THE CONTRIBUTION OF SIALIC ACID TO THE SURFACE CHARGE OF FRAGMENTS OF PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM

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In previous publications (1–4) we have described the isolation from the microsomal fraction of Ehrlich ascites carcinoma (EAC) of two major membrane types, namely: (*a*) ER-type particles, arising primarily from the "smooth" endoplasmic reticulum, and (*b*) PM-type particles originating principally from the plasma membrane. We have also presented some details of the light-scattering properties of these membrane types, in particular the distinctive absorbance changes which accompany alterations of the ionogenic properties of the particles' surfaces (5). The present report is a continuation of these studies and deals with the contribution of neuraminic acid to the negative charge of PM- and ER-type membranes.

EXPERIMENTAL

ER- and PM-type membranes were prepared as described previously (2, 3, 5). Prior to incubation with neuraminidase the membranes were freed of the sucrose or polysucrose in which they were prepared, since these compounds interfere with the determination of neuraminic acid. For this, membrane suspensions, containing 1 to 2 mg of protein/ml, were diluted 5-fold with 0.01 M Tris-HCl (pH 8.2), 0.01 M $CaCl_2$ and the particles sedimented by centrifugation at 39,000 RPM (Spinco rotor SW 39) for 30 min. The pellets were washed once in the same medium by suspension and recentrifugation. Preparatory steps were at 1–4°C. Storage at the membranes was in sucrose or polysucrose at −28°C. Protein measurements were by a microvolumetric modification of the ninhydrin procedure (6).

Neuraminidase treatment was with crystalline neuraminidase from *Cholera vibrio* (Behring Werke, Marburg-Lahn, Germany). The activity of the enzyme preparation was 109 units/ml, using erythrocyte stroma as substrate. Membrane pellets were suspended to a concentration of about 1 mg protein/ml in 0.01 M acetate (pH 5.4), 0.01 M in

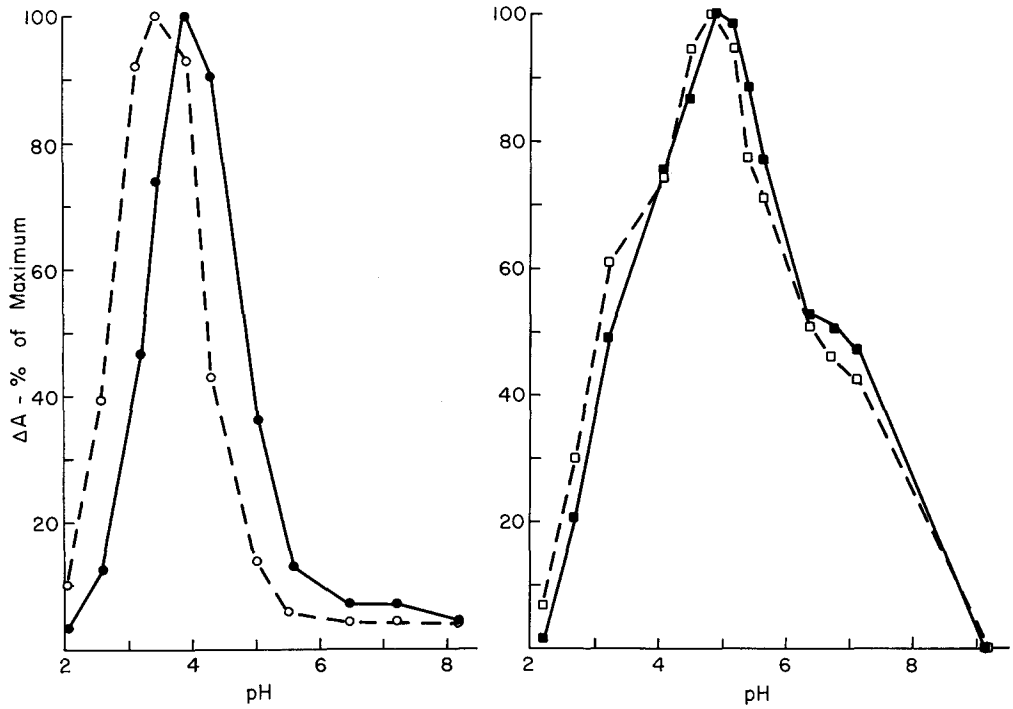

FIGURE 1 Effect of *Vibrio cholera* neuraminidase on the pH-absorbance profiles of PM and ER. *Left panel*, PM. *Right panel*, ER. Controls, O---O. Neuraminidase-treated, ●—●. Profiles determined as in reference 5. Details of neuraminidase treatment in text. The data pictured here are from representative experiments. ΔA is the increment in the absorbance at 400 mμ at a given pH over that at pH 9. ΔA at the isoelectric point has been set to 100%.

$CaCl_2$ and 0.05 M in NaCl, containing 25 units of neuraminidase/ml, and were incubated at 37°C for 30 min, by which time there is maximum neuraminic acid release. The samples were then centrifuged at 39,000 RPM (Spinco rotor SW 39) for 30 min. The supernatants were assayed for liberated neuraminic acid by the thiobarbituric acid procedure (7). The pellets were resuspended to a concentration of about 2 mg protein/ml in 0.01 M Tris-HCl (pH 8.2), 0.01 M EDTA and dialyzed against 200 volumes of the same medium for 1 hr, followed by two successive dialyses (at least 2 hr each) against 100 volumes of 0.001 M Tris-HCl (pH 8.2). Representative samples of the stable, dialyzed suspensions were taken for protein measurements and determination of pH-absorbance profiles. Two controls were included in each experiment, namely: (*a*) membrane samples carried through the entire procedure, except for omission of enzyme during the incubation step; (*b*) samples carried through the entire procedure, but incubated with enzyme that had been inactivated by heating at 100°C for 5 min. The protein concentrations of suspensions of membranes treated with active neuraminidase were identical with those of the controls.

pH-absorbance profiles were determined as described previously (5). 150-μl samples of the membrane suspensions in 0.001 M Tris-HCl (pH 8.2) were diluted 20-fold (for cuvettes with 1-cm optical path) or 200-fold (for 10-cm cuvettes) with appropriate Tris-acetate buffers (and dilute HCl for pH < 3) to give the desired final pH. Absorbance at 400 mμ and pH were measured 30 min after the pH change. The final ionic strength was 0.01. The final protein concentrations were 75 to 100 μg/ml in experiments using 1-cm cuvettes ($\frac{1}{10}$ of this with 10-cm cuvettes).

RESULTS

Three preparations of ER and PM were examined. Treatment with active neuraminidase led to the release of 0.016 ± 0.002 μmoles of neuraminic acid per mg protein from ER and 0.028 ± 0.002 μmoles per mg protein from PM. Incubation without neuraminidase or with heat-inactivated enzyme did not liberate detectable quantities of neuraminic acid.

The pH-absorbance profiles of the two membrane types after incubation with and without

active neuraminidase are shown in Fig. 1. The curves obtained on membranes incubated without enzyme or with heat-inactivated enzyme were identical. The pH values for maximum absorbance—corresponding to the apparent isoelectric points of the external surfaces of PM particles and both outer and inner surfaces of ER vesicles (5)—were 3.6 ± 0.15 in the case of PM and 4.5 ± 0.2 in the case of ER. The shapes of the pH-absorbance curves and the values of the "isoelectric" pH's did not differ significantly from those previously reported for untreated membranes (5), nor did the values of specific absorbance.

In the case of ER, there is no significant change in the pH-absorbance profile (Fig. 1), or in specific absorbance, after enzymatic liberation of neuraminic acid. Moreover, as in the case of untreated ER membranes (5), the isoelectric point and shape of the pH-absorbance curve do not change when the measurements are performed at $\frac{1}{10}$ the usual protein concentration (to minimize the contribution of aggregation to absorbance changes).

In contrast to ER, liberation of neuraminate from PM gives a clear shift of the pH-absorbance profile to higher pH, the new isoelectric point locating at pH 4.01 ± 0.07 (Fig. 1). The specific absorbance at the new isoelectric point is identical with that at the isoelectric point of the controls. There is still no indication of the presence of groups titrating between pH 6 and 9.

DISCUSSION

As shown previously (5), the pH-absorbance profiles of PM reflect titration of charged groups fixed on the outer surfaces of these vesicles, but in the case of ER these curves are due not only to aggregation, but also to refractive index changes which accompany alterations in vesicle volume with pH.

The effects of enzymatic removal of neuraminate ions from PM vesicles must be considered in the light of the influence of neuraminidase on the electrokinetic properties of intact EAC, reported by Cook, Heard, and Seaman (8). These authors showed that the electrophoretic mobility of untreated EAC is zero at pH about 3.8. (Other values for the isoelectric point of EAC range between pH 3 and 4 (9–11).) With decreasing pH below 3.8, the cells acquire increasing positive charge to pH about 2. With increasing pH between 3.8 and 5, the electrophoretic mobility of the cells becomes increasingly negative, but there is only slight further increase in negative charge between pH 5 and 10. After neuraminidase treatment, the isoelectric point of the cells shifts to pH about 4 and the cells acquire increasing negative charge between pH 4 and 6. There is little further increase in mobility above pH 6.

Both the pH-mobility curves of Cook et al. on intact EAC and our pH-absorbance profiles on PM vesicles derived therefrom describe surfaces whose charge characteristics arise from the action of three ionogenic systems, namely: (*a*) basic groups of pK 9; (*b*) unidentified anionogenic groups of pK about 4; (*c*) the neuraminate carboxyl of pK 2.6. The surfaces described are the external layers of the intact and vesiculated plasma membranes, respectively, of EAC. However, other experiments (5) indicate that the internal surfaces of PM also have ionogenic groups which titrate between pH 7 and 5. Since the fragmented plasma membranes of ruptured EAC sediment with the microsomal fraction and since ER vesicles, the other major membrane component of this fraction, have ionogenic properties quite distinct from those of the surface of intact EAC, the parallel between the pH-mobility curves of the intact EAC and the pH-absorbance profiles of PM vesicles represents further evidence that PM-type vesicles are indeed fragments of the disrupted surface membrane.

To the previously discussed differences between the pH-absorbance curves of PM and ER (5) we now add the lack of detectable effect of neuraminidase on the light-scattering properties of ER vesicles, despite unequivocal chemical evidence that neuraminate is, in fact, liberated. This situation is comparable to the observations of Naaman, Eisenberg, and Doljanski (12), who found that the isoelectric point (pH 4.4) and pH-mobility curves of isolated, normal liver cells are not altered by neuraminidase treatment. One explanation for our findings could be that the proportion of neuraminate ions among the acidic groups fixed on ER surfaces is much less than on PM surfaces and that removal of neuraminate affects the stability of ER vesicles too little to be detected by the methods employed. This may, indeed, also be the explanation for the observation that the electrophoretic mobilities of a number of cell types are not altered detectably by removal of neuraminate from the intact cells (13–16). However, some of the other possibilities suggested in explanation of the latter phenomenon—e.g. presence of neuraminic acid esters or amides, appearance of new anionic

groups, reorientation of existing charges, etc. (16)—may also apply here.

It should be noted that the presence of neuraminate in ER cannot be attributed to contamination with PM. Indeed, the ER fraction contains only traces of cell surface antigens (2) and, even if it is assumed that the small concentration of Na^+-K^+-activated ATPase in ER (2) is due to contamination with PM, this would not account for the fact that the neuraminate concentration in ER is almost 60% that of PM.[1] On the contrary, the present results suggest that this substance is a component of many diverse membrane systems and do not support our previous conjecture (17) that neuraminic acid is primarily a plasma membrane component. Our present view is also supported by the elegant, recent experiments of Marcus et al. (18), which show that neuraminate is definitely a component of the nuclear membranes of HeLa cells.

[1] About ⅔ of the membrane-associated neuraminate of EAC microsomes is in the ER fraction.

SUMMARY

The action of *Cholera vibrio* neuraminidase on purified fragments of endoplasmic reticulum of Ehrlich ascites carcinoma liberates 0.016 μmoles of neuraminic acid per mg of protein, but does not alter the charge properties of these membranes detectably. In contrast, neuraminidase action on plasma membrane fragments releases 0.028 μmoles of neuraminic acid per mg of protein and shifts the surface isoelectric point from pH 3.6 to 4.0.

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