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Sialic Acid Uptake by BHK Cells and Subsequent Incorporation Into Glycoproteins and Glycolipids

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BHK cells can be grown in the presence of growth medium to which radiolabeled sialic acid has been added. After 24 h, 85% of the radioactivity in the cells is covalently bound to glycoproteins and glycolipids. No metabolism of the radiolabeled sialic acid could be detected.

Key words: sialic acid uptake, sialoglycoproteins, sialoglycolipids

We recently reported that BHK cells grown in monolayers were permeable to sialic acid (1). It was also observed that after a 1-h incubation of the cells with sialic acid approximately 18% of the sialic acid that was associated with the cells was insoluble in phosphotungstic acid. This led us to speculate that a portion of the sialic acid became covalently bound to glycoproteins and glycolipids subsequent to its entry into the cell. In addition no metabolism of the radiolabeled sialic acid could be detected.

These observations stimulated a study to determine whether BHK cells could be grown for relatively long periods (24 h) in the presence of radiolabeled sialic acid to specifically label the cellular sialoglycoproteins and sialoglycolipids (2).

MATERIALS AND METHODS

Cells

BHK 21/13 cells were a gift of Dr. M. Green (St. Louis University). Cells were grown in Dulbecco's modification of Eagle's minimal essential medium (MEM) containing in addition 4 times the normal concentration of amino acids and vitamins (3). Cells were free of mycoplasma based on their level of uridine phosphorylase activity (4) and were counted in a Celloscope particle counter.

[^{3}H]-Sialic Acid

N-acetyl-[^{3}H]-neuraminic acid was synthesized enzymatically from [G-^{3}H]-N-acetyl D-mannosamine as previously described (1). The specific activity was 2.3 Ci/mmol. Radiochemical purity was at least 99.5% based on the criteria previously described (1).

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Time Course of Sialic Acid Uptake

Cells (3.9×10^5/35-mm plate) were grown in growth medium (1.3 ml; MEM containing 10% fetal calf serum) to which 12.5 μCi of [^{3}H]-sialic acid was added. At different times the medium was removed and the cells were rinsed 7 times with 2 ml of solution A (0.8% NaCl, 0.05% KCl, 0.001 M KPO_4, pH 7.4). Cold (4°C) phosphotungstic acid (PTA; 0.5 ml; 1% in 0.5 N HCl) was then added to each plate and the mixture was allowed to remain on ice for 15 min. The cells were then scraped with a rubber "policeman" into a 12-ml conical glass tube, and the plate was rinsed with an additional 0.5 ml of PTA. After centrifugation in a clinical centrifuge at maximum speed for 5 min the supernatant was removed and saved. The pellet was washed twice, each time with 0.5 ml of PTA. The pellet was dissolved in protosol (New England Nuclear Corporation) and the radioactivity in it and each supernatant fraction was determined as previously described (1). The cell number was determined at each time point in a parallel grown plate. After 32 h there were 2.0×10^6 cells/plate.

Identification of the Radioactivity Within Cells After 24 Hours Labeling

7.5×10^5 cells/35-mm plate were grown in growth medium (1.3 ml) to which [^{3}H]-sialic acid (12.5 μCi) had been added. After 24 h (9.4×10^5 cells/plate) the medium was removed and the cells were rinsed 7 times with 2 ml solution A. The 7th wash contained 200 dpm. Two milliliters of cold PTA was then added to the plate and allowed to remain on ice for 15 min. Cells were then scraped into a conical glass tube and the plate rinsed with an additional 1 ml of cold PTA. After centrifugation the supernatant (3,000 dpm) was removed and the pellet was washed with 1 ml of PTA. This supernatant contained 310 dpm. The pellet was washed with an additional 1 ml of PTA and after centrifugation this supernatant contained no radioactivity. The pellet was then washed twice, each time with 2 ml of water.

The pellet was suspended in H_2SO_4 (0.1 N; 0.75 ml) and heated for 1 h at 80°C with occasional stirring. After centrifugation at top speed in a clinical centrifuge, the supernatant was removed and saved. The pellet was washed with 0.25 ml of 0.1 N H_2SO_4 and after centrifugation the 2 supernatants were combined (42,000 dpm). The pellet was dissolved in protosol and the radioactivity (4,040 dpm) was determined as previously described (1). The supernatant was diluted 10-fold with water and applied to a Dowex-formate column. At least 90% of the radioactivity was characterized as sialic acid based on its behavior on this column and by ascending paper chromatography (1). The radioactivity which was soluble in PTA was applied to a Dowex-formate column after a 30-fold dilution with water. At least 90% behaved as sialic acid.

Biogel P-10 Chromatography

A PTA-insoluble pellet (700 μg of protein) was dissolved in 0.4 ml of sodium dodecyl sulfate (1% in 0.1 M phosphate buffer, pH 7.0; 0.5 ml) by boiling for 5 min. An aliquot of the sample (150 μl) was then applied to a Biogel P-10 column (13 × 1.0 cm) packed in the same buffer. We collected 0.5-ml fractions. For pronase treatment, 300 μg of pronase were added to an aliquot of the solubilized sample and the mixture was incubated at 37°C for 72 h. Every 12 h, an additional 300 μg of pronase was added to the reaction mixture.

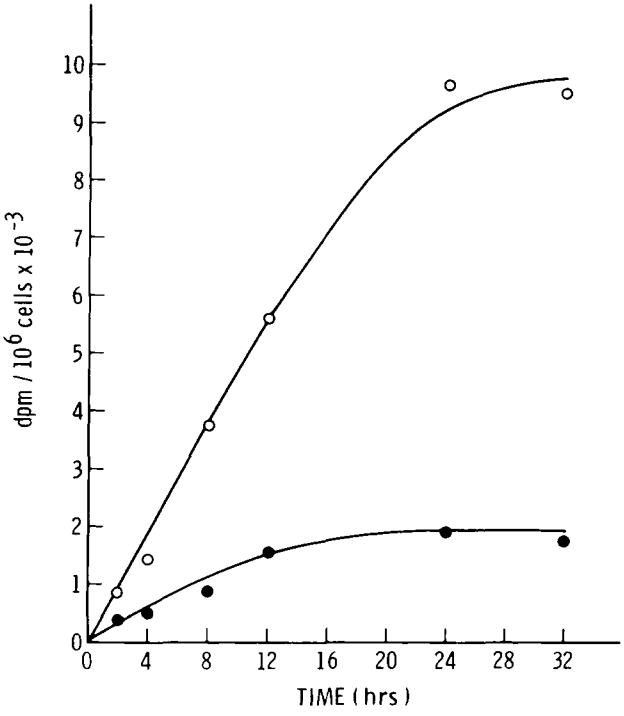

Fig. 1. Time course of [^{3}H]-sialic acid incorporation into BHK cells. Cells (3.9 X 10^5/35-mm plate) were grown in the presence of 12.5 μCi of [^{3}H]-sialic acid. The cells were washed and phosphotungstic acid (1% in 0.5 N HCl) soluble (•---•) and insoluble (○---○) radioactivity was determined as described under Methods.

SDS-Acrylamide Gel Electrophoresis

A PTA-insoluble sample (0.25 mg of protein) was solubilized in 50 μl of a mixture containing 0.05 M Na_2CO_3, 0.05 M dithiothreitol, 2% (wt/vol) sodium dodecyl sulfate, 12% (wt/vol) sucrose and 0.04% (wt/vol) bromophenol blue. Electrophoretic separation and subsequent staining, drying, and counting of gel slices (2 mm each) was performed as previously described (5).

Lipid Extraction

A PTA-insoluble pellet, from cells labeled for 24 h with radiolabeled sialic acid was dried under N_2. The pellet was extracted twice with 2 ml of chloroform-methanol 2:1. An aliquot was applied to a thin layer plate and developed in chloroform-methanol-water, 60:35:8, as previously described (6). Standard hematoside was obtained by growing cells with 1-[^{14}C]-palmitate (7).

RESULTS

Kinetics of [^{3}H]-Sialic Acid Incorporation Into Cells

Figure 1 shows that the PTA-insoluble radioactivity increased linearly for about 16 h

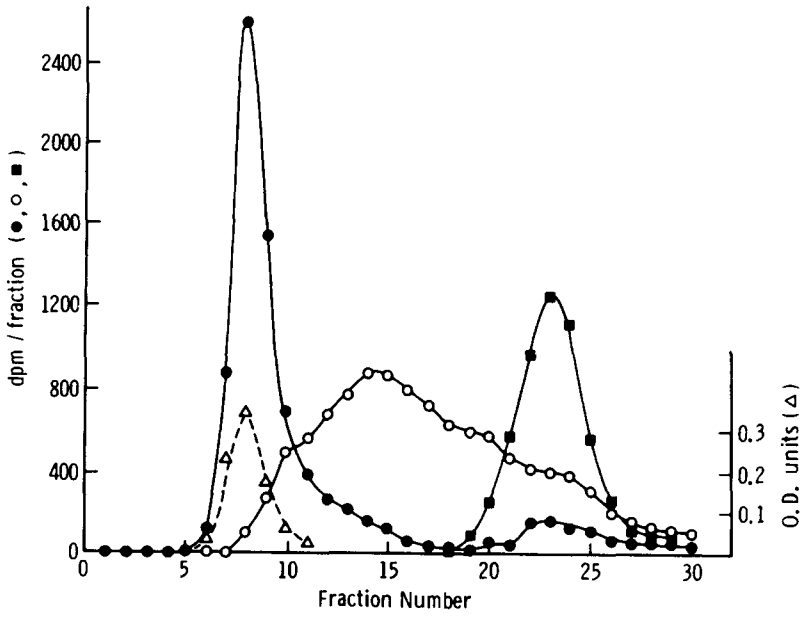

Fig. 2. Biogel P-10 chromatography. A phosphotungstic acid-insoluble pellet obtained from BHK cells which had been grown for 24 h in the presence of radiolabeled sialic acid was solubilized in 1% sodium dodecyl sulfate. An aliquot was applied to a Biogel P-10 column as described under Methods. Another aliquot was first treated with pronase (as described under Methods) and applied to the column. ●---●) phosphotungstic acid-insoluble, sodium dodecyl sulfate-soluble radioactivity; ○ ---○) the previous material incubated with pronase before being applied to the column; ■) standard [^{3}H]-sialic acid; △---△) blue dextran.

and became constant after 24 h. The soluble radioactivity had a similar profile. Most of the radioactivity within the cells was insoluble in phosphotungstic acid after 2 h incubation with 85% being so after 24 h.

Total radioactivity within cells was defined as that radioactivity which remained within the cells after removing the radioactive growth medium from the monolayer, followed by 7 washes with buffer. The radioactivity in the last 2 washes was constant and was never more than 3% of the total radioactivity that remained with the cells. An 8th wash with buffer to which 1 mM nonradiolabeled sialic acid had been added did not remove any additional radioactivity, suggesting that soluble radiolabeled sialic acid was not loosely bound to the cell surface. An additional correction was done to eliminate trapped radioactivity or insufficient washes as a source of error by subtracting from all time points the radioactivity obtained after a very short (less than 5 sec) incubation. This value was negligible for all time points longer than 2 h.

Identification of the Radiolabel Within the Cells

The phosphotungstic acid-soluble fraction of cells that had been grown for 24 h in the presence of radiolabeled sialic acid was analyzed for any metabolism of the radiolabel. At least 90% of the radioactivity was characterized as sialic acid based on its behavior on Dowex-formate chromatography (1). This suggests that if any metabolism of the radiolabel did occur, it must have been very small. It is possible the phosphotungstic acid-

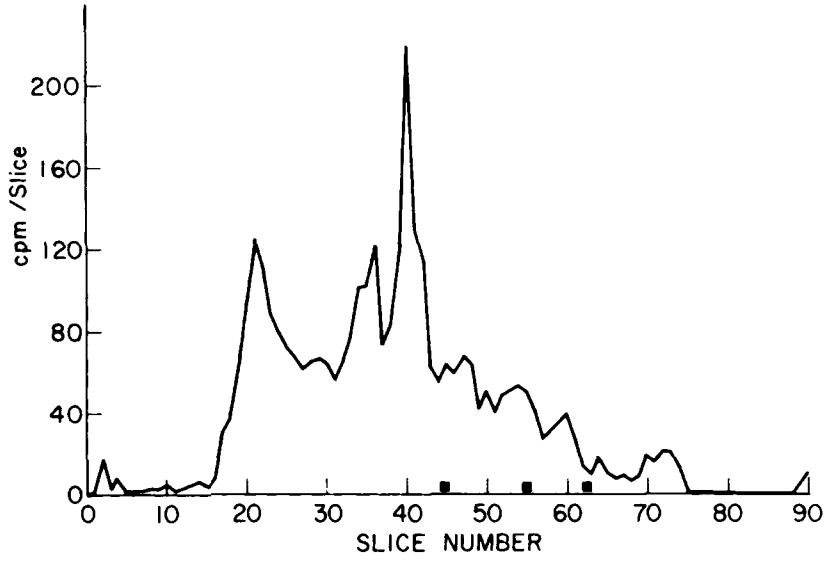

Fig. 3. SDS-acrylamide gel electrophoresis of a phosphotungstic acid-insoluble pellet of BHK cells which had been grown for 24 h in the presence of [^{3}H]-sialic acid. Slices 1–12 were the stacking gel. The dye marker ran in slice 88. The migration of the following standards is shown: bovine serum albumin, 68.5 × 10^3 daltons; ovalbumin, 43 × 10^3 daltons; and DNase, 31 × 10^3 daltons.

soluble radioactivity was originally present not only as free sialic acid but in another sialic acid-containing compound since the HCl, as part of the PTA reagent, may have cleaved a labile bond of such a compound yielding free radiolabeled sialic acid. Incubation of the PTA-insoluble pellet (obtained from cells grown in the presence of [^{3}H]-sialic acid for 24 h), with 0.1 N H_2SO_4 at 80°C for 60 min, solubilized over 90% of the radioactivity. These are the conditions which cleave the glycosidic linkage between sialic acid and other sugars (8). No solubilization of the radioactivity occurred if prior to heating the pellet was incubated for 1 h in cold acid.

Characterization of the Phosphotungstic Acid Insoluble Pellet

When a pellet (from cells that had been grown for 24 h in the presence of radiolabeled sialic acid) was solubilized in 1% sodium dodecyl sulfate (SDS) and subsequently applied to a Biogel P-10 column, all the radioactivity eluted with the void volume (Fig. 2). Pronase digestion of the material caused a shift of the radioactivity profile with most of the radioactivity now being retained by the column although not as long as standard free sialic acid (Fig. 2). SDS-acrylamide gel electrophoresis of the SDS-solubilized (PTA-insoluble pellet) showed comigration of the radioactivity with numerous protein bands throughout the gel (Fig. 3). Free sialic acid runs slightly behind the dye marker in this system. Extraction of the dried, PTA-insoluble pellet with chloroform-methanol 2:1 and subsequent thin layer chromatography of the extract, indicated that approximately 5% of the radioactivity, that was originally in the pellet, comigrated with hematoside. No radioactivity remained at the origin of the plate, strongly suggesting that the sample did not contain free sialic acid.

DISCUSSION

This paper describes the specific labeling of sialoglycoproteins and sialoglycolipids of BHK cells grown in the presence of radiolabeled sialic acid. The major drawback of the method is the relatively low efficiency of uptake of the radiolabel. Radiolabeled glucosamine has been extensively used in other studies to label cellular sialoglycoproteins and sialoglycolipids (9–11). However, this procedure is nonspecific towards sialoglycoproteins, particularly if the labeling is done for long periods. Radiolabeled N-acetylmannosamine has been shown by Harms et al. (12) to be a more specific precursor for labeling sialic acid moieties although significant metabolism to other compounds was also observed. A chemical procedure which has been successfully used to label sialoglycoproteins of red blood cells (13) has (as yet) not been reported to be applicable to other cells.

One important aspect of this study is the observation that no significant metabolism of the radiolabel in sialic acid (both soluble and in glycoproteins and glycolipids) could be observed, even after relatively long labeling, such as 24 h. The possibility that the observed sialic acid binding is not covalent but rather of a very tight, nonspecific nature seems unlikely for the following reasons: a) the PTA-insoluble radioactivity is solubilized by treatment with acid under conditions which are known to cleave sialic acid covalently bound to glycoproteins and glycolipids (8) (0.1 N H_2SO_4 80°, 60 min); cold sulfuric acid did not solubilize the radioactivity; b) pronase treatment of the PTA-insoluble pellet causes a shift of the radioactivity to a profile (on Biogel P-10) expected for a heterogeneous mixture of glycopeptides, rather than free sialic acid which could be expected if sialic acid were only tightly bound; c) the mobility of the chloroform-methanol soluble radioactivity on thin layer chromatography in a solvent system where free sialic acid does not migrate, is also incompatible with a noncovalent linkage of sialic acid (in this case to glycolipids); d) the radioactivity profile on SDS-acrylamide gels argues against noncovalent binding of free sialic acid to one particular protein. The fact that this behavior of sialic acid has also been observed with very different cells, mammalian and avian, established cell lines and secondary chick embryo fibroblasts and more recently in liver and kidney of mice (unpublished results) strongly suggests that this is a general phenomenon occurring in a variety of eukaryotic cells.

Evidence that most of the covalently bound radiolabeled sialic acid is in sialoglycoproteins comes from several experiments. One is the insolubility of the radioactivity in a solvent which is known to precipitate glycoproteins (1% PTA in 0.5 N HCl); another is the result of the pronase treatment of the PTA-insoluble pellet and the subsequent behavior of the radioactivity on Biogel P-10. The resulting profile is consistent with this being a heterogeneous mixture of glycopeptides of smaller size than the non-pronase-treated glycoproteins. In addition the radioactivity profile on SDS-acrylamide gel electrophoresis was similar to the labeling patterns obtained in other studies where cells were grown in the presence of radiolabeled sugars and the proteins subsequently separated by the above procedure (14–16).

Although analyses of the PTA-soluble material have shown radiolabeled sialic acid as the sole product when cells are grown in the presence of radiolabeled sialic acid, we cannot completely rule out the possibility that cleavage by a sialic acid aldolase (17) followed by resynthesis may have occurred. However, for such a mechanism to take place one would have to postulate a very low concentration of the intermediate radiolabeled N-acetylmannosamine. An unequivocal answer to this problem can be obtained by using a mixture of [1-^{14}C]- and [4,5,6,7,8,9-^{3}H]-N-acetyl neuraminic acid as precursor; such

an experiment is currently in progress.

The physiological significance of both the uptake and the subsequent incorporation of sialic acid into cells is not clear. A recent study (18) has shown the existence of neuraminidase activity in serum. This enzyme could act upon surrounding sialoglycoproteins and sialoglycolipids liberating free sialic acid which may then enter the cells. However, at this time such a mechanism is only speculative.

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NOTE ADDED IN PROOF

Recent results with a mixture of 1-[^{14}C]-sialic acid and 4,5,6,7,8,9-[^{3}H]-sialic acid strongly suggest that there is no cleavage of the sialic acid backbone in the above described experiments.

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