Action of Vibrio cholerae Neuraminidase (Sialidase) upon the Surface of Intact Cells and Their Isolated Sialolipid Components*

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SUMMARY

The effect of strong electrolyte upon the activity of Vibrio cholerae neuraminidase (VC neuraminidase) toward the surface of intact cells and toward their isolated, purified sialolipid components, was studied in detail. A linear dependence of the logarithm of initial velocity on the square root of ionic strength was observed for purified GM3 over a concentration range of 0 to 0.05 M NaCl in 0.01 M Tris-acetate, pH 6.5. The rate of decrease of the logarithm of the optimum initial velocity, obtained in 0.01 M Tris-acetate alone, with increasing square root of ionic strength was 3 times more rapid with GM3, GD1a, GD1b, and GT1 as substrate mixture than with the smaller sialolipid, GM1, as substrate. These data are consistent with previous results which indicate that ionic interactions upon the enzyme conformationally govern steric availability of the catalytic site. The effect of strong electrolyte upon the activity of neuraminidase toward the surface of intact cells was small in comparison with that observed with purified sialolipid substrates. Analysis of lipid extracts from untreated control and VC neuraminidase-treated samples by thin layer chromatography revealed no detectable difference between the sialolipid content of untreated control samples and samples treated with VC neuraminidase. These findings suggest that the sialolipid components in the intact cell surface are relatively unavailable to the extracellular action of VC neuraminidase. This is supported by evidence obtained by detailed analysis of the sialyl components in four cell types (Green monkey kidney cells, Morris hepatoma, B-16 melanoma, and human neuroblastoma) before and after VC neuraminidase treatment. The total cellular sialic acid content of these cell types, untreated, varied from 2.3 to 7.1 μg of sialic acid per mg of protein; lipid-bound sialic acid content varied from 0.7 to 1.6 μg of sialic acid per mg of protein. Progress curves for sialic acid release from the surface of intact cells were hyperbolic and had reached a plateau within approximately 30 min. In the plateau region approximately 20% of the total cellular sialic acid had been released consistently, in each case. Analysis of the lipid-bound sialic acid content of the VC neuraminidase treated intact cells revealed no major changes, all values being greater than 90% of the untreated, control values. The sialolipid composition of each cell type was examined by thin layer chromatography and found nevertheless to consist predominately of VC neuraminidase-labile components. Therefore, the sialolipids of the intact cell surface appear to be more deeply localized so as to be sterically inaccessible to VC neuraminidase, and release of sialic acid from intact cells by VC neuraminidase appears to be from surface sialoglycoproteins.

The behavior of complex sialolipids (gangliosides) as substrates for the exoglycohydrolase, Vibrio cholerae sialidase, or neuraminidase (VC neuraminidase), has been studied in detail. VC neuraminidase is active toward ganglioside mixtures as substrate regardless of whether the substrate is present in a monomeric, disperse form, or an aggregated, micellar form (1). The importance of ionic strength in controlling the activity of VC neuraminidase toward various substrates has been described (2). The activity of VC neuraminidase toward a complex sialolipid substrate mixture, GM1, GD1a, GD1b, and GT1, decreased rapidly with increasing ionic strength, but no such effect of ionic strength upon enzymatic activity was observed when the small non-lipid molecule, sialyllactose, served as substrate. These data were interpreted to indicate that ionic interactions on VC neuraminidase conformationally govern the sterical availability of its catalytic site to various substrates.

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1 The abbreviations used are: VC neuraminidase, Vibrio cholerae neuraminidase, more properly called sialidase since the sialic acids are naturally occurring N- and O-acetyl and glycolyl derivatives of the parent compound, neuraminic acid, which does not appear to occur in nature as such; GM1, monosialotetraglucosylceramide; GD1a and GD1b, disialotetraglucosylceramides; GT1, trisialotetraglucosylceramide; GM2, monosialotriglucosylceramide; GM3, monosialodiglycosylceramide; TAS, 0.01 M Tris-acetate-0.150 M NaCl; TAP, 0.01 M Tris-acetate-0.300 M pentaerythritol; % Amax, enzymatic activity, A, expressed in terms of the relative percentage of the optimum initial velocity, the latter being obtained in 0.01 M Tris-acetate, pH 6.5, without the addition of strong electrolyte and being represented in value as 100%; GL1, gloeosylceramide; GL2a, lactoepyleramidase; GL2b, digalactosylceramide; CP neuraminidase, Clostridium perfringens neuraminidase.
In the present investigation, the effects of ionic strength upon the activity of VC neuraminidase toward GM3, the predominant sialolipid of extraneural tissues, are described. Following from the experiments carried out with purified sialolipid substrates, a detailed investigation is made of the action of VC neuraminidase upon the surfaces of intact cells. The information gained from these studies has bearing upon present concepts of the structure of the plasma membrane of mammalian cells. It may also be of use in understanding the enhancement of the immunogenicity of tumor cells which, reportedly, takes place after VC neuraminidase treatment of the intact cell surface (3, 4).

EXPERIMENTAL PROCEDURES

MATERIALS

Sialolipid Substrates—Bovine brain grey matter served as a source of the mixed substrate GM1, GD1a, GD1b, and GT1. Human spleen served as a source of GM3. Tissue samples were homogenized in a Waring Blender in chloroform-methanol, 2:1 (v/v). The lipid extract was filtered through Whatman No. 2 filter paper. The filtrate was subjected to partition-dialysis as described by Folch et al. (5). Aqueous phases of the dialysate from bovine brain grey matter were pooled and reduced to dryness on a rotary flash evaporator. This preparation consisted of crude gangliosides, it was purified by silicic acid chromatography according to the method of Dain et al. (6). Fractions containing GM3, GD1a, GD1b, and GT1 were pooled and lyophilized. The final substrate preparation consisted of GM3, GD1a, GD1b, and GT1 in respective mole percentages of 9.2, 60.7, 14.5, and 15.6. Chloroform phases of the dialysate from human spleen were pooled, concentrated, and separated into neutral, glyco-, and phospholipid fractions as described by Vance and Sweeley (7). Crude glycolipid fractions were pooled and reduced to dryness on a rotary flash evaporator. This crude material was dissolved in a small volume of chloroform-methanol, 9:1 (v/v), and placed on a silicic acid column (30 x 4 cm). Glycolipids were eluted with chloroform-methanol-water, 65:25:4 (v/v/v). Column fractionation of individual glycolipids was developed for studying the effect of strong electrolyte on enzymatic activity (9). Finally, the washed, calcium ion-loaded cell pellets were suspended in 0.01 M Tris-acetate and 0.300 M urea, pH 7.4. Cell pellets for electrophoresis were washed twice in TAP, containing 2.5 to 3.0 mg of protein (~107 cells), were pooled and washed twice in TAS, pH 7.4, containing 5 mM CaCl2. This removed excess EDTA and also served to load the cell membranes with calcium ion, which is necessary for VC neuraminidase activity (9). Finally, the washed, calcium ion-loaded cell pellets were suspended in 0.01 M Tris-acetate and 0.300 M pentaerythritol, pH 6.5 (TAP). This suspension medium was used for the study of the effect of strong electrolyte on enzymatic activity toward intact, whole cells. Cells in the final preparation, suspended in TAP, appeared intact by phase-contrast microscopy. Cell counts were performed with a hemocytometer.

Protein concentration was determined by the procedure of Lowry et al. (10).

Effects of Strong Electrolyte on VC Neuraminidase Activity toward Intact Cell Surfaces—Strong electrolyte was studied over a concentration range of 0 to 0.05 M NaCl in 0.01 M Tris-acetate, pH 6.5. Osmolarity was consistently adjusted to 0.310 osm by addition of pentaerythritol to the Tris-acetate-saline solution. In preparing the assay mixtures, aliquots of the cell suspension in TAP, containing 2.5 to 3.0 mg of protein (~106 cells), were pelleted and resuspended in 1 ml of the appropriate salt solution. Experimental samples received 5 units of VC neuraminidase. Assay mixtures were incubated at 37° for 5 min, a length of time experimentally determined to be within the duration of the initial reaction velocity. At the termination of the incubation period the assay mixtures were cooled in ice and the cells were pelleted by centrifugation for 3 min at room temperature. The supernatant were removed with a Pasteur pipette and analysed for enzymatically released, free sialic acid. All analyses were run in duplicate with corresponding reagent, enzyme, and substrate blanks. The total lipid extracts of untreated control and VC neuraminidase-treated cells were prepared by sequential extraction with 5 ml of chloroform-methanol, 2:1 (v/v), followed by 5 ml of chloroform-methanol, 1:1 (v/v). In order to remove
insoluble debris, the extracts were filtered with the aid of suction through medium porosity sintered glass filters. This filtration system allowed less than 2% of the total quantity of cellular protein to pass through with the lipid extract. The filtrates were evaporated under a stream of N₂, and the lipid profiles were examined by analytic thin layer chromatography on Silica Gel Q5W thin layer glass plates (Quantum Industries). Plates were developed in chloroform-methanol-water, 70:35:7 (v/v/v). Sialolipids were visualized with the resorcinol spray reagent of Svensson-Holm (11). Identification of sialolipids was made by comparison with authentic reference compounds.

Enzymatically Released, Free Sialic Acid and Sialic Acid Content of Intact Cells before and after VC Neuraminidase Treatment—Progress curves for the release of sialic acid from the intact cell surface by VC neuraminidase were obtained by incubating 1 ml of cell suspension (2.0 to 3.2 mg of protein) in TAP with 5 units of VC neuraminidase for specified periods of time. At the termination of the incubation period, the reaction was quenched by addition of the acid-periodate reagent of Warren (8). Cells were pelleted by centrifugation. Supernatants were removed and analyzed for enzymatically released, free sialic acid. All analyses were run in duplicate with corresponding reagent, enzyme, and substrate blanks. To correct for the possible hydrolysis of endogenous sialolipid substrate by endogenous membrane-bound sialidase (12-14), 1 ml aliquots of the cell suspensions in TAP were incubated under the conditions of the assay. Duplicate aliquots were held at 0° as a control. Free sialic acid was analyzed as described above. Lipid extracts from untreated control and VC neuraminidase-treated cell pellets were prepared by chloroform-methanol extraction and examined both by thin layer chromatography and by quantitative analysis for lipid-bound sialic acid by the procedure of Jourdian et al. (15). Total cellular sialic acid was also determined by the procedure of Jourdian et al. (15). For these analyses, an amount of cellular material containing 2.5 to 3.0 mg of protein was taken.

RESULTS

In studying the effect of strong electrolyte upon VC neuraminidase activity toward sialolipid substrates, the substrate concentration was chosen such that added strong electrolyte (NaCl) did not apparently affect the aggregative state of the substrate. Linear dependence of the logarithm of initial velocity on the square root of the ionic strength (μ) of the assay medium was observed over a concentration range of 0 to 0.05 m NaCl in 0.01 m Tris-acetate, pH 6.5. The rate of decrease of log (% A₀) with increasing square root of ionic strength was 3 times more rapid with the GM₁₄, GD₁₈, GD₅₄, and GT₁ substrate mixture than with GM₃ as substrate. Representative data are presented in Fig. 1. With concentrations of GM₁₄ above 0.5 × 10⁻⁴ m, deviations from linearity were observed. Plots of log (% A₀) versus μ½ became increasingly hyperbolic, particularly at higher ionic strengths. For example, at an ionic strength of 0.06, log (% A₀) was 1.88, 1.85, and 1.80, respectively, at substrate concentrations of 0.5 × 10⁻⁴, 1.0 × 10⁻⁴, and 3.0 × 10⁻⁴ m. Thus, at high ionic strength and high substrate concentrations, activity actually decreased with increasing substrate concentration.

The effect of strong electrolyte upon the activity of VC neuraminidase toward the intact cell surface, as indicated in Fig. 1, was small in comparison with that observed with the purified sialolipid substrates. By extrapolation, log (% A₀) of VC neuraminidase toward a B-16 melanoma cell suspension in 0.01 m Tris-acetate-buffered, physiological saline (μ₀ = 0.140) would be fully 80 to 85% of that observed in TAP in the absence of added NaCl (μ₀ = 0.100).

Thin layer chromatographic analysis of the lipid extracts from untreated control and VC neuraminidase-treated B-16 melanoma samples indicated the predominant sialolipid to be GM₃. No detectable difference in the sialolipid content of untreated control samples, as compared with VC neuraminidase-treated samples, was observable over the entire range of strong electrolyte concentrations employed in these studies. In combination, these findings suggest that the sialolipids in the intact cell surface are relatively unavailable to the extracellular action of VC neuraminidase. Enzymatic release of sialic acid from the intact cell by VC neuraminidase therefore appears to be from surface sialoglycoproteins.

FIG. 1. The effect of strong electrolyte upon the activity of Vibrio cholerae neuraminidase toward purified sialolipid substrates and the surface of intact cells. Enzymatic activity, A, is expressed in terms of the relative percentage of the optimum initial velocity. The latter was obtained in 0.01 m Tris-acetate, pH 6.5, without the addition of strong electrolyte and is represented in value as 100%. Strong electrolyte (NaCl) varied in concentration from 0 to 0.05 m. Assay conditions for the enzymatic release and analysis of free sialic acid are described in the text. Curve a, 1 × 10⁻⁴ m GM₁₄, GD₁₈, GD₅₄, and GT₁, substrate mixture; Curve b, 0.5 × 10⁻⁴ m GM₁₄; Curve c, intact B-16 melanoma cells, 2.53 mg of protein per assay mixture.

FIG. 2. a, the effect of VC neuraminidase treatment upon the sialic acid content of intact B-16 melanoma cells. Assay conditions are described in the text. Enzymatically released, free sialic acid and lipid-bound sialic acid are both expressed as micrograms of sialic acid per mg of protein (PR). Each assay mixture contained 3.16 mg of protein. Curve a, VC neuraminidase-releasable sialic acid; Curve b, lipid-bound sialic acid of untreated control samples; and Curve c, lipid-bound sialic acid of VC neuraminidase-treated samples. b, the effect of VC neuraminidase (VCN) treatment upon lipid-bound sialic acid of intact B-16 melanoma cells. Assay conditions are described in the text and in Plate a. Lipid-bound sialic acid content is expressed as the ratio of the VC neuraminidase-treated value to the untreated control value. C:M, chloroform-methanol.
and human neuroblastoma cell surfaces are presented in Figs. 2a and 3a, respectively. These curves are hyperbolic and have reached a plateau within approximately 30 min. As indicated in these figures, the sialolipid content was not significantly decreased by VC neuraminidase action as compared with untreated control samples. The ratio of sialolipid content in VC neuraminidase-treated samples to that in untreated control samples decreased slightly with time as indicated in Figs. 2b and 3b, but the decrease was very minor. After VC neuraminidase treatment of intact B-16 melanoma cells for 30 min, a relatively small amount of breakdown of GM₃, with a corresponding increase in GL₃b content, was demonstrable on thin layer chromatograms as shown in Fig. 4.

Cellular sialic acid content of four cell types before and after VC neuraminidase treatment is presented in Table I. The total cellular sialic acid content varied over a rather broad range, from 2.33 µg per mg of protein for the Morris hepatoma to 7.18 µg per mg of protein for the B-16 melanoma. After a 30-min incubation period with VC neuraminidase, approximately 20% of the total cellular sialic acid was released in each case. Under the conditions of these experiments, no sialic acid was found to be released by endogenous membrane-bound neuraminidase. The amount of chloroform-methanol-extractable sialic acid varied from approximately 15% of the total cellular sialic acid for the neuroblastoma sample, to approximately 30% for the melanoma and hepatoma samples. All chloroform-methanol-extractable sialic acid was identified as sialolipid by thin layer chromatographic comparison with authentic sialolipid standards. The predominant sialolipid of the B-16 melanoma, Morris hepatoma, and Green monkey kidney cells was GM₃; the major sialolipid component of the human neuroblastoma preparation was GD₁ with lesser amounts of GT₁ and GM₃, respectively. As indicated in Table I, after a 30-min incubation of the intact cell preparations with VC neuraminidase, no major change occurred in the content of chloroform-methanol-extractable sialic acid, all values being greater than 90% of the untreated controls.

**DISCUSSION**

A previous study (2) of the effects of ionic strength of the incubation medium on the activity of VC neuraminidase toward the sialolipid substrate mixture, GM₁, GD₁₆, GD₁₉, and GT₁, indicated that elevated concentrations of strong electrolyte reversibly inhibit enzymatic activity. The inhibitory effect
were pooled and analyzed for lipid-bound sialic acid by the procedure of Jourdian et al. (15). The release of cell surface sialic acid by VC neuraminidase activity was determined by the Warren procedure (8) and is reported as the average of duplicate determinations. For the determination of VC neuraminidase-releasable sialic acid, duplicate samples in TAP, pH 6.5, were incubated for 30 min at 37° with 5 units of VC neuraminidase. Controls were handled identically, but, without the addition of VC neuraminidase. Following incubation, the assay mixtures were separated into cell pellet and supernatant fractions as described in the text. VC neuraminidase-releasable, free sialic acid in the supernatants was determined by the Warren procedure (8) and is reported as the average of duplicate determinations. Endogenous membrane-bound neuraminidase activity was determined as described in the text. Total lipid extracts of the cell pellets of the duplicate samples were pooled and analyzed for lipid-bound sialic acid by the procedure of Jourdian et al. (15).

Table I

| Cell sample     | Total cellular sialic acida | VC neuraminidase-releasable sialic acidb, c | Sialic acid releasable by endosialidaseb, c | CHCl3-MeOH-extractable sialic acidb, d | Control VC neuraminidase-treated |
|-----------------|----------------------------|-----------------------------------------------|---------------------------------|---------------------------------|---------------------------------|
| B-16 melanoma   | 7.18 ± 0.38                | 1.41 ± 0.02                                    | None @ pH 6.5                   | 1.83                            | 1.76                            |
| Human neuroblastoma | 6.71 ± 0.15               | 1.01 ± 0.02                                    | None @ pH 6.5                   | 0.82                            | 0.76                            |
| Morris hepatoma  | 2.33 ± 0.07               | 0.42 ± 0.01                                    | None @ pH 6.5                   | 0.73                            | 0.67                            |
| Green monkey kidney cells | 5.29 ± 0.02 | 1.10 ± 0.07                                    | None @ pH 6.5                   | 0.80                            | 0.75                            |

a Enzymatically released, free sialic acid, and total and lipid-bound sialic acid content are expressed as micrograms of sialic acid per mg of protein. Total cellular sialic acid was determined as described in the text and is reported as the average of duplicate determinations.

For the determination of VC neuraminidase-releasable sialic acid, duplicate samples in TAP, pH 6.5, were incubated for 30 min at 37° with 5 units of VC neuraminidase. Controls were handled identically, but, without the addition of VC neuraminidase. Following incubation, the assay mixtures were separated into cell pellet and supernatant fractions as described in the text. VC neuraminidase-releasable, free sialic acid in the supernatants was determined by the Warren procedure (8) and is reported as the average of duplicate determinations.

Endogenous membrane-bound neuraminidase activity was determined as described in the text.

Total lipid extracts of the cell pellets of the duplicate samples were pooled and analyzed for lipid-bound sialic acid by the procedure of Jourdian et al. (15).

Operated to change V0 max. No such effect was observed with the small non-lipid molecule sialyllactose. The results were interpreted to indicate that ionic strength effects upon the enzyme conformationally govern the steric availability of the catalytic site. The data presented in this paper for sialolipid substrates are consistent with the above interpretation. The sialolipid substrates, GD1α, GD1β, and GT1, consist of molecules containing sialyl residues in branched, VC neuraminidase-labile, 2 → 3 sialylgalactosyl and 2 → 8 sialyllactosyl glycosidic linkages on a tetrasaccharide backbone. These oligosaccharidyl units are longer and bulkier than those in which the sialyl residues are in terminal 2 → 3 linkage on a disaccharide backbone, as in GM3. Thus, if strong electroativity conformationally alters the steric availability of the catalytic site, enzymatic activity expectedly would be inhibited to a greater degree with the sialolipid substrates containing the bulkier oligosaccharidyl units, as we have observed experimentally.

Substrate concentration is a critical factor in these experiments. For the unambiguous interpretation of enzymatic characteristics, the substrate concentration must be such that it exists predominately either in a monomeric, disperse form, or in an aggregated, micellar form, and there is the additional requirement that increase in strong electrolyte concentration should not unpredictably shift the equilibrium between the two forms. These considerations are important since, even though VC neuraminidase is active toward both micellar and disperse substrate forms (1), the apparent K_m for aggregated, micellar substrate is 5-fold greater than that for the monomeric, disperse substrate. If the substrate remains in predominantly one physical state as ionic strength increases, one observes a linear dependence of log (% A_opt) on μ1/2. The effect of ionic strength is seen to be exerted upon the enzyme. When the substrate changes its physical state with increasing ionic strength, then one observes deviations from linearity in log (% A_opt) versus μ1/2 plots, since the effect of ionic strength upon the physical form of the substrate is superimposed upon direct effects on the enzyme. These considerations have bearing on the results which we have obtained with GM3. At a substrate concentration of 0.5 × 10^{-4} M, a linear dependence of log (% A_opt) versus μ1/2 was observed. Although the critical micelle concentration of GM3 in aequous solutions has not yet been determined, it presumably is lower than that for GM1 (0.75 × 10^{-4} M) as reported by Yohe et al. (16), but undoubtedly far higher than that reported by Smith et al. (17) for L-α-dipalmitoylphosphatidylcholine (4.5 × 10^{-4} M). Thus at a concentration of 0.5 × 10^{-4} M, GM3 is presumed to be mainly in a monomeric or disperse form, although this concentration may be quite close to the critical micelle concentration. As the concentration of GM3 is increased above this level, deviations from linearity are observed in plots of log (% A_opt) versus μ1/2; the plots become increasingly hyperbolic. Such deviations probably result from physical changes in the form of the substrate. These deviations occur over a broad range of relatively high substrate concentrations. Such effects have not been observed with the GM1, GD1α, GD1β, and GT1 substrate mixture. Perhaps the greater aqueous solubility of this latter substrate mixture renders its physical form less sensitive to ionic strength effects in comparison with GM3. Clearly a greater knowledge of the physical properties of GM3 is required in order to interpret this apparently anomalous effect. Such knowledge may provide insight regarding the characteristics of this important sialolipid component of the plasma membrane of mammalian cells.

In extending the study of ionic strength effects from purified sialolipid substrates to intact cell surfaces, a low molecular weight, non-ionic, osmolarity-balancing agent was required. The requirements were well met by pentaerythritol, C(CH2OH)4.

As indicated in Fig. 1, ionic strength effects upon VC neuraminidase activity toward intact cell surfaces are slight. By extrapolation, the initial velocity of sialic acid release from intact cells, suspended in physiological saline, would be 80 to 85% of that observed in a low ionic strength, pentaerythritol-containing, medium. The absence of a significant ionic strength effect on the release of B-16 melanoma cell surface sialic acid coupled with the absence of demonstrable changes in the sialolipid profile of untreated control samples as compared to VC neuraminidase-treated samples suggested that the sialolipids of the intact cell surface were unavailable to VC neuraminidase action. As indicated in Figs. 2, 3, and 4, and Table 1, this appears to be verified experimentally. The release of cell surface sialic acid by VC neuraminidase was found to be hyperbolic with time, and had reached a plateau within approximately 30 min. Even though total cellular sialic acid varied considerably between the four cell types investigated, a rather constant proportion (20%) of the total was released in each case after 30 min incubation. The amount of VC neuraminidase-releasable sialic acid would have been sufficient to account for the entire content of lipid-bound
sialic acid, except in the case of the B-16 melanoma and Morris hepatoma, where there was slightly more lipid-bound than VC neuraminidase-releasable sialic acid. However, when the amount of lipid-bound sialic acid was quantitated in untreated control and in VC neuraminidase-treated samples, no significant changes were observed. The sialolipid content of the VC neuraminidase-treated samples was always greater than 90% of the value of untreated controls. These quantitative data were supported by thin layer chromatographic analyses of the sialolipids present in the four samples examined. The major sialolipids of these samples consisted of the VC neuraminidase-labile component, GM₂, in the case of B-16 melanoma, Morris hepatoma, and Green monkey kidney cells, and VC neuraminidase-labile GD₁, in the case of human neuroblastoma cells. As presented in Fig. 4, VC neuraminidase treatment of the B-16 melanoma cell surface resulted in a slight, but not very significant, decrease in the content of GM₂ along with a corresponding slight increase in the content of the asialo product, GL₃α₃. The simplest interpretation of these data, in combination, is that sialolipids of the intact cell surface are more deeply localized within the plasma membrane and thus are rendered sterically inaccessible to VC neuraminidase. In contrast, protein-bound sialic acid is accessible. Weinberg et al. (18) have arrived at similar conclusions in their study of the glycolipids of the mouse L cell and its surface membrane.

A point which requires clarification is the apparent low percentage (~20%) of the total cellular sialic acid releasable by VC neuraminidase in our studies. Other investigators (18–20) have reported release ranging from 50 to 90% of the total cellular sialic acid in their experimental systems. In unravelling these differences, three interrelated factors are relevant. Firstly, Clostridium perfringens neuraminidase (CP neuraminidase) has been employed to release cell surface sialic acid in some studies (20). Wenger et al. (21) have recently reported that CP neuraminidase is capable of releasing sialic acid from GM₂. In contrast, VC neuraminidase has not been found to be capable of releasing this internal sialic acid residue. Thus, CP neuraminidase may have a broader substrate specificity than VC neuraminidase. Therefore, treatment of the intact cell surface by CP neuraminidase could result in a greater release of sialic acid. Secondly, neuraminidase endogenous to the cell membrane itself ordinarily has been overlooked. No sialic acid was found to be released by endogenous neuraminidase under the conditions employed in the current study. Endogenous neuraminidase is a membrane-bound enzyme which occurs together with endogenous sialolipid substrate. The pH optimum of this enzyme is in the vicinity of pH 4.0 (12–14), but it is still measurably active even at pH 6. In addition, endogenous neuraminidase activity, directed toward exogenously supplied sialolipid substrate, becomes apparent as a result of onecogenic transformation (22) of tissue-cultured cells. The action of exogenously supplied microbial neuraminidase at the cell surface, combined with increasing activation of endogenous membrane-bound neuraminidase as a consequence of pH effects, coupled with the effects of oncogenic transformation, could readily account for the high percentages of sialic acid release presumed to be due to the action of VC neuraminidase on the cell surface as reported in the literature. Finally, the cell preparations utilized in the currently reported experiments had been preloaded with calcium ion, and the medium contained no excessive amount of this ion. VC neuraminidase requires calcium ion for maximal activity (9), and, although the high percentages previously described for sialic acid release by VC neuraminidase may partly have resulted from a more optimal calcium ion concentration, the data reported here and prior studies (1) indicate that our experimental conditions permit unimpaired activity for VC neuraminidase with sialolipid substrates without addition of more calcium ion. The effects of calcium ion in the bulk aqueous suspension medium upon the surface of the intact cell and the effect of hypo-osmotic rupture of the cell membrane in increasing the availability of plasma membrane sialoglycolipids, especially those of the sialolipid components, to VC neuraminidase attack will form the subject of a separate report.

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