# Sialic Acid Uptake by Fibroblasts†

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ABSTRACT: The existence of surface sialyltransferases that use cytidine monophosphate (CMP)-sialic acid as substrate has been postulated in previous studies. This is based on the assumption that if whole, viable cells can catalyze the transfer of sialic acid from CMP-sialic acid to endogenous acceptors, then the transferases carrying out the reaction must be on the cell surface, provided that (1) CMP-sialic acid does not enter the cells, and (2) CMP-sialic acid does not break down outside the cells, yielding free sialic acid which then may enter the cells, in amounts large enough to explain the incorporation. We now report evidence showing that after incubation of intact NIL, BHK, and 3T3 fibroblasts with CMP-sialic acid, at least 78% of the sialic acid incorporated by these cells is the result of free sialic acid uptake. When cells growing in a monolayer were incubated with a mixture of CMP-[$^{14}$C]sialic acid and [$^{3}$H]CMP-sialic acid with a ratio of $^{3}H/^{14}C = 0.60$, this ratio was found to be markedly increased in whole cells. Chemical analyses of the radioactive species in the incubation medium showed that a considerable portion of the radiolabeled sugar nucleotide had broken down to cytidine, phosphoric acid, and sialic acid. Upon incubation of cells with doubly labeled sugar nucleotide in the presence of a large excess of both nonradiolabeled cytidine and sialic acid, the cells incorporated less than 6% of both isotopes. Incubation of cells with a mixture of CMP-[$^{14}$C]sialic acid and [$^{3}$H]sialic acid resulted in only 20–40% of the radioactivity within the cells being membrane bound, and 70–90% of this incorporation could be inhibited by the addition of 10 mM azide to the incubation medium. The possibility that a small fraction of the total incorporation of sialic acid by these cells is due to surface sialyltransferases cannot be completely ruled out. The uptake of free sialic acid by these fibroblasts is concentration dependent and a portion of it is incorporated into glycoproteins and glycolipids. Considerable loss of cell integrity was observed when fibroblasts grown on plates were removed by (ethylenedinitrilo)-tetraacetic acid or trypsin and subsequently incubated in buffer, indicating that these preparations are not suitable for intact cell studies.

Sialic acid is found in fibroblasts predominantly, if not solely, in the plasma membrane. Within this membrane it occurs as the terminal sugar moiety of glycoproteins and glycolipids rather than in free form. Several studies have demonstrated differences in the total sialic acid content of whole normal and transformed cells (Ohta et al., 1968; Grimes, 1970, 1973). Differences have also been reported for the sialo glycoproteins and sialo glycolipids of normal and transformed cells (Hakomori et al., 1968; Mora et al., 1969; Sakiyama et al., 1972; Warren et al., 1972, 1973).

The site(s) within the cell where the addition of sialic acid to glycoproteins and glycolipids occurs is not known, although in vitro experiments have pointed towards the Golgi as being at least one of them (Roseman, 1970). It has also been suggested that sialytransferases exist on the cell surface; several studies have suggested that the difference in the sialic acid content between normal and transformed cells is caused, at least in part, by differences in these surface sialyltransferases (Bosmann, 1972; Patt and Grimes, 1974, 1975; Datta, 1974; Sasaki and Robbins, 1974).

Previous studies have shown that in the lcl clone of NIL cells (a line of hamster fibroblasts) the sialic acid containing glycolipid "hematoside", the principal glycolipid of this cell line, increases two- to threefold in amount when sparse cells become confluent (Sakiyama et al., 1972; Hirschberg et al., 1975). Since such an increase could be related to the presence of surface sialyltransferases in these cells, we decided to investigate whether these enzymes could be demonstrated in NIL lcl cells.

The existence of surface sialyltransferases has been previously postulated on the basis of the following rationale: if whole viable cells can catalyze the transfer of sialic acid from exogenous CMP-sialic acid to endogenous acceptors, then the transferases carrying out this reaction must be on the cell surface. This interpretation requires that (1) CMP[1]-sialic acid does not enter the cell and (2) CMP-sialic acid does not break down outside the cell to free sialic acid which may then enter cells, in amounts large enough to explain the incorporation. It is also most important to rule out that as a consequence of cell damage some cytoplasmic sialyltransferases may be present in the incubation medium.

We now report evidence demonstrating that at least 78% of the sialic acid incorporation from CMP-sialic acid, which had been previously attributed to surface sialyltransferases, is due to the uptake by these cells of free sialic acid resulting from chemical and enzymatic breakdown of CMP-sialic acid in the incubation medium; we cannot completely rule out the possibility of a small amount of surface sialyltransferase activity. The uptake of free sialic acid by NIL, BHK, and 3T3 fibroblasts shows a linear concentration dependency up to approximately 5 mM. Once inside, some of the sialic acid is incorporated into glycoproteins and glycolipids.

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[1] Abbreviations used are: CMP-sialic acid, cytidine monophosphate-sialic acid; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; FCS, fetal calf serum; MEM, minimal essential medium; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

## Materials and Methods

*Cells.* NIL lcl cells are a clone of hamster fibroblasts isolated by Sakiyama et al. (1972). BHK 21/13 and 3T3 cells were gifts of Drs. G. Eliceiri and M. Green (St. Louis University). The cells were grown in Dulbecco's modification of Eagle's minimal essential medium containing in addition four times the normal concentration of amino acids and vitamins (Sakiyama et al., 1972). Cells were counted in a Celloscope particle counter. Cells were free of mycoplasma based on their level of uridine phosphorylase activity (Levine, 1972).

*Buffers.* The principal buffers used were Tris (tris(hydroxymethyl)aminomethane) buffered saline (0.15 M NaCl and 20 mM Tris-HCl, pH 7.4), Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffered saline (0.15 M NaCl and 20 mM Hepes, pH 7.4), PBS (0.15 M NaCl and 20 mM phosphate buffer, pH 7.4), and solution A (0.8% NaCl, 0.05% KCl, 0.001 M $KPO_4$, pH 7.4).

*Radiolabeled Compounds.* All radiolabeled compounds with the exception of [$^3$H]sialic acid and [$^3$H]CMP-sialic acid were purchased from New England Nuclear. CMP-sialic acid ([4,5,6,7,8,9-$^{14}$C]sialic acid; 217 mCi/mmol used in all cases except Table VII, experiment 2); CMP-sialic acid ([4-$^{14}$C]-sialic acid; 0.9 mCi/mmol); $Na_2[^{51}Cr]O_4$ (367 Ci/$\mu$g of chromium); [5-$^3$H]CTP (26 Ci/mmol); [G-$^3$H]*N*-acetyl-D-mannosamine (10 Ci/mmol).

*Liquid Scintillation Spectrometry.* All radiolabeled liquid samples (with the exception of those containing $^{51}$Cr) were counted in a Packard Liquid Scintillation spectrometer. Samples in water or NaOH were counted in 90% Aquasol (New England Nuclear). Protein pellets were dissolved in Protosol (New England Nuclear) and counted in toluene-Omnifluor (New England Nuclear).

*Nonradiolabeled Compounds.* Sialic acid (Type III from egg and from *E. coli*), phosphoenolpyruvate and CTP were purchased from Sigma Chemical Co.

*Preparation of [$^3$H]CMP-Sialic Acid.* The radiolabeled sugar nucleotide was prepared by an enzymatic condensation of [5-$^3$H]CTP with sialic acid. The partially purified CMP-sialic acid synthetase was obtained from hog submaxillary glands by the procedure previously described by Kean and Roseman (1966). The protein fraction that was eluted from the DEAE-cellulose column with 0.075 M KCl was used as enzyme. The reaction mixture contained [5-$^3$H]CTP (10 nmol), CTP (90 nmol), sialic acid (300 nmol) in 0.5 M Tris-acetate buffer, pH 8.4 (20 $\mu$l) containing 0.2 M magnesium acetate and 100 $\mu$l of enzyme (0.4 mg of protein/ml). After incubation in a rotatory bath at 37 °C for 2 h, an additional 100 $\mu$l of enzyme was added for 60 min. The reaction was stopped by the addition of 140 $\mu$l of acetone. After centrifugation in a clinical centrifuge, the supernatant was removed and applied to Whatman 3MM paper for ascending preparative chromatography in a solvent system of ethanol–ammonium acetate (1 M, pH 7.4) 70:30. [$^3$H]CMP-sialic acid was visualized by its ultraviolet absorption and by scanning with a Packard radiochromatogram scanner. The region of the paper where the sugar nucleotide migrated was cut out, eluted with water, and the sample was lyophilized. Upon chromatography on paper the material showed one uv positive spot in the region where authentic CMP-sialic acid migrated. Over 99% of the radioactivity was in this region. The overall yield of the enzymatic synthesis was 8%. Nonradiolabeled CMP-sialic acid was obtained by the same procedure. The final product had a molar ratio of CMP/sialic acid of 1/1.1.

*Preparation of [$^3$H]Sialic Acid. N-*[$^3$H]Acetylneuraminic acid was synthesized enzymatically from [G-$^3$H]*N*-acetyl-D-mannosamine and phosphoenolpyruvate as previously described by Warren and Glick (1966). The specific activity was 2.3 Ci/mmol. Radiochemical purity was at least 99.5% based on behavior on Dowex-formate column chromatography and the following paper chromatography systems: ethanol–ammonium acetate (1 M, pH 7.5, 6:4), butanol–pyridine–water (6:4:3), and pyridine–ethyl acetate–acetic acid–$H_2O$ (5:5:1:3).

TABLE I: Effect of Buffers on Cellular Growth Rates.[a]

| | Cells/Plate |
|---|---|
| Prior to incubation | $3.4 \times 10^5$ |
| After 2-h incubation with Hepes buffer | $3.6 \times 10^5$ |
| After 2-h incubation with TBS | $3.9 \times 10^5$ |
| After 2-h incubation with growth medium | $4.4 \times 10^5$ |
| After 2-h incubation with Hepes buffer + 22 h growth medium | $11.6 \times 10^5$ |
| After 2-h incubation with TBS + 22 h growth medium | $11.3 \times 10^5$ |
| After 24-h incubation with growth medium without incubation with buffer | $12.7 \times 10^5$ |

[a] NIL cells were grown in MEM + 10% FCS (3 ml) on 35-mm Falcon plates. After removal of the growth medium, the plates were rinsed with 2 ml of the appropriate buffer, and then incubated in 0.6 ml of buffer in a tissue culture incubator at 37 °C. After 2 h, the buffer was removed and fresh growth medium was added.

## Results

*Viability and Leakiness of Cells during Incubation.* An important requirement for proof of the existence of surface sialyltransferases, as outlined in the Introduction, is for the reaction to be catalyzed by whole intact viable cells, rather than leaky cells or cell fragments, thereby ruling out the presence of cytoplasmic sialyltransferases. Previous studies on these enzymes were done with two rather different cell preparations: (1) cells grown on a plate which were either removed with EDTA, trypsin, or scraping and then suspended in buffer (Bosmann, 1972; Patt and Grimes, 1974, 1975; Sasaki and Robbins, 1974) or (2) cells still attached to the plate (Datta, 1974). However, since in most of these studies cell viability and cell leakiness were not monitored, we decided to investigate these parameters in these two cell preparations. Table I shows that incubating NIL lcl cells on the plate with Hepes saline buffer, pH 7.4, for 2 h did not cause cell detachment nor loss of viability. During this period the cells grew more slowly than in growth medium, but regained their normal rate of growth, *without apparent lag,* upon removal of the buffer and readdition of growth medium. Very similar results were obtained with Tris-buffered saline, pH 7.4. Microscopic examination also indicated no detachment of cells during 2-h incubations with buffer.

The viability of NIL lcl cells that had been removed from the plate with EDTA and suspended for 1 h in TBS is shown in Table II. Only 20% of these cells had reattached to the plate 4 h after removal of the buffer and resuspension in growth medium; in contrast, cells that had been trypsinized, centrifuged, and resuspended in growth medium without a 1-h incubation with buffer, showed 80% attachment at the same time. After 24 h, these cells had tripled in number (consistent with a doubling time of approximately 16 h (Hirschberg et al., 1975), while the EDTA-treated cells had increased by only 20%. Numerous cell fragments could be observed under microscopic examination of these preparations. A similar effect,

TABLE II: Viability of NIL and 3T3 Cells after Removal from the Culture Plate with EDTA and Subsequent Incubations with Buffer.[a]

| Removed from Plate with | | Incubated | NIL Cells × $10^{-5}$/Plate | | | 3T3 Cells × $10^{-3}$/Plate | | |
|---|---|---|---|---|---|---|---|---|
| Trypsin | EDTA | with Buffer | 0 h | 4 h | 24 h | 0 h | 4 h | 24 h |
| − | − | − | 6.3 ± 0.3 | | | 92 ± 5 | | |
| + | − | − | | 5.5 ± 0.1 | 14.0 ± 0.1 | | 57 ± 1 | 87 ± 17 |
| − | + | + | | 1.4 ± 0.2 | 1.8 ± 0.1 | | 5 ± 1 | 6 ± 2 |

[a] NIL and 3T3 cells grown in 35-mm dishes were trypsinized for 10 min with 0.1% trypsin in solution A (1 ml). After 5-min centrifugation at medium speed in a clinical centrifuge, the supernatant was removed and the cells were suspended in 3 ml of growth medium and replated. Other plates were rinsed with solution A containing 1 mM EDTA (1.5 ml) and incubated for 10 min with solution A containing 10 mM EDTA (1 ml) in a cell incubator at 37 °C. The cells were then transferred to a conical tube, the plate rinsed with solution A containing 1 mM EDTA (1 ml) and the combined suspension was centrifuged for 5 min at medium speed in a clinical centrifuge. The supernatant was removed and the pellet was suspended in 1 ml of TBS, pH 7.5, and recentrifuged. The supernatant was removed and the pellet was resuspended in 0.5 ml of TBS, pH 7.5, and the suspension was incubated for 60 min. at 37 °C. At that time the suspension was centrifuged as before, the supernatant was removed, and the cells were resuspended in 3 ml of growth medium and transferred to a culture plate.

TABLE III: Leakage of $^{51}Cr$ by NIL lcl Cells Attached to Plates.[a]

| Incubation Medium | Cells/Plate | | cpm in Medium | | cpm in Cells |
|---|---|---|---|---|---|
| | Before Incubation | After Incubation | 0 min | 120 min | 120 min |
| MEM + FCS | $1.40 \times 10^6$ | $1.70 \times 10^6$ | 80 | 2890 | 25 410 |
| Hepes buffer | $1.40 \times 10^6$ | $1.72 \times 10^6$ | 130 | 2470 | 26 370 |
| TBS | $1.40 \times 10^6$ | $1.68 \times 10^6$ | 190 | 2050 | 25 180 |

[a] Cells were incubated in 35-mm plates for 2 h with $Na_2[^{51}Cr]O_4$ (7.5 μCi) in 3 ml of MEM. After removal of the radioactive medium they were washed seven times with nonradioactive MEM and once with 3 ml of buffer or MEM. We determined that after five washes with MEM the radioactivity remaining in the medium was constant. Then 0.6 ml of buffer or MEM was added for 0 min or 2 h and the cells were incubated in a tissue culture incubator at 37 °C. The radioactivity was determined in the medium and in the cells. For this the cells were dissolved in 0.5 N NaOH. Radioactivity was determined in a γ-counter. Cell number was determined in a parallel experiment.

although more drastic, was observed with 3T3 cells (Table II).

Leakage by cells of macromolecules during these treatments was monitored by measuring the release of $^{51}Cr$ into buffer by cells that had previously taken up $^{51}Cr$ from the growth medium. Table III shows that approximately 10% of the cellular $^{51}Cr$ was released when cells on a plate were incubated for 2 h with growth medium. A similar value was obtained when Tris-buffered saline was used instead of growth medium, suggesting that incubation of cells on the plate with buffer did not increase their permeability to macromolecules. When identically labeled NIL lcl cells were instead removed from the plate by EDTA and suspended in Hepes-buffered saline, 40% of $^{51}Cr$ was released in 60 min (Table IV). A similar increase in the release of $^{51}Cr$ after removal of the cells with EDTA and subsequent incubation with buffer was observed with both 3T3 and BHK cells (Table IV). An increase of cell permeability to macromolecules was also observed when the cells were removed from the plate with trypsin and subsequently incubated (while in suspension) with buffer (Table IV). On the basis of the results described in Tables I–IV, we proceeded in subsequent studies to do our incubations (with buffer) with cells on the plate.

*Are Cells Permeable to CMP-Sialic Acid and Is There CMP-Sialic Acid Breakdown?* Another important requirement for proving the existence of surface sialyltransferases is to show that cells are not permeable to CMP-sialic acid. To determine whether this was true we incubated NIL cells attached to the tissue culture plate with a mixture of [$^3H$]-CMP-sialic acid and CMP-[$^{14}C$]sialic acid. The initial incubation solution had a $^3H/^{14}C$ ratio of 0.60. After 90 min the buffer containing the radiolabeled CMP-sialic acid was removed, the cells were washed, and the amount of each isotope associated with the cells was determined. A ratio of the two isotopes within the cells equal to that in the incubation buffer would have been suggestive of CMP-sialic acid uptake by the cells, while a decrease in the $^3H/^{14}C$ ratio (over that in the incubation buffer) would have been consistent with surface transfer of [$^{14}C$]sialic acid. It was found that the ratio of the two isotopes inside the cells had greatly increased to 35.0, when compared to the incubation buffer (Table V). A similar large increase in the ratio of the two isotopes between the inside and outside of cells was found with 3T3 and BHK cells (Table V). These results suggested breakdown of CMP-sialic acid and subsequent entry of the breakdown products with different rates of transport. Uptake of CMP-sialic acid could have also occurred, but must have been very low in comparison to the uptake of free nucleoside and sugar. In addition, some differential loss of radioactivity from the cells due to inside-outside transport or to experimental workup cannot be completely ruled out, although we have no evidence for this.

Direct proof for exogenous breakdown of CMP-sialic acid was obtained by incubating CMP-[$^{14}C$]sialic acid in Hepes buffer (without cells) for 2 h at 37 °C. Approximately 10% of the radiolabel was recovered as [$^{14}C$]sialic acid rather than CMP-[$^{14}C$]sialic acid (based on paper chromatography). When [$^3H$]CMP-sialic acid was incubated with buffer approximately 10% of the radiolabel was recovered as [$^3H$]CMP. This nonenzymatic cleavage of CMP-sialic acid was also observed with TBS and PBS (Figure 1), the same buffers used in some other studies on surface sialyltransferases (Patt and Grimes, 1974; Datta, 1974; Sasaki and Robbins, 1974). When [$^3H$]CMP-sialic acid was incubated for 2 h with Hepes buffer in the presence of NIL cells, 12% of the radiolabel was recov-

TABLE IV: Leakage of $^{51}Cr$ by Cells after Removal from the Plate with EDTA or Trypsin and Subsequent Incubation with Buffer.[a]

| | NIL (cpm) | | 3T3 (cpm) | | BHK (cpm) | |
|---|---|---|---|---|---|---|
| | Plate | Suspension after EDTA | Plate | Suspension after EDTA | Plate | Suspension after EDTA |
| Plate supernatant | 7 500 | | 1 600 | | 2 370 | |
| EDTA supernatant | | 6 000 | | 4 500 | | 5 790 |
| TBS wash | | 7 300 | | 6 400 | | 9 300 |
| TBS supernatant | | 23 200 | | 4 100 | | 11 600 |
| Cell pellet or cells on plate | 88 500 | 54 300 | 22 700 | 10 500 | 52 500 | 43 400 |
| % leakage | 8 | 40 | 7 | 59 | 4 | 38 |

| | NIL Cells (cpm) | | BHK Cells (cpm) | |
|---|---|---|---|---|
| | Plate | Suspension after Trypsin | Plate | Suspension after Trypsin |
| Radioactivity released | 4 900 | 58 500 | 1 470 | 17 600 |
| Radioactivity remaining on plate/in pellet | 121 800 | 50 300 | 28 900 | 12 400 |
| % leakage | 4 | 54 | 5 | 59 |

[a] In the EDTA-treated cell suspensions, NIL cells ($2.6 \times 10^6$/plate), 3T3 cells ($1.5 \times 10^6$/plate), and BHK cells ($2.3 \times 10^6$/plate) were incubated in 35-mm plates for 2 h with $Na[^{51}Cr]O_4$ (12 $\mu$Ci) in 1 ml of MEM. The incubation medium was then removed and the cells were washed seven times with 1.5-ml aliquots of MEM. One plate (for cells in suspension) was then washed with solution A containing 1 mM EDTA and the cells were removed with solution A containing 10 mM EDTA as described in Table II. The combined EDTA supernatant was called EDTA supernatant. The cell pellet was washed with TBS as described in Table II and the supernatant obtained was called TBS wash. Finally, the cells were incubated for 1 h at 37 °C in 0.5 ml of TBS. Following centrifugation, TBS supernatant was obtained. The radioactivity in the pellet was determined as described in Table III. Leakiness of cells on the plate was determined by incubating them with 1 ml of TBS for 1 h at 37 °C. At that time the buffer was removed (plate supernatant) and the radioactivity in the buffer and the cells was determined as described in Table III. In the trypsin-treated cell suspensions NIL cells ($6.2 \times 10^6$/plate) were incubated in 50-mm plates for 2 h with $Na[^{51}Cr]O_4$ (20 $\mu$Ci) in 5 ml of MEM. The medium was removed and the cells were washed seven times with nonradioactive MEM (1.5 ml each time) and once with 3 ml of solution A. To one plate 1.5 ml of Hepes saline buffer, pH 7.4, was added and the cells were incubated for 1 h at 37 °C in a tissue culture incubator. At that time the radioactivity was determined in the medium and the cells as described in Table III. To the other plate 1.5 ml of 0.1% trypsin in solution A was added and after cell detachment the suspension was centrifuged as described in Table II. The pellet was washed once with 1.5 ml of Hepes saline buffer, pH 7.4, and then incubated in 0.5 ml of the same buffer for 1 h at 37 °C. The suspension was centrifuged and the supernatant was removed. The radioactivity in the supernatant and in the cell pellet (dissolved in 0.5 N NaOH) was determined in a $\gamma$-counter. BHK cells ($2.4 \times 10^6$ cells/plate) were incubated in 35-mm plates for 2 h in 1.5 ml of MEM with $Na[^{51}Cr]O_4$ (7.5 $\mu$Ci). Cell leakage was then determined as described for NIL cells.

TABLE V: Incubation of NIL, BHK, and 3T3 Cells with a Mixture of [$^3$H]CMP-Sialic Acid and CMP-[$^{14}$C]Sialic Acid.[a]

| Plate | NIL Cells $^3$H (dpm) | NIL Cells $^{14}$C (dpm) | NIL Cells $^3H/^{14}C$ | Medium $^3H/^{14}C$ |
|---|---|---|---|---|
| 1, [$^3$H]CMP-sialic acid + CMP-[$^{14}$C]sialic acid | 15 560 | 420 | 34.9 | 0.60 |
| 2, as above + 0.5 mM cytidine + 20 mM sialic acid | 40 | 20 | 2.0 | |
| 3, [$^3$H]CMP-sialic acid + CMP-[$^{14}$C]sialic acid + 0.5 mM cytidine | 490 | 460 | | |

| | BHK Cells $^3$H dpm | BHK Cells $^{14}$C dpm | BHK Cells $^3H/^{14}C$ | Medium $^3H/^{14}C$ |
|---|---|---|---|---|
| 1, [$^3$H]CMP-sialic acid + CMP-[$^{14}$C]sialic acid | 2830 | 560 | 5.1 | 0.63 |
| 2, as above + 0.5 mM cytidine + 20 mM sialic acid | 40 | 0 | | |

| | 3T3 Cells $^3$H dpm | 3T3 Cells $^{14}$C dpm | 3T3 Cells $^3H/^{14}C$ | Medium $^3H/^{14}C$ |
|---|---|---|---|---|
| 1, [$^3$H]CMP-sialic acid + CMP-[$^{14}$C]sialic acid | 25500 | 220 | 117.0 | 0.84 |
| 2, as above + 0.5 mM cytidine + 20 mM sialic acid | 30 | 0 | | |

[a] NIL cells ($3.8 \times 10^6$/35-mm plate) were incubated with a mixture of [$^3$H]CMP-sialic acid ($1.1 \times 10^6$ dpm) and CMP-[$^{14}$C]sialic acid ($1.85 \times 10^6$ dpm) in Hepes saline buffer, pH 7/4 (0.7 ml), for 90 min. After removal of the buffer containing the radiolabeled substrates, the cells were washed seven times with 1.5 ml of buffer and scraped off the plate. The suspension was centrifuged, the supernatant was removed, and to each pellet was added 0.4 ml of protosol. The mixture was heated for 2 h at 50 °C. After cooling, toluene omnifluor was added for liquid scintillation counting. At 0 time there were 61 dpm of $^3$H and 9 dpm of $^{14}$C associated with the cells. The $^3H/^{14}C$ ratio in the medium at 0 time was 0.61. 3T3 cells ($3.4 \times 10^5$/35-mm plate) were incubated with a mixture of [$^3$H]CMP-sialic acid ($1.7 \times 10^6$ dpm) and CMP-[$^{14}$C]sialic acid ($2.0 \times 10^6$ dpm); BHK cells ($8.4 \times 10^5$/35-mm plate) were incubated with [$^3$H]CMP-sialic acid ($1.5 \times 10^6$ dpm) and CMP-[$^{14}$C]sialic acid ($2.3 \times 10^6$ dpm) as described for NIL cells.

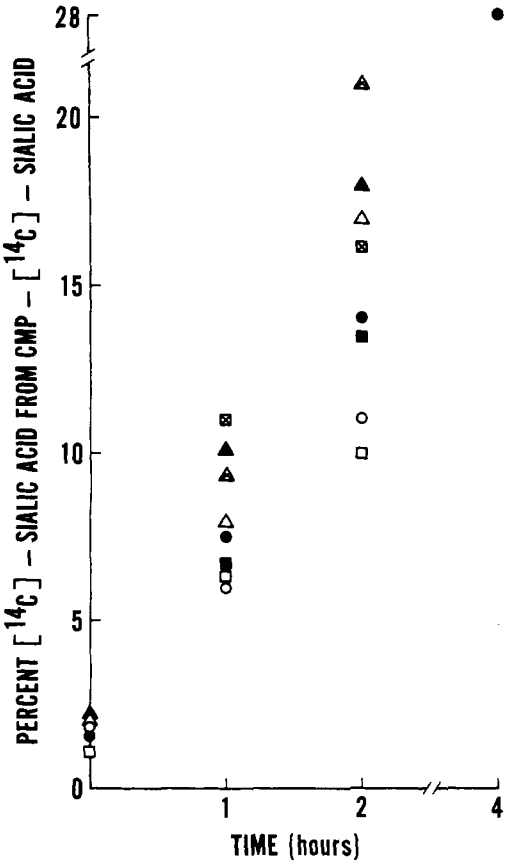


FIGURE 1: CMP-[$^{14}$C]sialic acid breakdown in buffer. For incubations without cells, CMP-[$^{14}$C]sialic acid (0.05 $\mu$Ci) was incubated with buffer, pH 7.4 (Hepes, Tris, or phosphate; 0.1 ml) in a small conical tube for 0, 1 or 2 h at 37 °C. At that time an aliquot of the mixture was applied to Whatman 3MM chromatography paper for ascending chromatography in ammonium acetate (1 M, pH 7.4)-ethanol (40:60). The radioactivity in CMP-[$^{14}$C]sialic acid and [$^{14}$C]sialic acid was determined with a Packard strip counter. For incubations with cells, CMP-[$^{14}$C]sialic acid (0.1 $\mu$Ci) was added to 0.6 ml of buffer and the mixture incubated with a confluent layer of cells on a 35-mm plate (NIL: $3.2 \times 10^6$ cells/plate; BHK: $2.6 \times 10^6$ cells/plate; 3T3: $8 \times 10^5$ cells/plate). An aliquot of the radiolabeled buffer was then applied to paper and developed in the solvent system previously described; (○) Hepes no cells, (●) Hepes + NIL cells; (△) TBS no cells, (▲) TBS + NIL cells; (□) PBS no cells, (■) PBS + NIL cells; (⊠) PBS + BHK cells; (▲) PBS + 3T3 cells.

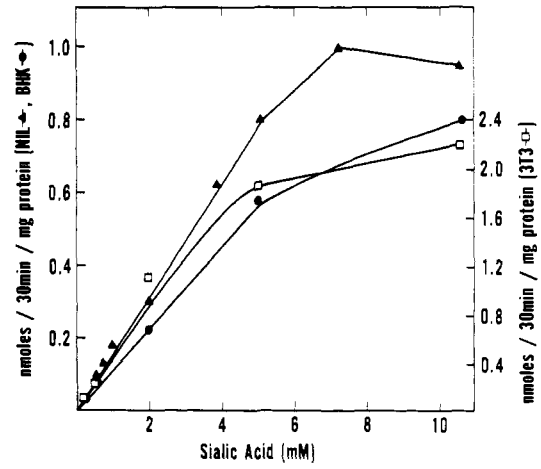


FIGURE 2: Uptake of sialic acid by NIL, BHK, and 3T3 cells. NIL cells ($2.2 \times 10^6$/35-mm plate) were incubated for 30 min with [$^3$H]sialic acid ($7.2 \times 10^6$ dpm) to which nonradiolabeled sialic acid was added to a final concentration as indicated in 0.6 ml of Hepes buffer. After removal of the buffer, the cells were rinsed on the plate seven times with nonradiolabeled buffer. NaOH was added (0.5 N, 0.5 ml, 80 °C) to dissolve cellular proteins and the samples were counted in Aquasol. BHK cells ($2.5 \times 10^6$/35-mm plate) were incubated with [$^3$H]sialic acid ($5.0 \times 10^6$ dpm) as described for NIL cells. 3T3 cells ($7.7 \times 10^5$/35-mm plate) were incubated with [$^3$H]sialic acid ($4.7 \times 10^6$ dpm) as described for NIL cells.

ered as [$^3$H]cytidine, strongly suggesting that after the initial cleavage of the sugar nucleotide, the resulting CMP was converted to cytidine, probably by cell surface phosphatases. While considerable cleavage of the sugar nucleotide to CMP and sialic acid occurred in the absence of cells, we have consistently found a somewhat higher value when cells were present (Figure 1), suggesting that a surface phosphodiesterase may also participate in the sugar nucleotide cleavage, although probably at a slower rate than the chemical breakdown.

These experiments therefore indicated that [$^3$H]cytidine and [$^{14}$C]sialic acid were present in these incubations in addition to the radiolabeled sugar nucleotide. The subsequent experiments demonstrated that the radioactivity associated with the cells was derived to a large extent, if not solely, from [$^3$H]cytidine and [$^{14}$C]sialic acid uptake by the cells rather than a surface transfer of [$^{14}$C]sialic acid. If the radioactive species entering the cells were [$^3$H]cytidine and [$^{14}$C]sialic acid, then one would expect that the addition of a large excess of nonradiolabeled cytidine and sialic acid to the incubation medium containing a mixture of [$^3$H]CMP-sialic acid and CMP-[$^{14}$C]sialic acid, would result in considerably less radioactivity entering the cells. An important condition for such a prediction to be valid is that the final concentration of each species be above their $K_m$ of uptake by these cells. On the other hand, one would not expect a priori that surface transfer of sialic acid from CMP-sialic acid should be affected by the addition of these nonradiolabeled compounds into the medium. To test which of these phenomena was occurring we repeated the previous incubation with a mixture of [$^3$H]CMP-sialic acid and CMP-[$^{14}$C]sialic acid but in the presence of 500 $\mu$M nonradiolabeled cytidine and 20 mM nonradiolabeled sialic acid (both concentrations above the apparent $K_m$ of uptake of each compound; 6-24 $\mu$M for cytidine (Plagemann and Richey, 1974) and 10 mM for sialic acid (see Figure 2). As can be seen in plate 2, Table V, this resulted in an inhibition of incorporation of both isotopes of 94%. The ratio of the two isotopes inside the cell was 2.0, again quite different from the incubation 0.60. This strongly suggested that this residual incorporation could not have been due to a small amount of radiolabeled CMP-sialic acid that had entered the cell. Addition of nonradiolabeled cytidine alone only inhibited the uptake of tritium (cytidine) and not [$^{14}$C] (sialic acid) as shown in Plate 3, Table V.

Taken together, these two experiments are consistent with our hypothesis that most of the radioactive species entering the cells were [$^3$H]cytidine and [$^{14}$C]sialic acid, and argue against (1) significant transport of CMP-sialic acid into the cell and (2) that the reaction was catalyzed by surface sialyltransferases to a large extent. Similar results were observed with 3T3 and BHK cells (Table V).

*Uptake, Distribution, and Identification of the Radioactivity within Cells after Incubation with [$^3$H]Sialic Acid.* The previous experiments were our first indication that cells were permeable to free sialic acid. As can be seen in Figure 2 this uptake of free sialic acid was linear with concentration up to approximately 5 mM. From the data shown in Figure 2 one can calculate the apparent $K_m$ for free sialic acid uptake to be 10 mM. Similar results were obtained with BHK and 3T3 cells (Figure 2).

The distribution of the radioactivity within NIL cells after

TABLE VI: Distribution of Radioactivity within NIL Cells after Incubation with [$^3$H]Sialic Acid.[a]

| Experiment 1 | cpm | Experiment 2 | cpm |
|---|---|---|---|
| PTA supernatant | 480 | Supernatant | 570 |
| PTA and TCA wash | 70 | Pellet | 290 |
| Pellet | 150 | | |

[a] In experiment 1, cells ($4.8 \times 10^6$/35-mm plate) were incubated with [$^3$H]sialic acid ($1.49 \times 10^6$ cpm, 1.0 mM) in Hepes buffer (0.7 ml, 0.1 M, pH 7.4) for 60 min at 37 °C. After removal of the buffer, the cells were washed on the plate with approximately 1.5 ml of Hepes buffer seven times. The radioactivity of the seventh wash was essentially background (7 cpm). To the plate was then added phosphotungstic acid (1.0 ml, 1% in 0.5 N HCl) and the mixture was allowed to stand for 15 min. The cells were scraped with a rubber policeman and the suspension was transferred into a conical centrifuge tube. The scraping was repeated once more with phosphotungstic acid (1.0 ml) and the combined suspensions were centrifuged. The supernatant was removed and called PTA supernatant. The pellet was washed once with phosphotungstic acid (1 ml) and once with trichloroacetic acid (1 ml; 5%). The latter two supernatants were combined and called PTA and TCA wash. The pellet was dissolved in protosol (0.40 ml) and the radioactivity was counted. In experiment 2, the same incubation and washing of cells with Hepes buffer was done. Hepes buffer (1 ml) was then added and the cells were scraped with a rubber policeman and the suspension was transferred to a Dounce homogenizer. The scraping and transfer was repeated once more with Hepes buffer (1 ml). The suspension was "dounced" 20 times with a tight-fitting pestle and the broken cells were centrifuged at 105 000$g$ for 2 h. The supernatant was removed and the radioactivity was determined in the supernatant and in the pellet. For this purpose the pellet was dissolved in protosol.

a 60-min incubation with [$^3$H]sialic acid is shown in Table VI. In experiment 1, glycoproteins together with glycolipids were precipitated with phosphotungstic acid as described by Patt and Grimes (1974). Approximately three-fourths of the radioactivity remained soluble. In order to exclude the possibility that this soluble radioactivity had been previously bound to glycoproteins and glycolipids, but had become soluble as a result of the acid treatment, we repeated the experiment as described but used sedimentation to separate particulate from soluble material. As can be seen in experiment 2, approximately 66% of the radioactivity within the cells was soluble, similar to the value obtained in experiment 1.

In order to identify the radiolabel within the cells and ascertain whether metabolism had occurred after a 60-min incubation, the cells were scraped (after seven washes with nonradiolabeled buffer) with a "rubber policeman" and transferred to a conical centrifuge tube. After centrifugation the pellet was incubated for 60 min with 0.1 N sulfuric acid at 80 °C (Patt and Grimes, 1974). Eighty-five percent of the radioactivity was soluble at the end of such incubation. At least 90% of it was characterized as sialic acid on Dowex-formate and ascending paper chromatography in ethanol–ammonium acetate (1 M, pH 7.5).

*Subcellular Distribution of Radioactivity Derived from CMP-[$^{14}$C]Sialic Acid.* Direct transfer of sialic acid from CMP-[$^{14}$C]sialic acid on the cell surface should result in all the $^{14}$C radioactivity associated with the cell being membrane bound. However, if CMP-[$^{14}$C]sialic acid was first broken down to [$^{14}$C]sialic acid and subsequently the free sialic acid was entering the cells, one would expect only 20% of the radioactivity associated with the cells to be membrane bound, the remainder being soluble since this was the distribution of free sialic acid (see Table VI). To test which of these events was occurring, NIL cells were incubated with a mixture of CMP-[$^{14}$C]sialic acid and [$^3$H]sialic acid. The subcellular distribution of both isotopes is shown in Table VII. In experiment 1, approximately 75% of each isotope was soluble. In experiment 2, where a two hundred-fold higher concentration of CMP-[$^{14}$C]sialic acid and [$^3$H]sialic acid was used than in experiment 1, with a corresponding decrease in specific activity of each compound, 80–90% of the radioactivity of each isotope was soluble. The results of a similar experiment with 3T3 and BHK cells incubated in Hepes-buffered saline are also shown in Table VII. In both cell lines most of the radioactivity was soluble although the percentage of the soluble tritium was somewhat higher than the $^{14}$C.

*Effect of Azide on the Incubation with CMP-[$^{14}$C]Sialic Acid.* Table VIII shows that 10 mM azide caused considerable

TABLE VII: Distribution of Radioactivity within NIL, BHK, and 3T3 cells after Incubation with a Mixture of CMP-[$^{14}$C]Sialic Acid and [$^3$H]Sialic Acid.[a]

| | NIL Cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Expt 1 | | Expt 2 | | BHK Cells | | 3T3 Cells | |
| | $^3$H (dpm) | $^{14}$C (dpm) | $^3$H (dpm) | $^{14}$C (dpm) | $^3$H (dpm) | $^{14}$C (dpm) | $^3$H (dpm) | $^{14}$C (dpm) |
| PTA supernatant | 2100 | 840 | 2220 | 850 | 2530 | 1320 | 1040 | 220 |
| PTA wash | 240 | 130 | 260 | 70 | 130 | 40 | 90 | 0 |
| Pellet | 780 | 370 | 650 | 120 | 570 | 920 | 210 | 100 |
| Percent in Pellet | 25 | 28 | 21 | 12 | 18 | 40 | 16 | 32 |

[a] In experiment 1, NIL cells ($4.8 \times 10^6$/35-mm plate) were incubated with CMP-[$^{14}$C]sialic acid ($2.2 \times 10^6$ dpm) and [$^3$H]sialic acid ($3 \times 10^6$ dpm) in Hepes buffered saline, pH 7.4 (0.7 ml), for 90 min at 37 °C. After removal of the buffer, the cells were washed seven times with nonradiolabeled buffer (1.5 ml). Phosphotungstic acid (1.5 ml, 1% in 0.5 N HCl) was then added and allowed to stand on the plate 15 min on ice. The cells were then scraped with a rubber policeman and the suspension was transferred to a conical centrifuge tube. After centrifugation at top speed in a clinical centrifuge, the supernatant was removed and called PTA supernatant. The pellet was washed once with 0.5 ml of phosphotungstic acid and the supernatant was called PTA wash. The pellet was dissolved in protosol. In experiment 2, the only difference with experiment 1 was that the incubation mixture contained CMP-sialic acid ([4-$^{14}$C]sialic acid, $2.4 \times 10^6$ dpm) and sialic acid (1 mM; $2.9 \times 10^6$ dpm). BHK cells ($3.0 \times 10^6$/35-mm plate) and 3T3 cells ($5 \times 10^5$/35-mm plate) were incubated in Hepes saline buffer, pH 7.4 (0.7 ml), containing CMP-[$^{14}$C]sialic acid ($1.8 \times 10^6$ dpm) and [$^3$H]sialic acid ($4.7 \times 10^6$ dpm) for 90 min at 37 °C. The subsequent workup of each sample was the same as that described for NIL cells.

TABLE VIII: Incubation of NIL Cells with a Mixture of CMP-[$^{14}$C]Sialic Acid and [$^{3}$H]Sialic Acid: Effect of Azide.[a]

| | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| | $^{3}$H (dpm) | $^{14}$C (dpm) | $^{3}$H (dpm) | $^{14}$C (dpm) |
| CMP-[$^{14}$C]sialic acid + [$^{3}$H]sialic acid | 840 | 190 | 840 | 300 |
| As above + 10 mM azide | 80 | 40 | 120 | 80 |
| Percent inhibition | 90 | 81 | 86 | 73 |

[a] In experiment 1, NIL cells ($4.8 \times 10^6$/35-mm plate) were incubated in Hepes saline buffer, pH 7.4 (0.7 ml), with CMP-[$^{14}$C]sialic acid ($1.0 \times 10^6$ dpm) and [$^{3}$H]sialic acid ($3.0 \times 10^6$ dpm) for 90 min at 37 °C. The cells were washed and the radioactivity within the cell pellet was determined as described in Table VII. In experiment 2, NIL cells ($2.4 \times 10^6$/35-mm plate) were incubated with CMP-[$^{14}$C]sialic acid ($1.0 \times 10^6$ dpm) and [$^{3}$H]sialic acid ($3.4 \times 10^6$ dpm) as described in experiment 1.

inhibition of incorporation of radioactivity of both isotopes into a phosphotungstic acid-insoluble pellet when the incubation medium contained a mixture of CMP-[$^{14}$C]sialic acid and [$^{3}$H]sialic acid.

In Table VII the somewhat higher $^{14}$C counts over $^{3}$H counts that were membrane bound in 3T3 and BHK cells could be consistent with a low level of surface sialyltransferase activity. A similar conclusion could be drawn from the somewhat lower inhibition by azide of membrane-bound $^{14}$C counts over $^{3}$H counts in NIL cells. The *maximum* amount of incorporation by surface sialyltransferases would be 22% (40–18) for BHK cells (Table VII), 16% (32–16) for 3T3 cells (Table VII), and 13% (86–73) for NIL cells (Table VIII). However, the absence of an increase in membrane-bound $^{14}$C counts over $^{3}$H counts of NIL cells in Table VII, and the even slightly higher level of soluble $^{14}$C counts over $^{3}$H counts in experiment 2 (Table VII) (88 vs. 79%), probably within the experimental error, makes it very difficult to quantitate the contributions of hypothetical surface sialyltransferases.

## Discussion

One of the central issues in studies on surface sialyltransferases is the question of cell integrity during incubations; if reactions involving the transfer of sialic acid from CMP-sialic acid are to be ascribed to cell surfaces then the presence of cell fragments or leaky cells in the incubation mixture must be ruled out. Surprisingly, cell leakiness has not been monitored in any of the other studies on surface sialyltransferases (Bosmann, 1972; Patt and Grimes, 1974, 1975; Datta, 1974; Sasaki and Robbins, 1974; Porter and Bernacki, 1975) and in only one case has cell viability been tested by a method other than the permeability to tryptan blue (Datta, 1974), a rather insensitive method. It is clear from our studies that removing fibroblasts that have been grown in monolayers with EDTA or trypsin and subsequent incubation of the cells in buffer cause considerable loss of cell integrity. Therefore, it is reasonable to assume that in studies where cells were obtained by such procedures (Patt and Grimes, 1974, 1975; Sasaki and Robbins, 1974) there were numerous cell fragments and leaky cells during the incubation making it very difficult to ascribe the reactions to the cell surface.

Under our reaction condition, at least 95% of the cells were viable and the culture was no more leaky to macromolecules than cells grown in normal growth medium. We also measured cell leakage of these preparations to small molecules by using 2-deoxy[$^{3}$H]glucose. Both NIL and 3T3 cells showed considerably more loss of radiolabel when they were removed from the plate with EDTA and suspended in buffer as compared to cells that were incubated with buffer while on the plate (data not shown). With this cell preparation (on plates) we have shown direct evidence for the nonpermeability of cells to sugar nucleotides. Other studies (Roth and White, 1972) have attempted to rule out such uptake by the failure to detect intact sugar nucleotides within cells that had been incubated with sugar nucleotides radiolabeled in the sugar moiety. However, the observed absence of sugar nucleotides would have also been observed if sugar nucleotides were rapidly hydrolyzed inside the cell or could rapidly transfer the sugar moiety to some acceptor. The large difference in isotope ratio in the mixture of [$^{3}$H]CMP-sialic acid and CMP-[$^{14}$C]sialic acid between the outside medium and the cells strongly suggests that CMP-sialic acid does not enter cells as a unit. In addition, this experiment suggested to us that exogenous breakdown of the substrate had occurred. This was directly confirmed by chromatographic analysis of the incubation mixture in which we found that 10–20% of the CMP-sialic acid had been broken down to free sialic acid.

Recently, Deppert et al. (1974) have made the important observation that cell surface pyrophosphatases of BHK and 3T3 cells cleave sugar nucleotides such as UDP-galactose (and others) to UMP and galactose phosphate. In agreement with these authors we have found such enzymes to be less active on CMP-sialic acid.

In other studies on surface sialyltransferases, cells were incubated with Tris-buffered saline or phosphate-buffered saline (Patt and Grimes, 1974; Datta, 1974; Sasaki and Robbins, 1974). As shown in Figure 1 the amount of CMP-sialic acid breakdown in these buffers was very similar to that in Hepes buffer. The presence of cells did not seem to increase the breakdown to a large extent. Considerable breakdown of CMP-sialic acid in phosphate-buffered saline (19%) has also been observed by Sasaki and Robbins (1974). In other studies on surface sialyltransferases either no breakdown (Patt and Grimes, 1974), or less than 1% of free sialic acid (Porter and Bernacki, 1975) was reported to be present in the incubation medium at the end of the experiment. We find this surprising, since we have found that commercial preparations of radiolabeled CMP-sialic acid contain 2–4% of free sialic acid before incubation.

Our experiments clearly show that in our and previous studies with radiolabeled CMP-sialic acid, there was a mixture of radiolabeled sugar nucleotide and free sialic acid in the incubation medium. Since in our and all previous studies the radioactivity that was associated with the cells was less than 0.1% of the radioactivity in the medium, it is most important to rule out the possibility that at least some of this radioactivity may have originated from free sialic acid uptake by the cells.

The subsequent experiments were designed to determine whether the sialic acid associated with the cells was derived from a direct transfer of CMP-sialic acid (on the cell surface), or whether it was due to free sialic acid uptake by the cells, or both. One line of evidence suggesting that the radioactivity associated with the cells originated from exogenous [$^{3}$H]cytidine and [$^{14}$C]sialic acid, rather than CMP-sialic acid itself, was the inhibition of incorporation of radioactivity into cells when 0.5 mM nonradiolabeled cytidine and 20 mM nonradiolabeled sialic acid were added to the incubation mixture containing CMP-[$^{14}$C]sialic acid and [$^{3}$H]CMP-sialic acid.

In all other studies on surface sialyltransferases, the failure to observe a decrease of radiolabeled sialic acid associated with the cells when the incubation medium contained an excess of nonradiolabeled free sialic acid was used as strong evidence against uptake of free sugar. For such a conclusion to be valid, however, it is important that the final concentration of nonradiolabeled free sialic acid be above the apparent $K_m$ for uptake, which is approximately 10 mM (Figure 2). Since the highest concentration of nonradiolabeled sialic acid used in other studies was 1 mM (Patt and Grimes, 1974, 1975; Datta, 1974; Porter and Bernacki, 1975), it is not surprising that competition was not observed, since the addition of nonradiolabeled sialic acid probably caused an increase in the net velocity of uptake of radiolabeled free sialic acid which compensated for the effect of the dilution. Table VII, experiment 2, clearly shows that when a mixture of CMP-[$^{14}$C]sialic acid and [$^{3}$H]sialic acid was incubated with cells, addition of 1 mM nonradiolabeled sialic acid does not cause a reduction of radioactive sialic acid associated with the cells.

The argument could be made that 20 mM sialic acid may inhibit the transfer of sialic acid from CMP-sialic acid by a direct effect on surface sialyltransferases. Since 0.5 mM cytidine was slightly less effective as an inhibitor of [$^{3}$H]cytidine uptake than a mixture of cytidine and sialic acid (97.3 vs. 99.8%, Table V), it is possible that a sugar transferase or some other enzyme(s) sensitive to both nucleoside and sugar may be involved in uptake. This possibility can neither be ruled out or proven, since the properties of these hypothetical enzymes are unknown and cannot be necessarily inferred from properties of intracellular sialyltransferases. In any case, inhibition of these surface sialyltransferases would have to be occurring *in addition* to the inhibition of uptake of free sialic acid from the medium.

Another line of evidence that is difficult to reconcile with the presence of surface sialyltransferases is the subcellular distribution of radioactivity after incubating cells with a mixture of CMP-[$^{14}$C]sialic acid and [$^{3}$H]sialic acid. According to the surface sialyltransferase hypothesis most radioactive sialic acid from CMP-sialic acid should be membrane bound if incorporated by an ectoenzyme. As shown in Table VII, the distributions of both isotopes within the cell were rather similar: the majority of the counts were soluble while only approximately 30% were membrane bound. The possibility that the soluble radioactivity was due to insufficient washing of the cells seems to be ruled out by the fact that the seventh wash contained less than 10% of the radioactivity associated with the cells. The possibility that the phosphotungstic acid caused some solubilization of the counts seems to be ruled out by the experiment shown in Table VI where acid was not used to determine the subcellular distribution of the radioactivity. In one other study where the subcellular distribution of the radioactivity derived from CMP-[$^{14}$C]sialic acid has been reported, it was found that approximately 30% of the radioactivity was soluble after 1-h incubation (Datta, 1974).

One would predict from the surface sialyltransferase hypothesis, that in the experiment described in Table VII where cells were incubated with a mixture of CMP-[$^{14}$C]sialic acid and [$^{3}$H]sialic acid, that the $^{14}C/^{3}H$ ratio associated with the cells would be higher than that of the incubation medium, since most of the radioactivity in the cells would be derived from a surface transfer of [$^{14}$C]sialic acid from CMP-[$^{14}$C]sialic acid. On the other hand, if most of the radioactivity associated with the cells was derived from free sialic acid uptake by the cells (after cleavage of the CMP-[$^{14}$C]sialic acid) then one would expect the $^{14}C/^{3}H$ ratio of the cells to be lower than that of the medium. The reason for this is that the incubation solution initially contained a large excess of free [$^{3}$H]sialic acid over free [$^{14}$C]sialic acid; however, as the incubation proceeds, the uptake of free [$^{14}$C]sialic acid becomes more significant as a result of CMP-[$^{14}$C]sialic acid cleavage. As can be seen in Table VII, there was a two- to fourfold decrease in the $^{14}C/^{3}H$ ratio in the cells as compared to that in the medium. A similar decrease in this ratio was observed with 3T3 and BHK cells.

These experiments therefore suggest that at least part, if not all, of the [$^{14}$C]sialic acid associated with the cells was *not* incorporated via a surface transferase mechanism. An accurate quantification of the entry of free [$^{14}$C]sialic acid uptake relative to surface transfer is difficult for the following reasons: (a) the concentration of free [$^{14}$C]sialic acid (derived from CMP-[$^{14}$C]sialic acid) in the medium changes with time, (b) the possibility that part of the cleavage of CMP-[$^{14}$C]sialic acid may be due to surface phosphodiesterases yielding free [$^{14}$C]sialic acid, which may be taken up by the cells *without* all of it mixing with the entire free [$^{3}$H]sialic acid pool in the incubation medium, and (c) the uptake of radiolabeled sialic acid is no longer linear after 90-min incubation.

Previous studies (Patt and Grimes, 1974; Sasaki and Robbins, 1974) have used the lack of inhibition by 10 mM azide of the incorporation of radioactivity derived from CMP-[$^{14}$C]sialic acid as strong evidence against uptake of free sialic acid by cells and thus as support for the existence of sialyltransferases on the cell surface. For this reasoning to be valid, however, it is most important to rule out that the observed reactions were not caused by cytoplasmic sialyltransferases that may have leaked out of the cell and that would not be expected to be inhibited by azide. Based on our studies regarding the integrity of cells that had been removed from the plate and then incubated with buffer it is very likely that in those studies where no inhibition by azide was observed (Patt and Grimes, 1974; Sasaki and Robbins, 1974) the reaction was catalyzed by cytoplasmic sialyltransferases. Our observation with intact cells that azide inhibits incorporation into membranes of radioactivity derived from both CMP-[$^{14}$C]sialic acid and [$^{3}$H]sialic acid is consistent with the hypothesis that most of the radioactivity in the cells originated from free sialic acid uptake.

We feel that evidence presented in previous studies (Bosmann, 1972; Patt and Grimes, 1974, 1975; Datta, 1974; Sasaki and Robbins, 1974; Porter and Bernacki, 1975) supporting the existence of surface sialyltransferases is insufficient; our studies clearly show that previous assumptions about cell integrity and the lack of uptake by cells of free sialic acid were incorrect. Since any incubation mixture of CMP-sialic acid contains a significant amount of free sialic acid (usually a 100-fold excess of what is finally associated with the cells), it is necessary to determine whether any of the sialic acid associated with the cells is derived from free sialic acid uptake. This has not been done to our satisfaction; however, failure to detect surface sialyltransferases that use CMP-sialic acid does not rule out the existence in these fibroblasts nor in other cells.

Indeed, Yogeeswaran et al. (1974) have recently suggested the existence of another class of surface sialyltransferases that do not use CMP-sialic acid as substrate but instead use another "unknown" sugar donor. We find a recent suggestion that surface sialyltransferases are cryptic but become exposed with EDTA (Patt and Grimes, 1975) to be less likely in view of our results concerning the leakiness of EDTA-treated cells.

To our knowledge this is the first report on the permeability of mammalian cells grown in tissue culture to sialic acid. This observation is consistent with a recent observation by Harms

et al. (1974) who reported that free sialic acid is incorporated into liver glycoproteins when injected intravenously into rats. Although we have observed apparent saturation of this uptake between 10 and 20 mM sialic acid, preliminary results indicate that at this concentration some nonspecific binding and trapping may occur. In addition, we wish to emphasize that the absolute values for the velocity of uptake should be viewed with caution, since the rate of cell growth is known to affect such parameters and we have not studied its effect on uptake.

The distribution of the radioactivity within the cells after 1-h incubations with free sialic acid deserves some attention. Approximately 25% of the radioactivity was precipitated with phosphotungstic acid, suggesting that the sialic acid had been reactivated and incorporated into glycoproteins and glycolipids. Whether this occurred through the intermediate formation of CMP-sialic acid within the cell is not known. Experiments are currently underway to further characterize this observation.

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