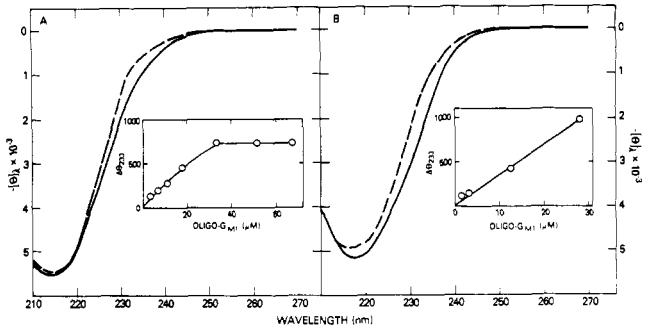

FIGURE 4: Effect of oligo-G_{M1} on circular dichroic spectra of choleragen and its B protomer. (A) The spectrum of choleragen was scanned between 270 and 210 nm without (—) and with (- - -) 66.7 μM oligo-G_{M1}. The insert is a plot of the change in $[\theta]_{233}$ vs. oligo-G_{M1} concentration; the concentration of choleragen was 530 without and 495 μg/mL with 33.3 μM oligo-G_{M1}. (B) The spectrum of B protomer was scanned without (—) and with (- - -) 27.7 μM oligo-G_{M1}. The insert is a plot of the change in $[\theta]_{233}$ vs. oligo-G_{M1} concentration; the concentration of B protomer was 417 μg/mL without oligo-G_{M1} and 391 μg/mL with 27.7 μM oligo-G_{M1}.

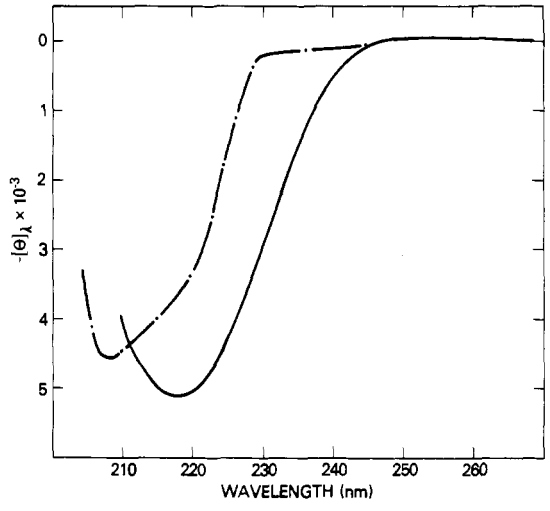

FIGURE 3: Circular dichroic spectra of the A and B protomers of choleragen. The proteins were dissolved in the same buffer described in the legend to Figure 2. The concentration of A (·—·) was 243 and of B (—), 417 μg/mL. The spectra were measured as described under Experimental Procedure.

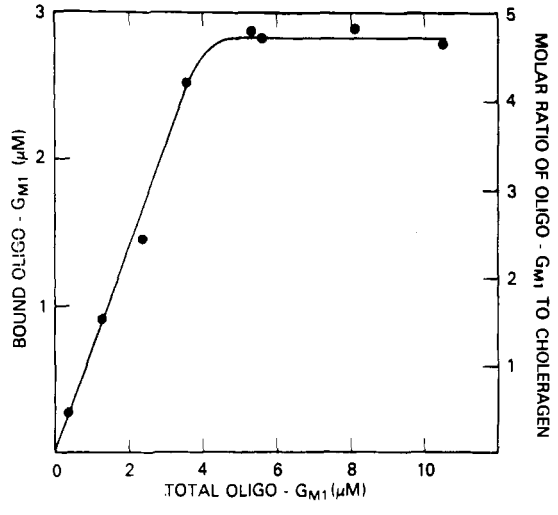

FIGURE 5: Equilibrium dialysis of choleragen and [^{3}H]oligo-G_{M1}. One chamber of each cell contained 50 μg/mL of choleragen (0.6 μM) and the other, [^{3}H]oligo-G_{M1} (58 000 cpm/nmol) at the indicated concentration. The buffer was that described in the legend to Figure 2. The cells were oscillated at 25 °C for 4 days and samples in triplicate were removed and counted. The volume in each chamber was 1 mL.

shift in the environment of the tryptophanyl residues of choleragen when each molecule has bound between 5 and 6 molecules of oligo-G_{M1}. In the presence of 8 μM oligo-G_{M1}, the λ_{max} of the B protomer shifted to 330 nm (Figure 2B); this 18-nm shift was slightly greater than the 15-nm shift that had been observed with the B protomer (0.236 mg/mL) and 33 μM G_{M1} (Moss et al., 1977a).

Effect of Oligo-G_{M1} on the Circular Dichroic Spectra of Choleragen and Its B Protomer. The circular dichroic spectra of choleragen and its A and B protomers are characteristic of proteins whose secondary structures are predominantly in the form of a β-pleated sheet (Figures 3 and 4). In the presence of oligo-G_{M1}, the mean residue ellipticity, $[\theta]$, of choleragen decreased between 220 and 240 nm (Figure 4A); when the decrease in $[\theta]_{233}$ was measured as a function of oligo-G_{M1} concentration, a plateau was reached at a molar ratio of 5.7 (Figure 4A, insert). Similar results were obtained with the B protomer (Figure 4B).

Direct Measurements of Oligo-G_{M1} Binding to Choleragen. Direct binding was determined by equilibrium dialysis using [^{3}H]oligo-G_{M1}. At saturation, 1 nmol of choleragen bound 4.8 nmol of oligo-G_{M1} (Figure 5). Similar results were obtained with unlabeled oligo-G_{M1} and choleragen concentrations of 1 mg/mL; a molar ratio between 5.4 and 5.7 was obtained.[4]

Direct binding was also determined by gel filtration column chromatography. An excess of oligo-G_{M1} was mixed with choleragen and the mixture was chromatographed on a Bio-Gel P-60 column (Figure 6). Oligo-G_{M1} and choleragen were eluted together in a molar ratio of 6:1; a second peak of free oligo-G_{M1} was eluted later from the column. Recovery of both

[4] After we had submitted this paper, we became aware of the report by Sattler et al. (1977), who described the binding of oligo-G_{M1} to choleragen and the B protomer by equilibrium dialysis. Although they employed different conditions (6 °C, 0.2% ovalbumin and 0.1 M sodium phosphate buffer, pH 7.5) and lower concentrations of protein, their results and ours are in agreement. In both studies, a plot of the binding data by the method of Scatchard (1949) resulted in a nonlinear plot. Because of this, we did not attempt to calculate an association constant for ligand binding. Sattler et al. (1977) reported that half-saturation was obtained at an oligo-G_{M1} concentration of 65 nM and, at saturation, the molar ratio of oligo-G_{M1} to choleragen was 3.9.

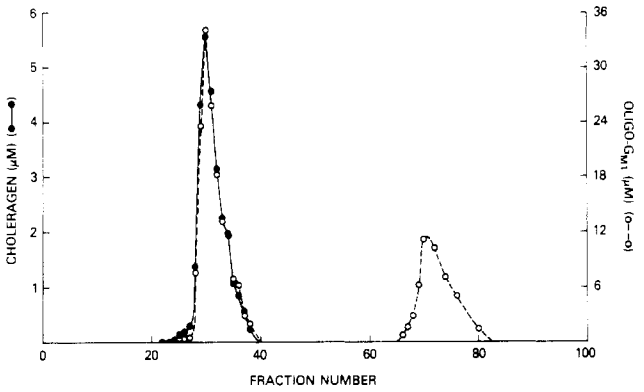

FIGURE 6: Gel filtration column chromatography of choleragen and oligo-G_{M1}. Choleragen (2 mg) and oligo-G_{M1} (230 nmol) in 0.55 mL of buffer containing 50 mM Tris(Cl^-), pH 7.4, 200 mM NaCl, 1 mM EDTA, and 3 mM NaN_3 were incubated for 30 min at 25 °C and chromatographed on a Bio-Gel P-60 (100–200 mesh) column (1 × 100 cm). The column was equilibrated and eluted with the same buffer; 1-mL fractions were collected. Choleragen was estimated by its absorbance at 280 nm and oligo-G_{M1} by its sialic acid content as described under Experimental Procedure. Total recovery of choleragen and oligo-G_{M1} was 2.1 mg and 222 nmol, respectively.

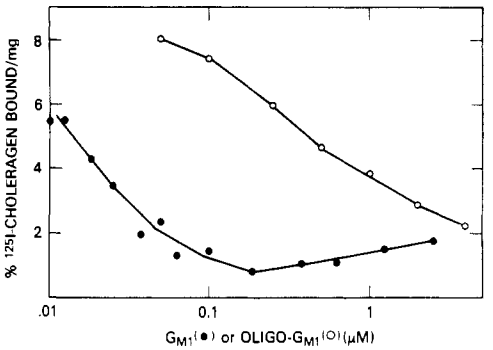

FIGURE 7: Effect of G_{M1} and its oligosaccharide on binding of [^{125}I]-choleragen to human fibroblasts. Monolayer cultures of human fibroblasts were incubated at 37 °C for 20 min with [^{125}I]choleragen (5 × 10^5 cpm) and G_{M1} (●) or oligo-G_{M1} (○) at the indicated concentrations in 10 mL of Hank's balanced salts solution. Binding was determined as described previously (Moss et al., 1977b).

compounds was >95%. Additional experiments with lower concentrations of toxin and [^{3}H]oligo-G_{M1} indicated that each choleragen molecule bound between 5 and 6 molecules of the labeled oligosaccharide (data not shown).

The apparent weight average molecular weight of choleragen and the B protomer was determined in the presence and absence of oligo-G_{M1} by conventional sedimentation equilibrium. The ln c vs. r^2 data obtained were linear throughout each cell and the difference between data in the presence and absence of the ligand (mol wt = 1021) was consistent with the binding of 5–6 molecules of oligosaccharide to choleragen and the B protomer (Table I).[5]

Inhibition of Choleragen Binding to Human Fibroblasts. [^{125}I]Choleragen can bind to G_{M1} receptors on the surfaces of cultured human fibroblasts (Moss et al., 1977b). Oligo-G_{M1} inhibited the binding of [^{125}I]choleragen to these cells but was a less potent inhibitor than G_{M1} (Figure 7). Analysis of a linear representation of the data (Bailey et al., 1967) indicated that 50% inhibition of binding occurred at 600 nM oligo-G_{M1} and 25 nM G_{M1}.

No uptake of [^{3}H]oligo-G_{M1} by the fibroblasts was observed. The cells bound only a very small fraction (<1%) of the [^{3}H]G_{M1} added to the medium (Fishman et al., 1977a). This small amount, however, represents a large increase in the number of G_{M1} molecules per cell (Fishman et al., 1977a) and may explain the increase in choleragen binding at high G_{M1} concentrations. Analysis of the media from these latter experiments indicated that over 95% of the radioactivity was recovered as [^{3}H]G_{M1} or [^{3}H]oligo-G_{M1}, respectively. Thus the cells appeared capable of removing or metabolizing at most only minute amounts of these inhibitors of choleragen binding.

Effect of Oligo-G_{M1} on the A Protomer of Choleragen. The A protomer of choleragen has NAD glycohydrolase activity (Moss et al., 1976b) and this activity was inhibited by several gangliosides including G_{M1} (Moss et al., 1977a). Oligo-G_{M1}, however, at 49 μM had no effect on the NAD glycohydrolase activity of A, whereas G_{M1} at 53 μM inhibited the activity of A by 70%.

TABLE I: Effect of Oligo-G_{M1} on the M_w^{app} of Choleragen and the B Protomer as Determined by Sedimentation Equilibrium.[a]

Protein	M_w^{app}	
	Buffer alone	+ 67 μM oligo-G_{M1}
Choleragen	75 700	82 060
B protomer	52 930	57 850

[a] Apparent weight average molecular weights (M_w^{app}) were obtained by sedimentation equilibrium measurements as described in Experimental Procedure. The initial concentrations of choleragen and the B protomer were 0.54 and 0.35 mg/mL, respectively. Each sample was centrifuged in the presence and absence of oligo-G_{M1} (67 μM) at 15 000 rpm and 21.5 °C. ln c vs. r^2 plots were linear throughout each cell and the values given are the mean of duplicate determinations that differed by no more than 3%.

Discussion

Binding of the oligosaccharide derived from G_{M1} to choleragen has been observed previously but the stoichiometry of the interaction was not reported (Holmgren et al., 1974; Staerk et al., 1974). In these studies oligo-G_{M1} did not precipitate choleragen and was less effective than G_{M1} in inhibiting toxicity. In our studies, oligo-G_{M1} was less potent than G_{M1} in inhibiting the binding of choleragen to cells. The K_i for G_{M1} that we observed (2.5 × 10^{-8} M) was similar to the value obtained by Cuatrecasas (1973a,b) for the inhibition of choleragen binding to fat cells and liver membranes, whereas the K_i for oligo-G_{M1} was 6 × 10^{-7} M. The affinity of choleragen for G_{M1} receptors on cells and membranes, however, is greater (10^{-9} M) than the K_i for G_{M1} inhibition of toxin binding (Cuatrecasas, 1973a,b; Holmgren et al., 1975).

As the specificity of the ganglioside for choleragen binding resides in the carbohydrate chain, the higher apparent affinity of choleragen for G_{M1} than for its oligosaccharide remains unexplained.[6] It may be related to either the ability of G_{M1} even in equimolar amounts to precipitate and inactivate the toxin or its ability to aggregate in solution. Although the

[5] Although the value we obtained for choleragen was somewhat lower than the values (82 000–86 000) reported by Finkelstein (1973) and Sattler et al. (1975), the conclusions we draw from the increase in M_w^{app} of choleragen in the presence of oligo-G_{M1} would be valid.

[6] Since the Scatchard plot of the binding of oligo-G_{M1} to choleragen was nonlinear and suggested positive cooperativity, a comparison of the relative affinities of choleragen for ganglioside and oligosaccharide may be difficult. In this regard, Sattler et al. (1977) reported that, using equilibrium dialysis, the affinity of choleragen for oligo-G_{M1} was higher than that for G_{M1}.

critical micellar concentration of G_{M1} is between 10^{-5} and 10^{-4} M (Yohe & Rosenberg, 1972; King et al., 1976), G_{M1} may form complexes below the critical micellar concentration (Yohe & Rosenberg, 1972) to which choleragen binds. Thus the microconcentration of G_{M1} in the vicinity of a choleragen molecule bound to such a complex would be higher than the concentration of the ganglioside in the bulk phase.

Several different experimental procedures provide evidence that choleragen and its B protomer contain multiple binding sites for oligo-G_{M1}. The shifts in the fluorescence and circular dichroic spectra of choleragen induced by the oligosaccharide indicated that each toxin molecule bound between 5 and 6 molecules of oligo-G_{M1}. The increase in apparent weight average molecular weight observed for choleragen and the B protomer in the presence of oligosaccharide also is consistent with 5-6 sites for oligo-G_{M1}. Similar values were obtained from equilibrium dialysis and gel permeation chromatography. An estimation of one binding site on each of the polypeptide subunits of the B protomer appears reasonable.[7]

The "blue shift" induced in the fluorescence spectrum of choleragen by G_{M1} indicated a change in the environment of tryptophanyl residues (Mullin et al., 1976). This change is due to an interaction between the B protomer of choleragen and the ganglioside (Moss et al., 1977a) and, as we have now demonstrated, between the B protomer and the oligosaccharide portion of G_{M1}. Such changes in fluorescence do not necessarily represent a conformational change in the protein molecule. The circular dichroic data provide additional evidence that the binding of oligosaccharide perturbs the toxin structure and further support the possibility of a conformational change. It is less likely that the changes in ellipticity which we observed were due to perturbation of the oligosaccharide structure as carbohydrates do not have a strong circular dichroic spectra in this region (Avigad et al., 1970).

The presence of multiple oligosaccharide binding sites on choleragen is consistent with the multivalent nature of its binding to lymphocytes as evidenced by patching and capping of the receptors (Révész & Greaves, 1975; Craig & Cuatrecasas, 1975; Sedlacek et al., 1976). It has been proposed that multivalent binding of toxin and its subsequent lateral movement in the plane of the cell membrane are involved in the activation of adenylate cyclase (Sahyoun & Cuatrecasas, 1975; Craig & Cuatrecasas, 1975). It has also been suggested that the multivalent binding of choleragen to the membrane promotes the penetration of the A protomer into the cell (Sattler et al., 1975; Gill, 1976). Our observations are consistent with either hypothesis.

Both choleragen and the B protomer bound to liposomes containing G_{M1} to a much greater extent than did the A protomer; only A, however, bound to G_{M1}-free liposomes (Moss et al., 1976c, 1977c). Thus the A protomer appears to be shielded in the intact toxin molecule but, once freed from the B protomer, it can interact with the lipid model membranes. The hydrophobic nature of the A protomer is further evidenced by the ability of several gangliosides, including G_{M1}, to inhibit its NAD glycohydrolase activity(Moss et al., 1977a) whereas oligo-G_{M1} was not inhibitory. Whether the perturbations in the choleragen molecule induced by binding of the B protomer to the oligosaccharide chain of G_{M1} also are involved in promoting the interaction of the A protomer with the cell membrane and the subsequent activation of adenylate cyclase by the A_1 subunit remains to be determined.

[7] Estimates of the number of polypeptide chains in the B protomer have ranged from 4 to 8. The best is 5 based on the cross-linking experiments of Gill (1976) and the complete amino acid sequence (Kurosky et al., 1977; Lai et al., 1977). Due to the limitations of our methods in determining low concentrations of choleragen and oligosaccharide, we made no attempt to distinguish between 5 and 6 subunits in the B protomer. In addition, the dissociation and reassociation of choleragen in solution to form complexes with different numbers of polypeptides in the B protomer have been reported (Gill, 1976; Lai et al., 1976).

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Diversity and Abundance of Polyadenylated RNA from *Achlya ambisexualis*†

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ABSTRACT: The diversity, abundance, and DNA sequence representation of poly(adenylic acid) containing RNA derived from cells of *Achlya ambisexualis* cultured in defined and undefined media have been determined. The kinetics of hybridization of polyadenylated RNA with complementary DNA were the same for both culture conditions and revealed the presence of three frequency classes containing 29, 220, and 3000 different sequences of an average length of 1150 nucleotides. Complexity estimates derived from experiments in which polyadenylated RNA was hybridized to unique sequence DNA were in good agreement with these results. The kinetics of hybridization of complementary DNA with an excess of nuclear DNA indicate that approximately 10% of the RNA is transcribed from reiterated DNA sequences while the remainder is transcribed from single copy sequences.

Achlya ambisexualis is an aquatic fungus in which the development of sexual reproductive structures and the coordination of the mating process are regulated by steroid hormones (Barksdale, 1969; Gooday, 1974; van den Ende, 1976). This organism has been the subject of recent investigations in our laboratory because of its potential usefulness as a model system for studying the steroidal regulation of gene activity. Recently we have made two observations which suggest that the mechanisms controlling mRNA transcription and accumulation in *Achlya* may perhaps differ significantly from those operating in metazoan cells. First, we have found that, while *Achlya* possesses both unique and repetitive DNA sequence components, these are arranged in the genome in a pattern different from the characteristic short period interspersion pattern of higher eucaryotes (Hudspeth et al., 1978; Davidson & Britten, 1973; Davidson et al., 1975). Indirect calculations based on quantitative aspects of our data suggest that the repetitive and single copy DNA have average genomic lengths in the range of 2.7×10^4 and 1.4×10^5 nucleotide pairs, respectively, much longer even than those observed in higher organisms, such as *Drosophila,* with long period DNA sequence interspersion (Manning et al., 1975; Crain et al., 1976). Second, the processes of mRNA transcription and posttranscriptional modification in *Achlya* appear to be more straightforward than has been found in advanced eucaryotic forms. The nuclear and polysomal poly(A^+)RNA populations in *Achlya* are indistinguishable with respect to size distribution, DNA sequence representation, and sequence complexity (Timberlake et al., 1977). This is in contrast to animals in which the hnRNA is typically larger and considerably more complex than mRNA active in translation (Lewin, 1975a,b). However, in several general respects the mRNA of *Achlya* is similar to that of higher eucaryotes (e.g., polyadenylated and monocistronic).

The novel features of genome organization and mRNA synthesis which we have observed in *Achlya* raise the question of how the mechanisms of gene regulation in this organism are related to those in other eucaryotes and to those in procaryotes. Here we report the results of a study in which the distribution of poly(A^+) RNA into abundance classes was determined. This investigation was prompted by the observation that, in all eucaryotic cells thus far examined, the poly(A^+) mRNA has been found to occur in classes with different relative cellular concentrations (abundance classes) (Bishop et al., 1974; Hastie & Bishop, 1976; Monahan et al., 1976; Levy & Dixon, 1977; Hereford & Rosbash, 1977a; Galau et al., 1976). Different abundance classes may have specialized metabolic roles and

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