The determination and localization of sialic acid in guinea-pig granulocytes

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When intact guinea-pig granulocytes (polymorphonuclear leucocytes) disrupted by sonication or with detergent were treated with neuraminidase from *Vibrio cholerae*, 3.1–3.2 nmol of sialic acid/10⁷ cells was released. By using a chromatographic procedure for the specific determination of total cell sialic acid, this releasable portion was found to constitute 70% of the total sialate. All of the neuraminidase-releasable sialic acid of the cells could be removed by enzymic treatment of intact cells with neuraminidase. It thus seemed likely that the neuraminidase-releasable sialic acid is all on the cell surface. To make sure that the result was not due to entry of neuraminidase into the cells, the enzyme was bound covalently to Sepharose 6B, and intact polymorphonuclear leucocytes were treated with the bound enzyme. All of the neuraminidase-releasable sialic acid could still be removed, though more slowly. The cells remained intact and only 1.5–2% of the bound enzyme was released from the Sepharose during incubation. Freed enzyme could have been responsible, at the very most, for release of 18% of the sialic acid. Fractionation studies showed that the nucleus and cytoplasm contain low amounts of sialic acid and that the neuraminidase-releasable sialic acid distributes in a manner similar to the distribution of 5'-nucleotidase, an unambiguous marker for the plasma membrane in these cells. Thus neuraminidase-releasable sialate constitutes a clear marker for the membrane of polymorphonuclear leucocytes. Most of the neuraminidase-insensitive sialate was present in the granule fraction. Removal of sialic acid from intact polymorphonuclear leucocytes did not affect their ecto-AMPase, -ATPase and -*p*-nitrophenyl phosphatase activities.

Removal of sialic acid from intact cells by treatment with neuraminidase is often taken as evidence that this substance is located on the outside of the plasma membrane. The assumption is that the large size of neuraminidase molecules prevents them from getting into intact cells, but this assumption may well be unwarranted, as detailed in the Discussion section. We have previously reported (Noseworthy *et al.*, 1972) that treatment of intact guinea-pig polymorphonuclear leucocytes with neuraminidase removes only 35–40% of the total cell sialic acid. Similar results were obtained by others (e.g. Tsan & McIntyre, 1976). However, the value for total sialic acid content obtained in these studies may have been overestimated because of the high background due to 2-deoxyribose, unsaturated lipids, and perhaps other substances.

We have investigated these uncertainties by

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measuring the total and neuraminidase-releasable sialic acid in whole cells with a number of different techniques to correct for interfering substances. We have found with these cells that a method combining chromatographic separation of sialate from interfering substances and dichromic measurement is essential (Svennerholm, 1963). We have also treated intact cells with neuraminidase covalently bound to Sepharose 6B beads to ensure non-penetration. The findings from these experiments were in agreement with those from experiments with unbound enzymes, and are further supported by determination of the distribution of neuraminidase-releasable sialic acid in subcellular fractions.

Materials and methods

Chemicals

Unless otherwise stipulated, all chemicals used were purchased from commercial sources and were of analytical grade. For chemicals of particular

importance, the source is given where the substance is mentioned for the first time.

Animals and cells

Guinea pigs of either sex (Charles River Breeding Laboratories Inc., Wilmington, MA, U.S.A.) weighing 500–1000 g were used. The animals were given chow pellets (containing vitamin C) and water *ad libitum*. The guinea pigs were injected intraperitoneally with 30 ml of a sterile solution of 12% (w/v) sodium caseinate (Difco Laboratories Inc., Detroit, MI, U.S.A.) in normal saline (0.9% NaCl) and 15–20 h later cells were harvested from the peritoneal cavity as described previously (DePierre & Karnovsky, 1974a). Cells were quantified either by counting or by protein determination. One guinea pig yielded $(5-12) \times 10^8$ cells, including 95% polymorphonuclear leucocytes, 2% monocytes and 2% lymphocytes (DePierre & Karnovsky, 1974a). Ten million cells contained about 0.7 mg of protein. These cells were in good condition as indicated by their appearance under the phase-contrast microscope, by the fact that more than 98% exclude Trypan Blue (Fallon *et al.*, 1962; Chodirker *et al.*, 1968; Geczy & Baumgarten, 1970) and by their ability to phagocytose with the expected metabolic concomitants (Karnovsky, 1962).

Monolayers and phagocytosis

Monolayers were formed and phagocytosis was measured by procedures developed previously in our laboratory (Michell *et al.*, 1969).

Homogenization and subfractionation of cells

Polymorphonuclear leucocytes were homogenized and the nuclear fraction was isolated as described previously (DePierre & Karnovsky, 1973). Further purification of the nuclei was based on a method developed by Chauveau *et al.* (1956) for the isolation of rat liver nuclei. Briefly, the nuclear pellet was re-suspended in 0.34 M-sucrose containing 3 mM-CaCl₂ and then re-sedimented by centrifugation at 1500 g for 15 min (all g values are g_{av}). The resulting pellet was re-suspended in 15 vol. of 2.4 M-sucrose/3 mM-CaCl₂ by gentle homogenization. This suspension was subsequently centrifuged in a 40 rotor in a Spinco model L ultracentrifuge at 45 000 g for 1 h. Finally, the supernatant fluid was removed by aspiration, the inside of the tube wiped carefully with a laboratory tissue and the pellet containing the purified nuclei was re-suspended in Krebs–Ringer phosphate (KRP) buffer and dialysed against KRP buffer to remove remaining sucrose.

The supernatant fluid from preparation of the original nuclear pellet (supernatant without nuclei) was treated in one of two ways. (a) It was

centrifuged at 25 000 g for 20 min in the fixed-angle rotor (catalogue number 870) in the International model B-20 centrifuge. The resulting pellet was designated 'granule fraction', and the supernatant 'supernatant without granules'. Both were dialysed against KRP buffer. (b) It was layered over a two-layer discontinuous sucrose gradient containing 8.5% sucrose in the upper layer, 45% sucrose in the lower layer, and 40 mM-NaCl/100 mM-KCl/5 mM-MgSO₄/20 mM-Tris/HCl, pH 7.6, throughout the gradient. After centrifugation at 90 000 g for 2 h in an SW 25.1 rotor, the interphase material (membrane) and pellet were collected and dialysed against KRP buffer.

Sonication when required was carried out on an MSE 100W ultrasonic disintegrator (Measuring and Scientific Equipment, London, U.K.) at a setting of 4–6 μ m. The sonication vessel was immersed in an ice/water bath and, unless otherwise indicated, samples were sonicated for 90 s.

Covalent linkage of neuraminidase

Attachment of neuraminidase to Sepharose beads was accomplished by a modification of the method of Cuatrecasas (Cuatrecasas *et al.*, 1968; Cuatrecasas, 1969; Bing, 1971; Royer & Green, 1971); 10 ml of the enzyme (from *Vibrio cholerae*; Behring Diagnostics, Inc.) was freeze-dried and re-suspended in 5 ml of 0.1 M-NaHCO₃, pH 8.1. Ten mg of bovine serum albumin (Sigma, A7638; crystallized and freeze-dried; essentially globulin-free) was added to this enzyme solution as 'carrier' protein. Packed Sepharose 6B (Pharmacia) (2.5 ml) was washed with several hundred ml of 0.1 M-NaHCO₃, pH 8.1, on a sintered-glass funnel. The Sepharose 6B was then re-suspended in 5 ml of the bicarbonate solution and titrated to pH 10.5–11.0 with 4 M-NaOH. CNBr (200 mg) in 3 ml of water was then added to the suspension of Sepharose at room temperature. This mixture was stirred constantly and maintained at pH 10.5–11.0 with drops of 4 M-NaOH. When the pH stopped falling (approx. 8 min after initiation of the reaction), 5 ml of ice was added to the reaction vessel. The contents were quickly poured on to a sintered-glass funnel and washed with 1 litre of 0.1 M-NaHCO₃, pH 8.1. The dry Sepharose was then scraped into the enzyme solution and this mixture was stirred at 4°C overnight. The Sepharose was finally washed with 1.5 litres of 0.1 M-NaHCO₃ and 1 litre of water on a sintered-glass funnel, re-suspended in KRP buffer, and stored at 4°C. Well over half of the enzyme activity became attached to Sepharose beads in an active form. Two observations suggest that this binding is covalent. First, if the preparation that had already been washed with 1000 vol. of KRP buffer and water was assayed, washed with an additional 2500 vol. of KRP buffer, and then re-assayed, the washing was found to cause

no detectable decrease in the activity associated with the beads. Secondly, if the CNBr activation step was eliminated, no detectable neuraminidase was associated with the beads.

Enzyme assays

5'-Nucleotide (AMPase), ATPase, p-nitrophenyl phosphatase and alkaline phosphatase. These were assayed by published procedures (DePierre & Karnovsky, 1974a; Michell *et al.*, 1970).

Neuraminidase activity. This was assayed by incubating the sample with 2 mM-*N*-acetylneuraminyl-lactose (Sigma, Grade I, from bovine colostrum, 95% pure) in KRP buffer at 37°C. The reaction was terminated by the addition of sufficient 0.4 M- or 2.0 M- H_2SO_4 to give a final concentration of 0.2 M. Free sialic acid was then determined (see below). This assay procedure was found to be linear with time and enzyme concentration under conditions where 25% or less of the substrate present was hydrolysed. The background due to non-enzymic hydrolysis of substrate was routinely determined and corrected for. One unit of neuraminidase is defined as the amount that hydrolyses 1 μmol of *N*-acetylneuraminyl-lactose/h at pH 7.4.

Chemical assays

Protein. This was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Sialic acid. This was assayed basically by the thiobarbituric acid method of Warren (1959). The standards used were a crystalline pure sample kindly provided by Dr. L. Svennerholm, and *N*-acetylneuraminic acid obtained from Sigma and designated 'type IV, synthetic crystalline, 95% pure'. Neuraminidase-susceptible sialic acid was determined on a portion after treatment of cells or fractions with the enzyme (for detailed conditions, see the legends to the appropriate Figures and Tables); total sialic acid was determined after hydrolysing the samples in 0.2 M- H_2SO_4 at 80°C for 1 h. Certain substances present in biological material, including 2-deoxyribose from DNA, and unsaturated lipids, interfere with the Warren assay (sucrose also interferes, but was removed from samples by dialysis before assay). A number of different procedures were used to correct for such interference.

(a) *A_{549} method.* In this case it was assumed that no substance other than sialic acid is contributing to the absorption at 549 nm and the amount of sialic acid present was calculated simply from Beer's law [μmol of sialic acid = $0.075 \times A_{549}$ (Warren, 1959)].

(b) *Minimal-interference method.* In this case it was assumed that only a small portion of the absorption at 549 nm comes from substances other than sialic acid, and the amount of sialic acid present

was calculated from the formula: sialic acid (μmol) = $(0.090 \times A_{549}) - (0.033 \times A_{532})$ (Warren, 1959). Rosenberg & Einstein (1972) have shown that this method corrects adequately up to 100 μg of DNA/5 nmol of sialate (see *d* below).

(c) *Large-interference method.* In this case it was assumed that a large portion of the absorption at 549 nm comes from substances other than sialic acid and the amount of sialic acid present was calculated from the formula: sialic acid (μmol) = $(0.138 \times A_{562}) - (0.009 \times A_{532})$ (Warren, 1959).

(d) *DNA-correction method.* The amount of DNA present in the sample was determined by the Burton procedure (Burton, 1956) with calf thymus DNA as a standard (Sigma type V, sodium salt, highly polymerized). Different amounts of calf thymus DNA were subjected to the Warren procedure and their apparent content of 'sialic acid' was calculated by using the formula applied in the minimal-interference method (Fig. 1). The apparent amount of sialic acid in a sample of polymorphonuclear leucocytes was calculated by using the formula, and the background due to measured DNA present was subtracted. This method would be useful in cases of contamination by DNA in excess of those that may be compensated for in method (b) above.

(e) *Chromatographic method.* Free sialic acid was separated from the sample by chromatography on the ion-exchanger Dowex 2 (X8) (Svennerholm, 1963) and sialic acid was subsequently determined

Fig. 1. Interference by DNA in the thiobarbituric acid assay for sialic acid

Various amounts of calf thymus DNA were subjected to the thiobarbituric acid assay for sialic acid and the apparent content of sialic acid was calculated by using the formula $(0.090 \times A_{549}) - (0.033 \times A_{532})$. Two experiments were performed. For further details, see the text.

by the Warren assay (minimal-interference method). In these experiments, to test the efficiency of the method, free sialic was applied to the ion-exchange columns, and recovery was $91 \pm 1\%$.

Results

Total and neuraminidase-releasable sialic acid in whole cells

Neuraminidase from *Vibrio cholerae* was used in the present study because commercial preparations of this enzyme free of proteinase and phospholipase C activities are available (Nordling & Mayhew, 1966). Furthermore, even though the *Vibrio cholerae* enzyme has a pH optimum at 5.6, it maintains 50% of its maximal activity at pH 7.4 (Ada *et al.*, 1961), the pH at which it seemed advisable to carry out treatment of intact cells with the enzyme. This enzyme requires 1 mM- Ca^{2+} for maximal activity (Ada *et al.*, 1961) and Krebs-Ringer phosphate buffer contains about 1.5 mM- Ca^{2+} .

Table 1 shows that three different procedures for determining the amount of sialic acid that can be released by treatment of intact polymorphonuclear leucocytes with neuraminidase give identical results. On the other hand, different procedures for determining total sialic acid give very different results. This is not really surprising, since the incubation in 0.2 M- H_2SO_4 required for the determination of total sialic acid might be expected to release large amounts of interfering substances. In our judgement chromatographic separation of sialic acid from most of the other components of the hydrolysate gives the most reliable values for total cell sialic acid. Comparative measurements of the releasable sialic acid again show that recovery by the chrom-

atographic method is excellent ($> 90\%$; see Table 1). On the basis of the results obtained with this method, it appears that 70% of the total sialic acid in guinea-pig polymorphonuclear leucocytes can be released by treatment of the intact cells with neuraminidase.

Localization of the neuraminidase-releasable sialic acid

It seemed possible that all of the neuraminidase-releasable sialic acid of polymorphonuclear leucocytes might be on the outside surface, and experiments were designed to test this possibility. First, removal of sialic acid from intact cells with neuraminidase (set at 100%) was compared with removal from cells disrupted by sonication or by 0.1% Triton X-100. Under the conditions of Table 1, the amounts were $99.0 \pm 11.3\%$ ($n = 4$) and $96.1 \pm 2.6\%$ ($n = 2$) respectively.

The time course of release from intact cells and sonicated material is shown in Fig. 2. No detailed time course for the removal of sialic acid from cells disrupted with 0.1% Triton X-100 was performed, but this release was found to be maximal after 60 min under the conditions described in the legend to Table 1. In no case was the termination of sialic acid removal due simply to inactivation of the added neuraminidase. This was demonstrated in two ways. First, after 90 min of incubation with intact or disrupted cells, the neuraminidase present in the medium was assayed directly and was found to have retained all of its original activity. Secondly, if a fresh portion of neuraminidase equal to the amount originally added was pipetted into the medium after 90 min of incubation, no further release of sialic acid occurred. In addition, it was determined that the concentration of sialic acid present in the medium at

Table 1. *Total and neuraminidase-releasable sialic acid in whole granulocytes*

For neuraminidase-releasable sialic acid, 10^8 intact cells were treated with 1.99 units (neuraminyl-lactose standard) of soluble neuraminidase. Before use, the soluble enzyme, which comes from the supplier in 0.06 M-acetate buffer, pH 5.0, was either dialysed against KRP buffer, or freeze-dried and resuspended in KRP buffer. Incubation was carried out at 37°C for 90 min in a total volume of 1.0 ml of Krebs-Ringer phosphate buffer. Control tubes without neuraminidase were also incubated, and all samples were shaken throughout the incubation period. At the end of the incubation period, enough KRP buffer was added to each tube to bring the total volume of the contents to 6 ml, and the tubes were spun at 100g for 10 min. The supernatants were removed, dried down under a stream of N_2 at 40°C, and assayed for free sialic acid. Values are means $\pm$ s.d. for the numbers of determinations given in parentheses. For details of assay procedures, see the Materials and methods section.

Assay procedure	Total sialic acid (nmol/ 10^7 cells)	Neuraminidase-releasable sialic acid (nmol/ 10^7 cells)
(a) No correction applied (A_{549})	—	3.25 ± 0.44 (6)
(b) Minimal interference	12.22 ± 3.38 (9)	3.22 ± 1.54 (9)
(c) Large interference	7.00 ± 1.51 (5)	—
(d) DNA correction	8.37 ± 1.34 (5)	—
(e) Chromatography	4.42 ± 1.14 (3)	3.09 ± 0.08 (3)

the end of 90 min of incubation had no effect on neuraminidase activity. Sialic acid is known to inhibit *Vibrio cholerae* neuraminidase, but only at rather high concentrations (10 mM inhibits by 40% at pH 7.4; Holmquist, 1969).

Thus soluble neuraminidase releases as much sialic acid from intact cells as from disrupted cells. In order to conclude from this observation that all of the neuraminidase-susceptible sialic acid of guinea-pig polymorphonuclear leucocytes is on the surface, it must be assumed that neuraminidase is not getting into the cells. Unfortunately, this assumption may not be valid (see the Discussion section).

However, neuraminidase covalently bound to Sepharose beads removes as much sialic acid from intact cells as does the soluble enzyme. Fig. 2 illustrates that the time course of release with the bead-bound enzyme was much slower. This may be due to the fact that the amount of enzyme bound to the surface of the beads and available for interaction with intact cells may be only a fraction of the total neuraminidase bound. In addition, it is almost certainly more difficult for the bound enzyme molecules to make intimate contact with a cell surface to remove exposed sialic acid residues than it is for the soluble enzyme.

Fig. 2. Time course for removal of sialic acid from intact granulocytes by neuraminidase

Conditions for the soluble enzyme were as described in the legend to Table 1. Those for the Sepharose-bound enzyme, washed on a Millipore filter with 100 vol. of KRP buffer immediately before use, were: 1.63 (neuraminyllactose method) units of bound enzyme per 10^8 cells, at 37°C in 0.5 ml of KRP buffer. Control tubes with cells and an equivalent amount of untreated Sepharose beads were subjected to the same treatment without any observable release of sialate. All samples were shaken vigorously throughout the incubation. Symbols: □, intact cells, soluble neuraminidase; ▲, sonicated material, soluble neuraminidase; ●, intact cells, Sepharose-bound neuraminidase.

Two important series of controls were needed before it could be concluded from these results that all of the neuraminidase-susceptible sialic acid is on the surface of these cells. First, the cells must be shown to remain intact throughout the incubation period (3 h; the cells are shaken with large beads the whole time). Phase-contrast microscopy suggested that less than 3% of the cells were sufficiently disrupted for the Sepharose beads (40–210 μm) to make contact with intracellular organelles. Further, it was found that after the incubation, 87% of the cells still excluded Trypan Blue and 83% of their catalase (a soluble cytoplasmic enzyme in these cells) could still be pelleted with the cells. In addition, after the incubation period only about 1.5% of the total alkaline phosphatase was found in the medium. This enzyme is contained in cytoplasmic granules in the polymorphonuclear leucocytes, and it was reasoned that if the cells were disrupted enough to allow large Sepharose beads to get in, then they would be disrupted enough for alkaline phosphatase granules to get out. The possibility remained that even though the cells were not breaking up enough for large beads to make contact with intracellular organelles, they were becoming leaky enough for some form of bound sialic acid to diffuse out into the medium to be attacked by the bound neuraminidase. To check for this possibility cells were shaken at 37°C for 3 h with Sepharose beads having no attached neuraminidase. After this period, the cells and beads were spun down, and the resulting supernatant was assayed for total sialic acid; none was detected.

The second set of controls that was necessary was a demonstration that neuraminidase was not getting into the cells. It seems impossible that the beads could have been phagocytosed by the cells; no evidence for this was seen in the phase-contrast microscopy, where the smallest bead ever seen was at least two times the size of a polymorphonuclear leucocyte. On the other hand, it was discovered that after incubation 1.5–2.0% of the added bound neuraminidase could no longer be sedimented with the beads or removed from the solution by Millipore filtration. This release is not prevented by copious washing of the beads before use; and it is not clear why this small amount of the enzyme comes off the beads. When an amount of soluble enzyme equal to 1.5% of the amount of bound neuraminidase normally used was incubated with intact cells for 3 h (cf. Fig. 2), 18% of the neuraminidase-susceptible sialic acid was removed. It seems a reasonable assumption that susceptible sialic acid residues on the surface of the cells are at least as accessible to this soluble neuraminidase as are similar residues inside the cells. In the control experiment activity corresponding to 1.5% of the total bound enzyme used was present throughout the 3 h incubation,

Table 2. *Total and neuraminidase-releasable sialic acid in subcellular fractions*

For further details of the fractions see the Materials and methods section. Total sialic acid was determined by the chromatographic method for the nuclear fraction and by the minimal-interference method for the other fractions. For determination of neuraminidase-releasable sialic acid, enzyme treatment was carried out as described in the legend to Table 1, replacing 10^8 intact cells with each fraction from an equivalent number of cells. The values are means $\pm$ S.D. for the numbers of determinations given in parentheses.

Fraction	Total sialic acid (nmol/ 10^7 cells)	Neuraminidase-releasable sialic acid (nmol/ 10^7 cells)
Nuclear fraction	0.38	0.35
Granule fraction	3.75 ± 0.93 (10)	2.26 ± 0.43 (10)
Supernatant	0.74 ± 0.18 (10)	0.45 ± 0.31 (10)
Total	4.87	3.06

Table 3. *Comparison of the distribution of total and neuraminidase-releasable sialic acid and AMPase in subcellular fraction*

For further details of the fractions see the Materials and methods section. Values are percentages of the total recoveries of sialic acid and AMPase. Enzyme treatment and sialic acid determination were carried out as indicated in the legend to Table 2, with the fractions other than the nuclear fraction being handled in the same manner as the granule fraction and supernatant of Table 2. AMPase was assayed as described in the Materials and methods section. It should be noted that the cleanest membrane fraction and the pellet fraction (the granule fraction containing also the bulk of the plasma-membrane) are subfractions of supernatant fraction without nuclei.

Fraction	Sialic acid (%)		AMPase (%)
	Total	Releasable	
Nuclear fraction	8.6	11.4	9.0
Supernatant without nuclei	91.4	88.6	91.0
Membrane	19.8	28.1	31.9
Pellet	69.5	55.6	59.1

whereas when cells were treated with bound neuraminidase, the enzyme was released gradually from the beads to reach a final level of 1.5–2% at the end of the incubation period (3 h). The conclusion we draw from these results is that 82–100% of the neuraminidase-susceptible sialic acid of guinea-pig polymorphonuclear leucocytes is on their surface; the lower limit given is the most conservative estimate.

Subcellular distribution of total and neuraminidase-releasable sialic acid

When the polymorphonuclear leucocytes were fractionated into nuclear, granule and supernatant fractions, the distribution of total and neuraminidase-releasable sialic acid shown in Table 2 was

found. The nuclear fraction, which contains 97% of the total cell DNA and about 10% of the plasma membrane (DePierre & Karnovsky, 1973), contained about 10% of both total and releasable sialic acid, whereas the highest amounts of sialate were recovered in the granule fraction, which contains most of the plasma membrane, judging from AMPase titres. The recovery of total sialic acid was 110% of that found in whole cells, whereas the recovery of neuraminidase-susceptible sialic acid was 99% (cf. Table 1).

Table 3 reports somewhat more detailed sub-fractionation of the polymorphonuclear leucocytes. The distribution of neuraminidase-releasable sialic acid closely follows that of AMPase, which is exclusively a marker for the plasma membrane in these cells (DePierre & Karnovsky, 1974*a,b,c*). The distributions of total sialic acid and AMPase differ somewhat more. The former observation supports the conclusion that almost all of the neuraminidase-releasable sialic acid of these cells is on the plasma membrane.

Finally, in experiments in which the cleanest membrane fraction was specifically examined, 93% of the total sialate was neuraminidase-releasable. In the granule (pellet) fraction, which was heavily contaminated with membrane, the value was 53%. The bulk of neuraminidase-insensitive sialate is thus in the granule fraction.

Neuraminidase-releasable sialic acid and ecto-enzymes

It has been demonstrated in this laboratory (DePierre & Karnovsky, 1974*a*) that guinea-pig polymorphonuclear leucocytes have at least three ecto-enzymes, i.e. enzymes localized on the plasma membrane with their active sites facing the extracellular medium. Removal of sialic acid from intact cells with neuraminidase has no marked effect on the ecto-AMPase, -ATPase and -*p*-nitrophenyl phosphatase, the values being 123%, 111% and 88% of the control respectively, when the cells were maxi-

mally desialated. It has also been found previously in this laboratory (Noseworthy *et al.*, 1972) that removal of sialic acid from the cells does not affect phagocytosis.

Discussion

Several studies concerning the localization of sialic acid in mammalian cells have been reported. Eylar and his co-workers (Eylar *et al.*, 1962) demonstrated that neuraminidase removes 95–100% of the sialic acid found in chicken, pig, lamb, calf and human erythrocytes when the intact cells are treated with this enzyme. Similar treatment of Ehrlich ascites-carcinoma cells released 73% of the total sialic acid (Wallach & Eylar, 1961). Rosenberg & Einstein (1972) noted that *Clostridium perfringens* neuraminidase released 61–92% of total sialic acid of human lymphoid cell lines in culture. Trypsin may also be used to release sialopeptides from intact cells (Cook *et al.*, 1960; Mäkelä *et al.*, 1960; Eylar & Madoff, 1962; Seaman & Uhlenbruck, 1963; Winzler *et al.*, 1967; Shen & Ginsburg, 1968; Pepper & Jamieson, 1969; Weis & Narahara, 1969).

We have found in the present study that treatment of intact guinea-pig polymorphonuclear leucocytes with neuraminidase removes as much sialic acid as does treatment of disrupted cells. The methods used to disrupt the cells were drastic; in the presence of detergent, at least, there should be no remaining permeability barriers to the enzyme. Thus, it is tempting to conclude that all of the neuraminidase-releasable sialic acid of these cells is on the outside surface. Results from a chromatographic procedure for determining total cell sialic acid suggest that the neuraminidase-releasable portion represents 70% of the total sialic acid.

However, some evidence for the penetration of neuraminidase into intact cells has been reported. Wallach & Eylar (1961) stated that neuraminidase releases more sialic acid from intact Ehrlich ascites-carcinoma cells than is actually present on the plasma membrane; they reported that one-third to one-half of the sialic acid present in nuclei, mitochondria and the soluble cytoplasm is also released by such treatment. A similar finding was made by Glick and her co-workers (Glick *et al.*, 1970) for treatment of intact mouse fibroblasts with neuraminidase. The most detailed study along these lines has been carried out by Nordling & Mayhew (1966). By using a variety of cell types, including human sarcoma and Ehrlich ascites carcinoma cells, these investigators found that when fluorescent-labelled neuraminidase was added to the medium, 15–20% of the enzyme was taken up by the cells in 30 min. This uptake did not seem to be due to damaged cells. The nuclei and nucleoli were particularly fluorescent.

Furthermore, Nordling & Mayhew (1966) found that the electrophoretic mobility of isolated nuclei treated directly with neuraminidase was greatly decreased and that the electrophoretic mobility of nuclei isolated from intact cells that had been treated with the enzyme was similarly decreased. Because of these observations, it is not possible to come to a conclusion regarding the proportion of neuraminidase-releasable sialate that is on the external surface of the cell.

We have attached neuraminidase to a solid support by converting hydroxy groups on beads of Sepharose 6B into reactive imidocarbonate groups by using CNBr and subsequently allowing these imidocarbonate groups to react with free amino groups on the enzyme molecules. That this procedure was successful indicates that *Vibrio cholerae* neuraminidase has at least one free amino acid group that is not essential for activity. When intact polymorphonuclear leucocytes were treated with the bound enzyme, as much sialic acid was released as by treatment with the soluble enzyme. A small amount (1.5–2%) of the enzyme is released from the beads during the 3 h incubation period, perhaps as a result of the activity of some enzyme on the surface of the cells. Consequently, we could only conclude, perhaps over-conservatively, that 82–100% of the neuraminidase-releasable sialic acid of these cells is on the outside surface.

Subsequently, we subfractionated polymorphonuclear leucocytes to determine the distribution of neuraminidase-releasable and total sialic acid. Nuclei and cytoplasm contained relatively little sialic acid. The neuraminidase-releasable sialic acid was distributed in much the same manner as AMPase, a specific marker for the plasma membrane in these cells (DePierre & Karnovsky, 1974*a,b,c*). This is also consistent with a localization of this sialic acid on the outside surface of the cell. The 30% of the total sialic acid that is not releasable by neuraminidase had a somewhat different distribution and appeared in the granule fraction.

Polymorphonuclear leucocytes are perhaps a special case, i.e. not all cells necessarily have all their sialidase-releasable sialate on the membrane, and consequently would not yield the same results whether exposed to free sialidase or to immobilized enzyme.

The localization of neuraminidase-releasable sialic acid on the surface of guinea-pig polymorphonuclear leucocytes may make it possible to use this sialic acid as a specific marker for the plasma membrane. For example, during maximal phagocytosis by guinea-pig polymorphonuclear leucocytes 20% of the neuraminidase-releasable sialate was internalized (J. E. Smolen, unpublished work), indicating an internalization of one-fifth of the plasmalemma as phagosomes. This value compares

very well with observations with other markers (Smolen & Karnovsky, 1980).

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