Biochimica et Biophysica Acta, 467 (1977) 72—85

BBA 77694

DEMONSTRATION OF SIALYL TRANSFER FROM EXOGENOUS CMP-*N*-ACETYL-[^{14}C]NEURAMINIC ACID TO CELL MEMBRANE-BOUND ACCEPTOR MOLECULES AT THE SURFACE OF INTACT EHRLICH ASCITES TUMOUR CELLS

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(Received January 21st, 1977)

Summary

Intact Ehrlich ascites tumour cells were incubated with exogenous CMP-*N*-acetyl-[^{14}C]neuramine acid (AcNeu). The resulting incorporation of radioactive materials onto acid-precipitable acceptor molecules was hydrolyzed and quantitatively identified as [^{14}C]AcNeu by cochromatography with [^{3}H]AcNeu in three different solvent systems. The total amount incorporated varied fivefold when different cell preparations were compared. A similar degree of variability was observed for the kinetic parameters. The apparent K_m was 10.9 ± 4.3 μM and the apparent V 69.6 ± 38.5 pmol per 10^8 cells and 30 min. Treatment of the intact labeled cells with extracellular neuraminidase from *Clostridium perfringens* liberated 60—80% of the incorporated [^{14}C]AcNeu. A corresponding incubation in only buffer, lacking the enzyme reduced the label by 5—10%. 85% of the neuraminidase-susceptible incorporation was released within the first 5 min of incubation with this enzyme. Fixation of the labeled cells in glutaraldehyde did not alter and ultrasonication decreased the susceptibility to neuraminidase. 32—35% of the neuraminidase-nonsusceptible label were extracted with chloroform/methanol. Treatment of the cells with galactoseoxidase from *Polyporus circinatus* before incubation with CMP[^{14}C]-AcNeu diminished the incorporation of [^{14}C]AcNeu by at most 47.5%. Chase experiments and studies on the initial velocity during the first minutes of incubation failed to demonstrate any flow of activated sialic acid through different cellular compartments. Under conditions when the amount of cellular debris derived from the cell interior accumulated, the sialyltransferase activity was decreased. Autoradiographic experiments showed that all the cells were labeled.

Abbreviation: AcNeu, *N*-acetylneuraminic acid.

It is concluded that the incorporation was mediated by sialyltransferase(s) and acceptor molecules located at the cell surface of intact cells.

Introduction

Several lines of evidence indicate that part of the animal cell glycosyltransferases are located on the cell surface [1]. In some experiments, dealing with such ectoenzymes, intact cells are incubated with the precursor, a labeled nucleotide-linked sugar. It has been suggested that the resulting incorporation onto acid-precipitable acceptor molecules reflects the natural way of glycosylation [2]. Evidence has been obtained that the incorporation is not always due to enzyme and acceptor molecules located at the cell surface. In some systems the nucleotide-sugar is hydrolyzed at the cell surface, the sugar is transported into the cell, reattached to a nucleotide and used in a transferase reaction inside the cell [3]. Another aspect is that several glycosyltransferases operate through a lipid intermediate [4,5]. It has been shown that the sugar may be reversively transferred from the nucleotide to the lipid [4,5]. This will probably cause a rapid equilibration of the lipid-soluble sugar in various membrane systems irrespective of the initial site of transfer and the location of the ultimate acceptor molecules. The most suggestive evidence for incorporation of sugar from exogenous nucleotide-sugar onto the cell surface comes from studies on the ectosialyltransferases of BHK [6] and L-1210 mouse leucaemic [7] cells.

The present data indicate that all incorporation from exogenous CMP-*N*-acetyl-[^{14}C]neuraminic acid (AcNeu) into the acid-precipitable fraction of intact Ehrlich tumor ascites cells derives from sialyzed acceptor molecules at the cell surface. Furthermore, evidence is given that the enzymes mediating this transfer are located on the cell surface.

Experimental

Materials. Buffer salts were reagent grade from E. Merck AG. Darmstadt, Germany. Unlabeled CMP-AcNeu was prepared according to Kean et al. [8,9] from CTP and AcNeu, (Type IV, obtained from Sigma Chemical Co., St. Louis, Mo.). It was purified on a Dowex 1 × 2% column using a linear gradient of 0—1 M NH_4HCO_3 and lyophilized. The NH_4HCO_3 had been further purified from traces of heavy metals by passing it through a Dowex-50 column. The lyophilized CMP-AcNeu was dissolved in 0.01 M Tris · HCl, pH 7.4 (Trizma, Sigma) and filtered through a millipore filter, size 0.45 μm.

The concentration of CMP-AcNeu was adjusted using the thiobarbituric acid assay [10] and standard solutions composed of AcNeu. Radioactive [^{14}C]-AcNeu (specific radioactivity 50 Ci/mol), CMP-[^{14}C]AcNeu (200 Ci/mol) and CMP-[^{3}H]AcNeu (2.4 Ci/mmol) were purchased from NEN Chemicals, GmbH, Frankfurt-on-Main, Germany. All commercial enzyme preparations used were from Sigma. Galactoseoxidase was type I and neuraminidase was type VI (prepared from *Polyporus circinatus* and *Clostridium perfringes*, respectively). Glutaradehyde was from Kebo AB, Stockholm. Protosol and Aquasol were from NEN Chemicals. The cellulose thin-layer plates were from E. Merck AG.

Cells. About 10^7 Ehrlich ascites tumour cells (ELD strain) were inoculated into the abdomen of Swiss albino mice of both sexes obtained from Anticimex breeding farm, Norrviken, Stockholm. After 8—16 days the peritoneal cavity was opened and the cells collected. All subsequent preparatory steps were carried out at 0—4°C. The ascitic fluid was immediately diluted about ten-fold with Krebs-Ringer bicarbonate buffer. The cells were washed 3 times with Krebs-Ringer bicarbonate buffer and suspended to a concentration of $5.5 \cdot 10^7$ cells/ml.

Incubation of intact cells with CMP-AcNeu. $9.2 \cdot 10^7$ cells, suspended in 1.5 ml of Krebs-Ringer biocarbonate buffer were transferred to SS-34 propylene plastic centrifuge tubes placed in ice. 20 μl of the prepared CMP-AcNeu and 5 μl of the commercial CMP-[^{14}C]AcNeu dissolved in ethanol/water (v/v, 7 : 3) were added. The final concentration of CMP-[^{14}C]AcNeu was 10 μM with a specific activity of about 5 Ci/mol unless otherwise indicated. In incubations of more than 2 h, 1.5 mg of glucose in 20 μl of redistilled water were added in order to prevent depletion of intracellular ATP. The tubes were then capped and aerated with a humidified carbogen gas mixture composed of 93.5% O_2 and 6.5% CO_2. The incubation was started by transferring the tubes to a thermostated shaker and terminated by cooling the tubes in an ice bath and diluting the cell suspension with 25 ml of ice-cold Krebs-Ringer bicarbonate buffer, followed by centrifugation for 25 min at 4°C and 3000 $\times g$ except in those cases where treatment with neuraminidase was planned.

Neuraminidase treatment of labeled cells. After incubation with CMP-[^{14}C]-AcNeu and dilution as described above the cells were centrifuged to a loose pellet at 1000 $\times g$ for 15—25 min. Long centrifugation times were chosen in order to minimize aspiration of cells during the subsequent steps. The ^{14}C-labeled cells were then washed once with 15 ml of ice-cold Krebs-Ringer bicarbonate buffer followed by resuspension in 2 ml of a buffer, pH 6.5. Two buffers were used, one was an isotonic NaH_2PO_4/Na_2HPO_4 buffer, the other was a modified Krebs-Ringer phosphate buffer, prepared by replacing 9/10 of the $CaCl_2$ stoichiometrically with NaCl and adding isotonic NaH_2PO_4 to pH 6.5. 0.1 units (manufacturers definition) of neuraminidase were added and the mixture incubated for 45 min at 37°C. The incubation was terminated by adding 15 ml of the cold Krebs-Ringer bicarbonate buffer followed by centrifugation for about 25 min at 3000 $\times g$.

Ultrasonication of labeled cells preceding treatment with neuraminidase. An ultrasonication apparatus obtained from M.S.E., Crawley, U.K. was used. After suspension in the phosphate buffer as described above the cells were sonicated for 2 min (12 μm peak to peak) in an ice-bath. After sonication the tubes were placed in boiling water for 15 min in order to inactivate lysosomal enzymes.

Fixation of intact cells in glutaraldehyde. A 27% solution of glutaraldehyde was shaken with charcoal to remove impurities. It was made isotonic by dilution with water and neutralized with a few drops of isotonic $NaHCO_3$. Cells incubated with CMP-[^{14}C]AcNeu were washed and suspended in 5 ml of ice-cold Krebs-Ringer bicarbonate buffer. 14 ml of the cold isotonic glutaraldehyde solution were added and allowed to react with the cells for 30 min at 0°C. (The final concentration of glutaraldehyde was 2%). The cells were then centrifuged and resuspended in 15 ml of the ice-cold buffer and put in the ice-bath for

15 min. This procedure was repeated twice after which they were suspended in a buffer, pH 6.5, and finally treated with neuraminidase.

Treatment of the intact cells with galactoseoxidase. Treatment of intact cells with galactoseoxidase followed by reduction with Na[^{3}H]BH_4 has previously been used by Gahmberg et al. [11] to label cell surface components. In the present experiments galactoseoxidase was added to $9.2 \cdot 10^7$ cells suspended in 1.5 ml of Krebs-Ringer bicarbonate buffer. After incubation for up to 2 h the cell suspension was diluted with 15 ml of cold Krebs-Ringer bicarbonate buffer and centrifuged at $1000 \times g$ for 15 min. The supernatant was aspirated and the cells were resuspended in 1.5 ml of the buffer followed by incubation with CMP[^{14}C]AcNeu.

Control of cell viability. Microscopic examination was routinely used to control the viability of the cells. Two criteria, the formation of microagglutinates and trypan blue uptake were considered to indicate decreased cell viability. Unless indicated only preparations where at least 90% of the cells excluded trypan blue were used.

Assay of incorporation of [^{14}C]AcNeu in the cell pellet. 1 ml of the supernatant from incubated cells diluted with cold Krebs-Ringer bicarbonate buffer was transferred to a scintillation tube. The remaining supernatant was aspirated, the walls of the tubes were rinsed with the cold buffer and the cell pellet was frozen. After storage at −20° C it was washed three times with 10 ml of ice-cold 3% sulfosalicylic acid, each time with the addition of 0.25 μmol of unlabeled AcNeu. After centrifugation for 15 min at $3000 \times g$, three drops of water were added to the pellet followed by digestion in 3 ml of Protosol and after the addition of 150 μl of glacial acetic acid 1 ml of the digest was transferred to a scintillation vial containing 10 ml of Aquasol. The supernatant probe was solubilized in the same amount of Aquasol. A similarly treated cell pellet not contaminated by any radioactive materials or buffer in Aquasol was used for measuring the background.

Extraction of lipids with chloroform/methanol. The lipids were extracted after treatment with sulfosalicylic acid. 3 ml of chloroform/methanol (2 : 1) were added to the 0.2 ml pellet which was suspended by sonication (1 min, 12 μm peak to peak), 3 ml of methanol were added followed by centrifugation for 20 min at $3000 \times g$. This procedure was repeated twice with chloroform/methanol (2 : 1) and thereafter three times with chloroform/methanol/water (1 : 2 : 0.15) (cf. ref. 12). The resulting pellet was finally washed with 3% sulfosalicylic acid and assayed for radioactivity.

Identification of the acid-precipitable label as [^{14}C]AcNeu. $9.2 \cdot 10^7$ cells were incubated for 2 h with 2 nmol of CMP-[^{14}C]AcNeu with a higher than usual specific radioactivity, 197 Ci/mol. They were collected by centrifugation and washed 5 times with ice-cold sulfosalicylic acid and unlabeled AcNeu. 5 mCi of CMP-[^{14}C]AcNeu and 1.5 μmol of unlabeled AcNeu were added followed by acid hydrolysis in 10 ml of 0.05 M H_2SO_4 for 1 h at 80° C. After neutralization with 0.1 M NaOH and dilution with 80 ml of deionized water the mixture was transferred to a Dowex 1 × 2% column in NH_4^+ form (length 25 cm, diameter 1.2 cm) and eluted with a linear 200 ml H_2O/200 ml 1 M NH_4HCO_3 gradient [9]. The radioactive samples were pooled, lyophilized and dissolved in 1.5 ml of deionized water. They were further analyzed by thin-

layer chromatography on cellulose using two different solvent systems, 1-butanol/1-propanol/0.1 M HCl (1 : 2 : 1) and 1-butanol/pyridin/water (6 : 4 : 3) according to Svennerholm [13].

Determination of the degree of hydrolysis of CMP-[^{14}C]AcNeu. A modification of the method of preparing CMP-AcNeu [9] was used for this purpose. A Pasteur pipette was filled with Dowex 1 × 2% (NH_4^+ form) to a hight of 8 cm (total volume 1.8 cm^3). 1 ml of the supernatant from the incubated cell suspension, diluted with cold Krebs-Ringer bicarbonate buffer was diluted with 3 ml of water. 30 nmol of CMP-AcNeu were added as carrier. 3 ml of this solution were transferred to the column. After washing twice with 3 ml of water the AcNeu was eluted with 2 × 3 ml of 0.3 M NH_4HCO_3 followed by elution of the CMP-AcNeu with 2 × 3 ml of 1 M NH_4HCO_3. 1 ml of the eluates was solubilized in 10 ml of Aquasol and the radioactivity was measured in a Nuclear Chicago Unilux II scintillation equipment.

Autoradiography. $9.2 \cdot 10^7$ cells in 1.5 ml of Krebs-Ringer bicarbonate buffer were incubated for 2 h with 2 nmol of CMP-[^{14}C]AcNeu (specific activity: 197 Ci/mol) and washed 3 times in 3% sulfosalicylic acid containing 0.25 μmol of AcNeu. The pellet was suspended in 1.5 ml of sulfosalicylic acid and one drop was spread on a microscope slide, dried in air and covered with ilford Nuclear Research Gel emulsion, Type G5. Exposition was for 110 days followed by development for 30—120 s in Kodak D 11 developer, rinsing in 10% acetic acid and fixation in Kodafix solution.

Results

When whole Ehrlich ascites tumor cells are incubated with CMP-[^{14}C]AcNeu and assayed for radioactivity as described above the resulting incorporation is highly reproducible provided the same cell preparation is used. 98.5% of the label was hydrolyzed from the acid-precipitated material in 0.05 M H_2SO_4 for 1 h at 80°C. The ^{14}C-labeled material and added [^{3}H]AcNeu coeluted from a Dowex 1 × 2% column in NH_4^+ form using a linear H_2O/1 M NH_4HCO_3 gradient. The recoveries of the two isotopes were 83 and 85.6% of ^{14}C and ^{3}H, respectively. The ^{14}C-labeled product and [^{3}H]AcNeu also cochromatographed on thin-layer cellulose plates using two different solvent systems, 1-butanol/1-propanol/0.1 M HCl and 1-butanol/pyridin/water. In 29 experiments on different occasions incubation with 1 μM CMP-[^{14}C]AcNeu resulted in the incorporation of 4.3 ± 1.7 (S.E.M.) pmol of [^{14}C]AcNeu per 10^8 cells and 30 min. In the presence of 10 μM CMP-[^{14}C]AcNeu. The cells incorporated 33.2 ± 10.7 pmol of [^{14}C]AcNeu per 10^8 cells and 30 min. This label, corresponding to 40—200 cpm and less than 1% of the added substrate, was not detached from the intact cells upon washing them repeatedly in Krebs-Ringer bicarbonate buffer. When the denatured cells were washed several times with 3% sulfosalicylic acid all label remained in the pellet. 7% of the radioactive material was eluted when the sulfosalicylic acid-denatured cells were washed three times in isotonic KRB buffer and collected by centrifugation at 1500 × *g*. When ultrasonication preceded sulfosalicylic acid treatment no label was lost. These procedures indicate that the incorporated acid-insoluble ^{14}C-label is largely structure-bound and not confined to soluble cytoplasmic proteins. Control experiments were

made to exclude the possibility that soluble enzyme leaking from the cell interior or being eluted from the cell membrane contributed to the incorporation. Cells were suspended in fresh KRB buffer or in KRB buffer that was derived from incubating other cell suspensions in the absence of CMP-AcNeu for up to 60 min at 37°C. CMP-[^{14}C]AcNeu was then added followed by incubation for 30 min at 37°C (Table I). No significantly increased incorporation was seen.

Kinetic determinations. A Lineweaver-Burk plot of the incorporation of [^{14}C]AcNeu into whole Ehrlich cells is shown in Fig. 1. In 25 kinetic determinations on different occasions the mean apparent K_m value was 10.9 ± 4.3 μM and the mean apparent V 69.6 ± 38.5 pmol · 10^8 cells per 30 min. Less than 1.5% of the added CMP-[^{14}C]AcNeu were hydrolyzed under these conditions. Addition of the corresponding amounts of unlabeled CMP and/or AcNeu or even 100-fold excess of AcNeu [14] did not influence the sialyltransferase activity.

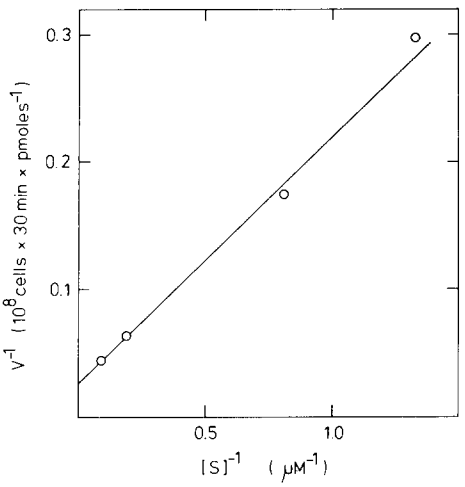
Treatment with neuraminidase. When cells incubated with CMP-[^{14}C]AcNeu were further treated with extracellular neuraminidase for 45 min, 60—80% of the incorporated [^{14}C]AcNeu were liberated. Incubation of the cells in the same buffer lacking the enzyme reduced the label by only 5—10%. Neuraminidase from *C. perfringens* is known to hydrolyze α-D-configurated bound sialic acid residues from terminal sites in glycoproteins and glycolipids [15]. The time course of that reaction on the surface of intact Ehrlich cells labeled with CMP-[^{14}C]AcNeu is given in Fig. 2. 85% of the incorporated [^{14}C]AcNeu available for neuraminidase were liberated within 5 min of incubation. AcNeu, a known competitive inhibitor of some bacterial neuraminidases [15] diminished the activity of the commersial enzyme preparation used (Table IIA). The hydrolyzing action of neuraminidase was dependent on the enzyme concentration as shown in Table IIA. Furthermore, its action was not abolished by fixation of the cells in glutaraldehyde before the enzyme treatment (Table IIB). The mere fixation of the cells in glutaraldehyde did not lead to any liberation of [^{14}C]AcNeu. Ultrasonication followed by boiling decreased the effect of neuraminidase by 16% (Table IIB). Two subsequent treatments of the labeled intact cells with neuraminidase was more efficient than a single one, liberating an extra amount of about 10% of the total incorporation (Table IIA). In no case was this amount exceeded when neuraminidase-treated cells were soni-

TABLE I

SIALYLTRANSFERASE ACTIVITY IN DIFFERENT SUSPENDING MEDIA

0.17 ml of packed cells were suspended in 1.5 ml of either fresh Krebs-Ringer bicarbonate buffer or in Krebs-Ringer bicarbonate buffer that had been the suspending medium for other cells at 37°C for the indicated time. CMP-[^{14}C]AcNeu was then added to 10 μM and the incubation was performed for 30 min at 37°C. (Mean of 2 determinations.)

Incubation medium	Incorporation (%)	Total cpm
Fresh buffer	100 ± 2	174
Buffer incubated with cells at 37°C for 30 min	101 ± 3	
Buffer incubated with cells at 37°C for 60 min	96 ± 1	



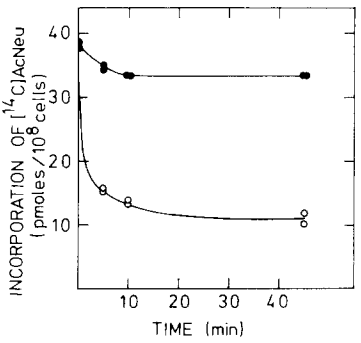


Fig. 1. A Lineweaver-Burk plot of the sialyl transfer on intact cells.

Fig. 2. The time-course of neuraminidase treatment of cells labeled with exogenous CMP-[^{14}C]AcNeu. Cells were incubated in a spinner culture equipment at 37°C for 1 h with 10 μM CMP-[^{14}C]AcNeu. After washing, aliquots of 1.67 ml were transferred to centrifuge tubes and incubated with neuraminidase in a modified Krebs-Ringer bicarbonate buffer for different times (open circles) or in that buffer only (closed circles).

cated followed by an extra treatment with neuraminidase (Table IIB). Obviously, sonication followed by boiling decreases rather than increases the availability for neuraminidase. The neuraminidase-nonsusceptible incorporation was not influenced by adding 1 mM of unlabeled AcNeu during the incubation with CMP-[^{14}C]AcNeu excluding the possibility of any significant adsorption in that pool. Chase experiments with unlabeled CMP-AcNeu (Table IIA) demonstrated that the "nonsusceptible" incorporation is not a precursor pool for the "susceptible" incorporation. Thus the same amount of incorporated [^{14}C]AcNeu was susceptible to neuraminidase regardless if this treatment was preceded by incubation with unlabeled CMP-AcNeu or not. Extraction with chloroform/methanol liberated 32—35% of the neuraminidase-nonsusceptible incorporation from the cell pellet (Table IIB).

Treatment of the cells with galactoseoxidase. The effect of pretreating the cells with galactoseoxidase before incubation with CMP-[^{14}C]AcNeu is illustrated in Fig. 3. This enzyme diminishes the incorporation of [^{14}C]AcNeu, probably by modifying acceptor sites (cf. ref 11). The mere incubation in buffer before addition of CMP-[^{14}C]AcNeu did not result in any diminished incorporation. The effect of galactoseoxidase was dependent on the enzyme concentration as shown in Table III.

Time course. Chase experiments were designed in order to investigate if the sialyltransferase enzyme was immediately available for the added CMP-AcNeu. Cells were incubated for up to 3 h in the presence of 10 μM unlabeled CMP-AcNeu. The cell suspensions were then chilled in an ice-bath, traces of CMP-[^{14}C]AcNeu were added and the cells were further incubated for 5—30 min at 37°C. Some cells were in addition treated with neuraminidase after these steps. These experiments which are illustrated in Fig. 4 indicate that the extracellular

TABLE II

NEURAMINIDASE ACTION UNDER VARIOUS CONDITIONS

The cells were first labeled with CMP-[^{14}C]AcNeu for 30—60 min at a cell density of $55 \cdot 10^6$ cells/ml. After washing once and diluting to the same cell density 1.67 ml were transferred to plastic tubes and treated as given in the columns. The enzyme units refer to the manufacturers definition. (Mean of 2 determinations.)

Type and sequence of posttreatment	Incorporation (%)	Total cpm
A. Expt. no. 1		
No	100 ± 2.8	105
Buffer 45 min	95.2 ± 1.5	
Neuraminidase (0.01 units)	46.9 ± 1.5	
Neuraminidase (0.02 units)	32.7 ± 4.4	
Neuraminidase (0.05 units)	29.8 ± 4.2	
Neuraminidase (0.1 units)	24.2 ± 4.2	
Expt. no. 2		
No	100 ± 1.7	239
Neuraminidase (0.1 units)	33.5 ± 0.6	
Neuraminidase (0.1 units) + AcNeu (1.5 μmol)	34.5 ± 0.6	
Neuraminidase (0.1 units) + AcNeu (15 μmol)	49.5 ± 0.6	
Expt. no. 3		
No	100 ± 0.3	173
Neuraminidase (0.1 units)	42.7 ± 2.3	
Neuraminidase (0.1 units) → neuraminidase (0.1 units)	31.9 ± 1.3	
Expt. no. 4		
No	100 ± 4.6	
Neuraminidase (0.1 units)	44.2 ± 2.5	
Neuraminidase (0.1 units) → neuraminidase (0.1 units)	36.4 ± 0.7	
Neuraminidase (0.1 units) → neuraminidase (0.1 units) → neuraminidase (0.1 units)	35.3 ± 1.4	
Expt. no. 4		
10 μM unlabeled CMP-AcNeu in Krebs-Ringer bicarbonate buffer for 45 min → neuraminidase (0.1 units)	45.2 ± 2.8	
B. Expt. no. 1		
No	100 ± 6.7	177
Neuraminidase (0.1 units)	40.9 ± 2.1	
Fixation in glutaraldehyde	101.6 ± 1.7	
Fixation in glutaraldehyde → neuraminidase (0.1 units)	41 ± 0.2	
Expt. no. 2		
No	100 ± 8.8	258
Neuraminidase (0.1 units)	31.4 ± 0.0	
Sonication → boiling → buffer 45 min	100 ± 2.3	
Sonication → boiling → neuraminidase (0.1 units)	41.9 ± 3.5	
Neuraminidase (0.1 units) → sonication → boiling → buffer 45 min	30.2 ± 1.2	
Neuraminidase (0.1 units) → sonication → boiling → neuraminidase (0.1 units)	22.9 ± 1.7	
Expt. no. 3		
No	100 ± 5.7	477
Neuraminidase (0.1 units)	34.6 ± 0.6	
Neuraminidase (0.1 units) → chloroform/methanol extraction	22.6 ± 2.5	
Expt. no. 4		
No	100 ± 1.0	153
Neuraminidase (0.1 units)	44.5 ± 0.5	
Neuraminidase (0.1 units) → chloroform/methanol extraction	30.3 ± 2.0	

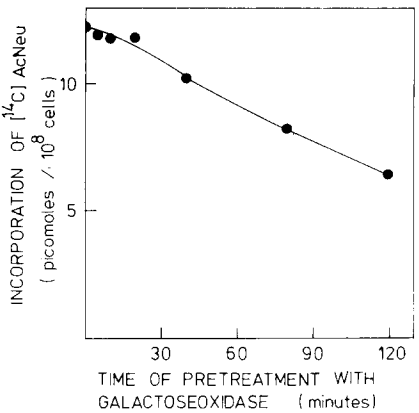


Fig. 3. The effect of treating the cells for different times with galactoseoxidase before incubation with CMP-[^{14}C]AcNeu. The enzyme concentration in this experiment was 7.5 units of galactoseoxidase according to the manufacturers definition. (Mean of 2 determinations.)

CMP-[^{14}C]AcNeu immediately joins to the pool of unlabeled activated sialic acid which is used in the reaction. If the enzyme were not immediately available for the added CMP-[^{14}C]AcNeu a time lag would be observed after the addition of radioactive substrate before the appearance of measurable incorporation. The same considerations apply to the very short incubations (30 s to 4 min) illustrated in Fig. 5. No lag of incorporation was observed in these experiments and the label was partly susceptible to neuraminidase.

Cells were incubated with CMP-[^{14}C]AcNeu also for extremely long periods (1—21 h) under conditions of decreased cell viability (Fig. 6). Less than 2% of the total CMP-[^{14}C]AcNeu were incorporated during this entire incubation. It is evident from the figure that the incorporation of sialic acid into both neuraminidase-susceptible and nonsusceptible bonds is roughly parallel and that it is not stimulated as the cell viability progressively decreases. The experiments illustrated in Fig. 4—6 also show that the initial reaction and that after the sialyl transfer has proceeded for several hours are of the same type as judged from the susceptibility to neuraminidase.

TABLE III

THE EFFECT OF INCUBATION WITH GALACTOSEOXIDASE ON THE SUBSEQUENT INCORPORATION OF [^{14}C]AcNeu

Intact cells were treated for 40 min with different concentrations of galactoseoxidase before incubation with CMP-[^{14}C]AcNeu for 30 min. (Units refer to the manufactures definition.) (Mean of 2 determinations.)

Type of preincubation	Incorporation (per cent)	Total cpm
No preincubation	100 ± 3.3	204
Galactoseoxidase (1.9 units)	106 ± 6.1	
Galactoseoxidase (3.8 units)	90.6 ± 1.2	
Galactoseoxidase (7.5 units)	83.6 ± 9	
Galactoseoxidase (15 units)	72.1 ± 0.0	

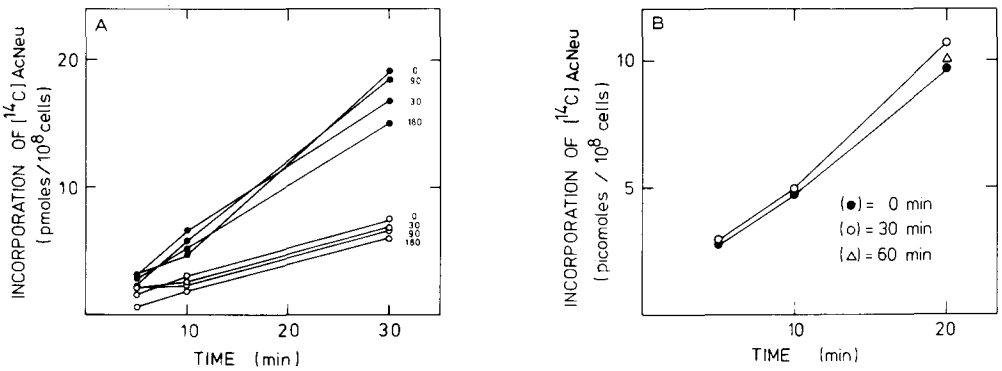


Fig. 4. Chase experiments with unlabeled CMP-AcNeu. Cells were incubated for up to 3 h in the presence of 10 μM unlabeled CMP-AcNeu and 5.6 mM glucose. After the indicated time (numbers in the figure refer to minutes) the tubes were chilled on ice, CMP-[^{14}C]AcNeu was added and the cells were further incubated for 5—30 min (closed circles). A parallel experiment was made but in addition the cells were treated with neuraminidase after the incubation with CMP-[^{14}C]AcNeu (open circles). The plots in (A) represent single tubes and in (B) the mean of three determinations. In the latter experiment 1 mM of AcNeu was added to each tube in order to minimize the influence of [^{14}C]AcNeu liberated by hydrolysis of CMP-[^{14}C]AcNeu. Closed and open circles and triangles in (B) refer to different times of preincubation.

Autoradiography. After washing with sulfosalicylic acid the cells appeared as partly confluent bodies of approximately the same size as intact cells. The association of silver grains with such cell-size bodies after different development times is recorded in Table IV. It appears that there are at least three different populations of cells characterized by different levels of associated [^{14}C]AcNeu. One population constituting 15—17% in this slide requires longer development for the appearance of associated grains and is thus weakly labeled while 7—11% is heavily labeled in a patchy non-uniform manner and the remain-

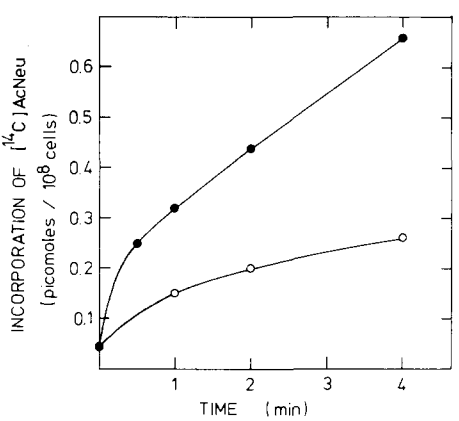


Fig. 5. Initial velocity of the sialyl transfer. $9.2 \cdot 10^7$ cells were prewarmed for 10-20 min and 20 μl of CMP-[^{14}C]AcNeu in ethanol/water were added. The final concentration was 1.3 μM with a specific radioactivity of 197 Ci/mol. The incubation was terminated by diluting with 5 ml of ice-cold Krebs-Ringer bicarbonate buffer containing 60 nmol of unlabeled CMP-AcNeu. 25 ml of the cold buffer were thereafter added followed by centrifugation. Closed circles represent total and open circles neuraminidase-nonsusceptible incorporation. (Mean of 3 determinations.) The zero time value was determined by adding the unlabeled CMP-AcNeu immediately before addition of labeled substrate.

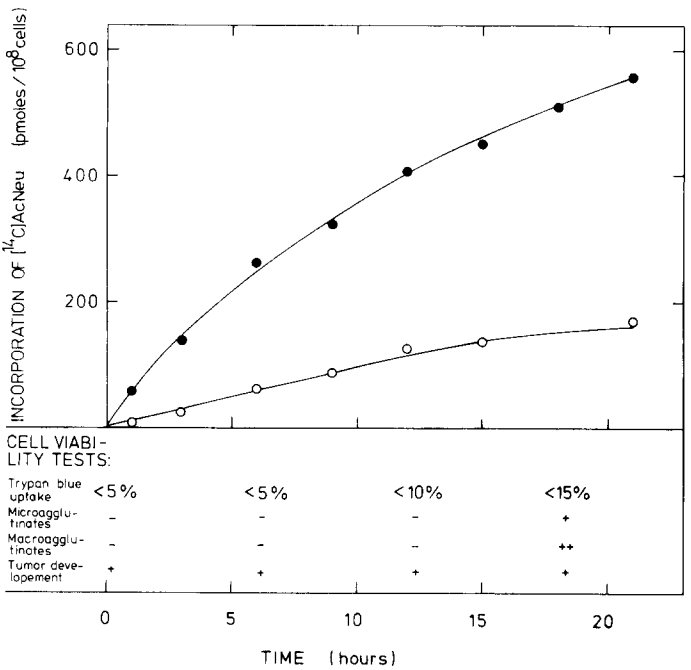


Fig. 6. Long term incubation of Ehrlich cells with CMP-[^{14}C]AcNeu. The cells were suspended to a concentration of 55 · 10^6 cells/ml in Eagles medium [19] with the addition of CMP-[^{14}C]AcNeu to a concentration of 20 μM and unlabeled AcNeu to 1 mM, and the incubation was performed in a spinner culture equipment. The medium and substrate were renewed after 12 h of incubation. After the indicated times two aliquots were taken, one series of samples were treated with neuraminidase at 37°C for 45 min (open circles) and the other were assayed directly for radioactivity (closed circles). The viability of the cells was checked every 6th hour by investigating the percentage of cells that excluded Trypan Blue, by assessing of the tendency for agglutination and by inoculating 1 · 10^7 cells into the abdomen of mice followed by estimation of tumor growth.

ing 72—78% is associated with intermediate amounts of uniformly spread [^{14}C]-AcNeu. When development was for 120 s or more this difference was not easily detected and all cells seemed to be uniformly labeled. Focusing the microscope indicated that the grains surrounded the cells but since the silverbromide gel was not shown to penetrate the cells and the radiation of ^{14}C extends longer than the thickness of a cell membrane this finding gave no information on the subcellular location of the label.

TABLE IV

THE ASSOCIATION OF SILVER GRAINS TO SULFOSALICYLIC-ACID WASHED CELL-SIZE BODIES AFTER DIFFERENT TIMES OF DEVELOPMENT

500 cells were counted and the figures are a percentage of the total number of cells.

Time of development (s)	Cells without associated grains	Cells involved in patchy distribution of grains	Cells uniformly surrounded by grains
30	15	7	78
60	17	11	72
120	0	0	100

Discussion

The rapid effect of neuraminidase in hydrolyzing [^{14}C]AcNeu from intact cells labeled with exogenous CMP-[^{14}C]AcNeu indicates that at least 60—80% of the incorporated sialic acid is exposed to the extracellular medium. Only the cell surface or possibly cellular debris offers this immediate availability to the extracellular enzyme provided the neuraminidase molecules do not rapidly penetrate the cell membrane. This interpretion is corroborated by the fact that fixation of the labeled cells in glutaraldehyde does not alter the efficiency of neuraminidase. Thus, pinocytosis of neuraminidase [16] is not necessary for the effect of removing the incorporated [^{14}C]AcNeu. The specificity of the commercial enzyme preparation in liberating cell surface-bound [^{14}C]AcNeu was investigated. Its hydrolyzing action was decreased by adding AcNeu, a known competitive inhibitor of some bacterial neuraminidases [15] suggesting that the effect is on terminal sialic acid residues. Since the incorporation of [^{14}C]AcNeu was distributed over most cells as determined by autoradiography and did not increase under conditions of decreased cell viability it is not mediated by cellular debris derived from the cell interior. These experiments demonstrate that at least 50—70% of the radioactivity representing the neuraminidase-susceptible pool in the acid precipitated intact cells after incubation with exogenous CMP-[^{14}C]AcNeu represent enzymatically incorporated [^{14}C]AcNeu bound to cell surface acceptor molecules.

The remaining label not released by a second treatment with neuraminidase, may constitute cell surface located [^{14}C]AcNeu covalently bound in linkages that are inaccessible or nonsusceptible to neuraminidase. The fact that the time course for the incorporation of "nonsusceptible" [^{14}C]AcNeu is linear and passes through the origin in long term incubations (Fig. 4 and 6) excludes the possibility of any significant unspecific adsorption in that pool. Furthermore, the linear time-dependent increase can not be ascribed to adsorption either, since 1 mM of unlabeled AcNeu did not influence the incorporation. Therefore, it is plausible that the "nonsusceptible" [^{14}C]AcNeu is also the result of enzymatic activity. Evidence that the neuraminidase-nonsusceptible pool of incorporated [^{14}C]AcNeu is located on the cell surface is afforded by long term incubations. A successive decrease of incorporation into both "susceptible" and "nonsusceptible" pools is then correlated with decreased cell viability and the appearance of agglutinates and increasing amounts of cell debris. If the incorporation were dependent on entrance of CMP-[^{14}C]AcNeu into the cells, the proportion of nonsusceptible to susceptible [^{14}C]AcNeu would be expected to change since the accessability of the intracellular organelles then involved is probably not the same when the functional state of the cell membrane is altered. The roughly parallel increase in the neuraminidase-susceptible and nonsusceptible pools indicates that similar mechanisms governing the saturation of these acceptors are operating and that the acceptors are equally accessible to the extracellular CMP-[^{14}C]AcNeu. Furthermore, ultrasonication although destroying the cell membrane decreased the susceptibility to neuraminidase. Therefore, if the nonsusceptible [^{14}C]AcNeu is located on the microsomal or nuclear membranes, this would lead to the rather improbable implication that all susceptible [^{14}C]AcNeu is located on the cell surface and the nonsusceptible

incorporation entirely confined to the cell interior. However, generally speaking, not all glycosidic bonds in which sialic acid participate can be expected to be attacked by neuraminidase from *C. perfringens* [15]. It is therefore highly probable that the "nonsusceptible" incorporation is located on the cell surface also. A minor fraction of the incorporation, extractable with chloroform/methanol may constitute gangliosides, a glycolipid fraction containing sialic acid that has been located to the plasma membrane in several other cell systems [17,18]. In conclusion these consideractions strongly support the view that all incorporated [^{14}C]AcNeu is bound to the cell surface.

Since participation of any extracellular soluble enzyme in the sialyltransferase reaction has been ruled out as discussed earlier (cf. Table I) two possible sites for the sialyltransferase remain. It may be intracellular, either soluble or structure-bound, or it may be located in the cell membrane as an ectoenzyme. A location inside the cell would implicate a flow of both activated [^{14}C]AcNeu and sialyzed acceptors because the ultimate acceptors are located on the cell surface. There are three lines of evidence against this possibility. Firstly, chase experiments failed to demonstrate any flow of activated sialic acid through cellular compartments. Secondly, there was no lag of incorporation even during the first minute of incubation. Thirdly, pretreatment with exogenous galactose-oxidase diminished the incorporation suggesting that the acceptors are situated on the cell surface not only after, but also before incubation with CMP-[^{14}C]-AcNeu. A sialyl transfer inside the cell would thus implicate a very complex mechanism probably involving endo and exocytosis. However, these combined processes would most probably require energy and it was observed that under some conditions even strong metabolic inhibitors such as 2 mM sodium arsenite only slightly inhibited the sialyl transfer. Hence, the present data strongly indicate that both the enzymes and acceptor molecules mediating the sialyl transfer in a suspension of whole Ehrlich tumour cells incubated with exogenous CMP-[^{14}C]AcNeu are located on the surface of intact cells.

Acknowledgements

This work was generously supported by Drs. G. Ronquist and G. Ågren through grants from the Swedish Medical Research Council (Project B75-13X-228-11), by Svenska Sällskapet för Medicinsk forskning, The University of Uppsala and a personal grant from Uddeholms AB.

References

1 Shur, B.D. and Roth, S. (1975) Biochim. Biophys. Acta 415, 473—512
2 Patt. L.M. and Grimes, W.J. (1974) J. Biol. Chem. 249, 4157—4165
3 Deppert, W., Werchau, H. and Walter, G. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3068—3072
4 Adamany, A.M. and Spiro, R.G. (1975) J. Biol. Chem. 250, 2842—2854
5 Peterson, P.A., Rask, L., Helting, T., Östberg, L. and Fernstedt, Y. (1976) J. Biol. Chem. 251, 4986—4995
6 Datta, P. (1974) Biochemistry 13, 3987—3991
7 Porter, C.W. and Bernacki, R.I. (1975) Nature 256, 648—650
8 Kean, E.L. (1972) in Complex Carbohydrates, (Ginsburg, V., ed.), Part B, pp. 413—422
9 Kean, E.L. and Roseman, S. (1966) J. Biol. Chem. 241, 5643—5650
10 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975

11 Gahmberg, C.G. and Hakomori, S. (1973) J. Biol. Chem. 248, 4311—4317
12 Egawa, K., Choi, Y.S. and Tanino, T. (1975) Japan, J. Exp. Med. 45, 155—159
13 Svennerholm, L. (1963) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. VI, pp. 453—459
14 Cervén, E., Ronquist, H. and Ågren, G. (1975) Upsala J. Med. Sci. 80, 58—60
15 Gottschalk, A. and Drzeniek, R. (1972) Glycoproteins (Gottschalk, A., ed.), Part A, pp. 381—402, Elsevier Publishing Company, Amsterdam
16 Nordling, S. and Mayhew, E. (1966) Exp. Cell. Res. 44, 552—562
17 Renkonen, O., Gahmberg, C.G., Simons, K. and Kääriäinen, L. (1970) Acta Chem. Scand. 24, 733—735
18 Brady, R.O. and Fishman, P.H. (1974) Biochim. Biophys. Acta 355, 121—148
19 Eagle, H.J. (1955) J. Exp. Med. 102, 595