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Preparation of 9-fluoro-9-deoxy-*N*-[2-^{14}C]acetylneuraminic acid

Activation and transfer onto asialo-α_1-acid glycoprotein

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9-Fluoro-9-deoxy-*N*-[2-^{14}C]acetylneuraminic acid has been prepared from 6-fluoro-6-deoxy-*N*-acetylmannosamine and [2-^{14}C]pyruvic acid for the first time, using *Clostridium perfringens* *N*-acetylneuraminate pyruvate-lyase (EC 4.1.3.3). The fluoro sugar was activated by CMP-*N*-acetylneuraminic acid synthase and CTP to yield CMP-9-fluoro-9-deoxy-*N*-[2-^{14}C]acetylneuraminic acid. Both products were obtained in good yield (60 and 30%, respectively). The radioactive sugar in its activated form is glycosidically attached to asialo-α_1-acid glycoprotein by sialyltransferase and can be removed by the action of *Vibrio cholerae* sialidase. The reaction rates of the enzymes studied are lower with the 9-fluoro derivatives than with the *N*-acetylneuraminic acid substrates.

*9-Fluoro-9-deoxy-*N*-acetylneuraminic acid* *Sugar activation* *Radioactive sugar analog* *Sialyltransferase* *Enzyme specificity* *Sialoglycoprotein*

1. INTRODUCTION

Sugar analogues as inhibitors of glycoprotein synthesis have recently attracted considerable interest. Blocking the *N*-glycosylation of viral glycoproteins at the level of lipid-linked oligosaccharide formation by carbohydrate analogues has been extensively studied and the analysis of their metabolism has been performed in some detail (review [1]).

No information, however, is available about substances interfering with the processing and terminal glycosylation of *N*-glycosidically bound oligosaccharides of proteins. Analogues of *N*-acetylneuraminic acid may provide a valuable tool to study the final assembly and destination of cellular sialoglycoproteins.

The 9-fluoro derivative of *N*-acetylneuraminic acid has been synthesized enzymatically [2] and chemically [2,3]. Its enzymatic activation to CMP-9-fluoro-*N*-acetylneuraminic acid has been reported [4] when our studies were in progress. We here report for the first time on the preparation of (2-^{14}C)-labelled 9-fluoro-9-deoxy-*N*-acetylneuraminic acid and provide further evidence for the biochemical activities of this fluoro sugar in vitro.

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; ManNAc, *N*-acetylmannosamine; TLC, thin-layer chromatography

2. MATERIALS AND METHODS

2.1. *Materials*

Crystalline *N*-acetylneuraminic acid was prepared in our laboratory. 6-Fluoro-6-deoxy-*N*-acetylmannosamine was synthesized as in [5] using a greatly simplified procedure (unpublished) and unlabelled 9-fluoro-9-deoxy-*N*-acetylneuraminic acid as in [2]. [2-^{14}C]Pyruvic acid (15.8 mCi/mmol)

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was obtained from Amersham Buchler (Braunschweig, FRG), CMP-*N*-[G-^{3}H]acetylneuraminic acid (18.9 Ci/mmol) was purchased from NEN (Dreieich, FRG). *N*-[G-^{3}H]Acetylneuraminic acid was prepared by mild hydrolysis from CMP-*N*-[G-^{3}H]acetylneuraminic acid in 0.1 M acetic acid for 1 h at 60°C and subsequent isolation of the free sugar by paper chromatography. *V. cholerae* sialidase (neuraminidase) was from Behringwerke (Marburg, FRG) and *Clostridium perfringens N*-acetylneuraminate pyruvate-lyase grade III was from Sigma (München, FRG). α_1-Acid glycoprotein was prepared by Drs G. Keilich and D. Ziegler in this laboratory. Desialylation was performed essentially as in [6]. CTP, lactate dehydrogenase and NADH were from Boehringer (Mannheim, FRG).

2.2. *Paper chromatography and TLC of labelled compounds*

Chromatographic separations were performed on silica gel sheets, cellulose plates and on Whatman 3 MM paper (descending direction) using the following solvent systems: solvent A, propanol–acetic acid–water (5:2:2, by vol.); solvent B, ethanol–0.5 M ammonium acetate, pH 7.4 (7:3, v/v); solvent C, ethanol–0.1 M NH_4OH (5:2, v/v). Radioactive material was detected by autoradiography on LKB Ultrofilm ^{3}H (LKB, Gräfeling, FRG) or by radioscanning with a Berthold TLC scanner. For quantitation, radioactive material was scrapped off from the plates, or paper chromatograms were cut in 1-cm strips and radioactivity was counted.

Elution of radioactive compounds from paper chromatograms was in descending direction, with 0.04 M NH_4OH for the nucleotide sugar and water for *N*-acetylneuraminic acid.

Purity was checked before assay, and repurification was achieved by the above methods when necessary.

2.3. *Enzymic synthesis of 9-fluoro-9-deoxy-*N*-[2-^{14}C]acetylneuraminic acid*

9-Fluoro-*N*-[2-^{14}C]acetylneuraminic acid was synthesized from 6-fluoro-*N*-acetylmannosamine and [2-^{14}C]pyruvic acid by a modification of the procedure in [7] for the synthesis of *N*-acetylneuraminic acid.

The reaction mixture contained in a final volume of 260 μl: 12.7 μmol [2-^{14}C]pyruvic acid (200 μCi), 3.1 μmol 6-fluoro-*N*-acetylmannosamine, 12.5 μmol Na-phosphate buffer (pH 7.0) and 0.33 units *C. perfringens N*-acetylneuraminate pyruvate-lyase. After incubation at 37°C for 2 h, this solution was treated with 5 ml ice-cold ethanol, and the precipitate was removed by centrifugation; 9-fluoro-9-deoxy-*N*-[2-^{14}C]acetylneuraminic acid was obtained from the supernatant after chromatography on a DEAE–Sephadex A-25 column and final purification by paper chromatography in solvent B. The overall yield after purification was 7.95 μmol 9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid (15.8 μCi/μmol, 60%, referring to the initial amount of pyruvate). Upon TLC on silica gel sheets in solvent A and autoradiography of the plates only one single radioactive spot was detected, co-migrating with authentic 9-fluoro-*N*-acetylneuraminic acid.

2.4. *Studies with CMP-acylneuraminate synthase*

The enzyme was partially purified from rat liver (unpublished). The synthase used here contained a CMP-*N*-acetylneuraminic acid synthesizing activity of 77 munits/mg protein. One unit is defined as the amount of enzyme producing 1 μmol CMP-*N*-acetylneuraminic acid per min under the following conditions in 0.2 ml: 2 μmol *N*-acetylneuraminic acid, 2 μmol CTP, 0.5 μmol dithioerythritol, 4 μmol $MgCl_2$, 40 μmol Tris–Cl (pH 9.1) and the enzyme at 37°C. Product formation was determined by the $NaBH_4$/thiobarbituric acid method [8].

For kinetic studies, the incubation mixtures contained the concentrations above, radiolabelled *N*-[9-^{3}H]acetylneuraminic acid or its 9-fluoro-[2-^{14}C]analogue and 0.135 mg enzyme (10 milliunits) in 0.1 ml. Enzyme activity was determined using a radiochemical test: the reaction was stopped by the addition of 0.2 ml cold ethanol, centrifuged and aliquots of the supernatant were analysed by TLC on cellulose sheets in solvents B or C. Areas corresponding to the free sugar and the CMP-derivative were scraped off and radioactivity was determined separately by liquid scintillation counting. Counts per min (cpm) were corrected for desintegrations per min (dpm) by adding [^{14}C]toluene or [^{3}H]toluene as internal standards.

For preparative activation of 9-fluoro-[2-^{14}C]acetylneuraminic acid, incubation mixtures contained, in 0.335 ml, 2.4 μmol of the sugar

analogue (37.5 μCi), 4 μmol CTP and 0.1 unit synthase. After 1 h 2 μmol CTP was added, incubation was continued for 2 h and the protein was precipitated with 2 ml ice-cold ethanol. The nucleotide sugar was isolated as in [9] and final purification was achieved by means of paper chromatography in solvent C. The purity of CMP-9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid was checked according to the criteria in [10] and by autoradiography.

2.5. *Sialyltransferase measurements*

As a source of sialyltransferase (EC 2.4.9.9) a rat liver microsomal fraction was prepared essentially as in [6], except that the final membrane preparation was dissolved in 20 mM Mes buffer (pH 6.4) plus 0.25% Triton X-100.

The transferase activity was measured at 37°C in 200 μl assay volume containing: 32.5 μM CMP-*N*-[^{3}H]acetylneuraminic acid (600000 dpm) or CMP-9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid (32.5 μM = 300000 dpm), 200 μg asialo-α_1-acid glycoprotein, 20 μM Mes buffer (pH 6.4), 0.25% Triton X-100 (by vol.) and enzyme preparation. After desired time periods, protein-bound radioactivity was determined after precipitation with ice-cold 1% phosphotungstic acid in 0.5 N HCl.

For further studies with *V. cholerae* sialidase, radiolabelled α_1-acid glycoprotein was isolated by gel filtration on a 1.6 × 30 cm column of Sephadex G-75.

2.6. *Studies with* V. cholerae *sialidase*

The action of *V. cholerae* sialidase on radiolabelled α_1-acid glycoprotein was measured in 0.3 ml incubation mixtures containing: 0.025 units *V. cholerae* sialidase (Behringwerke), 17.5 μmol sodium acetate (pH 5.5) and *N*-[9-^{3}H]acetylneuraminic acid or 9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid labelled α_1-acid glycoprotein isolated by gel filtration as described above. At given times (fig.4), aliquots were removed and the protein was precipitated with phosphotungstic acid (PTA) as described for the sialyltransferase assay. The protein pellet was washed 2 times with ice-cold PTA, the supernatants were combined and radioactivities associated with protein and the acid-soluble fractions were determined separately.

3. RESULTS

3.1. *Synthesis of radiolabelled 9-fluoro-*N*-acetylneuraminic acid*

9-Fluoro-*N*-[2-^{14}C]acetylneuraminic acid was obtained from 6-fluoro-*N*-acetylmannosamine and [2-^{14}C]pyruvic acid in the presence of *C. perfringens* acylneuraminate pyruvate-lyase in 60% yield. The final product was found pure based on autoradiographic analysis after TLC in various systems. Furthermore, cleavage of the compound with the *N*-acetylneuraminic acid pyruvate-lyase and subsequent trapping of the pyruvate with lactate dehydrogenase and NADH yielded virtually all radioactivity in L-lactate (isolated by paper chromatography).

3.2. *Enzymic activation of 9-fluoro-*N*-[2-^{14}C]-acetylneuraminic acid*

The activation of unlabelled 9-fluoro-*N*-acetylneuraminic acid by a calf brain CMP-sialate synthase has been reported only recently [4]. Here, a highly active enzyme preparation from rat liver was employed for the synthesis of radiolabelled CMP-9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid. The overall yield of CMP-9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid was 30% after purification. Cytidine, 9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid and phosphate were recovered at mol ratios of 0.95:1.0:1.02 from the final product. With the above quoted radiochemical enzyme assay, K_m values for *N*-acetylneuraminic acid and 9-fluoro-*N*-acetylneuraminic acid of 0.78 ± 0.05 and 4.35 ± 0.15 mM, respectively, were determined from initial velocity studies using Lineweaver-Burke plots ($1/v$ *vs* $1/s$, fig.1).

3.3. *Transfer to sialic acid acceptors*

Using CMP-9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid and CMP-*N*-[9-^{3}H]acetylneuraminic acid as substrates, and asialo-α_1-acid glycoprotein as acceptor along with the microsomal enzyme preparation, 9-fluoro-*N*-acetylneuraminic acid is transferred at a lower rate than *N*-acetylneuraminic acid at nearly saturating concentrations of CMP-*N*-acetylneuraminic acid (fig.3). Even at a 4 times higher nucleotide sugar concentration the transfer rate of the analogue is only 2/3 as compared to the naturally occurring compound. This difference is

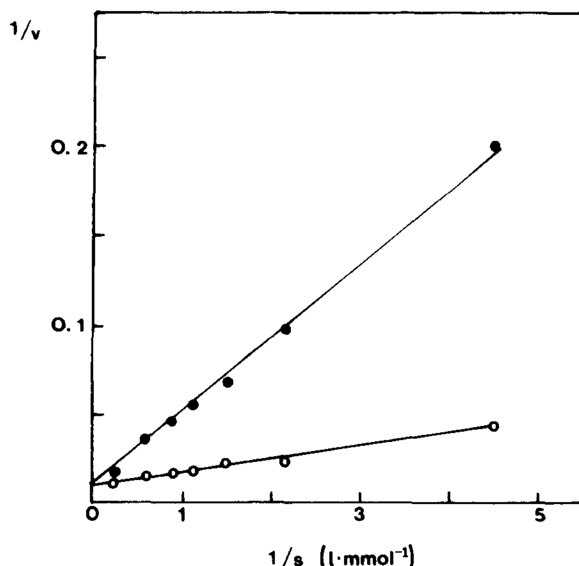

Fig.1. Kinetics of *N*-[^{3}H]acetylneuraminic acid and 9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid activation. Initial velocities are plotted as functions of *N*-[^{3}H]acetylneuraminic acid concentration (○—○) and 9-fluoro-*N*-[^{14}C]acetylneuraminic acid concentration (●—●). Product formation was measured by the radiochemical test. Velocity is expressed as product formed within 10 min in 0.1 ml at a protein concentration of 0.135 mg. The calculation of K_m for *N*-acetylneuraminic acid as substrate afforded a second plot with a reduced enzyme concentration (not shown).

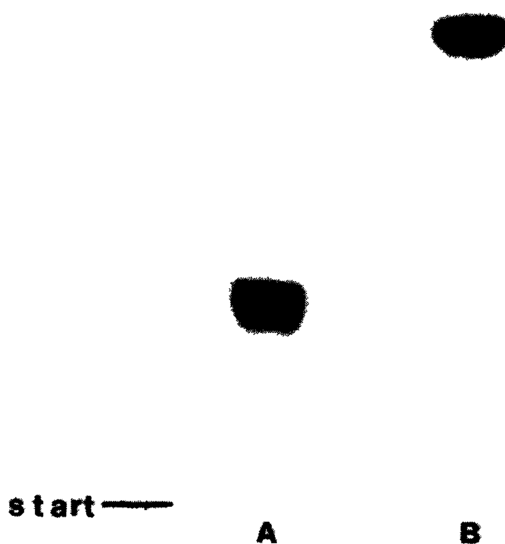

Fig.2. Autoradiography of a thin-layer chromatogram of purified 9-fluoro-*N*-[^{14}C]acetylneuraminic acid (A) and CMP-9-fluoro-*N*-[^{14}C]acetylneuraminic acid (B) run in solvent B. Authentic *N*-acetylneuraminic acid and its corresponding sugar nucleotide both have a slightly lower mobility as compared to the 9-fluoro derivatives.

not due to a greater instability of the activated fluoro-derivative under our reaction conditions. TLC analysis of reaction mixtures revealed that less than 8% *N*-acetylneuraminic acid or 9-fluoro-*N*-acetylneuraminic acid is liberated from the nucleotide sugar after 1 h of incubation.

3.4. *9-Fluoro-*N*-[2-^{14}C]acetylneuraminic acid α_1-acid glycoprotein as a substrate for* V. cholerae *sialidase*

When *N*-[9-^{3}H]acetylneuraminic acid or 9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid-labelled α_1-acid glycoprotein was subjected to acid hydrolysis in 0.05 N H_2SO_4 for 1 h at 80°C, 85% of the released radioactivity was identified as free *N*-acetylneuraminic acid or 9-fluoro-*N*-acetylneuraminic acid upon TLC in solvents A and B.

The action of *V. cholerae* sialidase on 9-fluoro-*N*-[2-^{14}C]acetylneuraminic acid- and *N*-[9-^{3}H]ace-

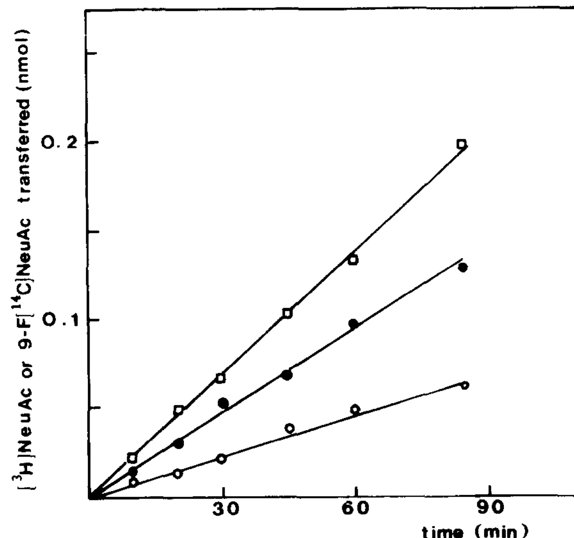

Fig.3. Incorporation of *N*-[^{3}H]acetylneuraminic acid and 9-fluoro-*N*-[^{14}C]acetylneuraminic acid in α_1-acid glycoprotein by rat liver sialyltransferase. 32.5 μM CMP-*N*-[^{3}H]acetylneuraminic acid (6 × 10^5 dpm) (□—□), 65 μM CMP-9-fluoro-*N*-[^{14}C]acetylneuraminic acid (6 × 10^5 dpm) (○—○), 130 μM CMP-9-fluoro-*N*-[^{14}C]acetylneuraminic acid (1.2 × 10^6 dpm) (●—●). After the indicated times, aliquots were removed from incubations and acid-precipitable radioactivity was determined.

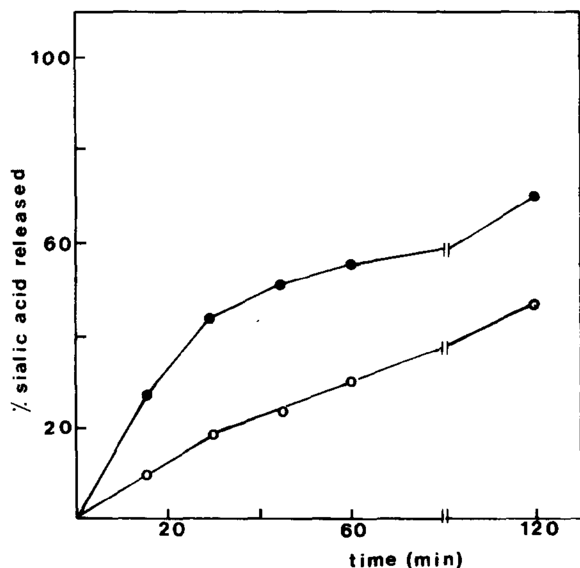

Fig.4. Susceptibility of glycosidically linked radioactivity of α_1-acid glycoprotein to *V. cholerae* sialidase. (○—○) 9-Fluoro-*N*-[^{14}C]acetylneuraminic acid-α_1-acid glycoprotein (200 μg) containing 170 pmol bound 9-fluoro-*N*-acetylneuraminic acid (7000 dpm); (●—●) *N*-[^{3}H]acetylneuraminic acid-α_1-acid glycoprotein (200 μg) containing 170 pmol *N*-acetylneuraminic acid (14000 dpm).

tylneuraminic acid-labelled α_1-acid glycoprotein is shown in fig.4. The ^{14}C-label is removed from the sialylated protein at 1/2 of the rate for *N*-[^{3}H]acetylneuraminic acid when the same substrate concentrations are present on the same amount of glycoprotein. Similar results were obtained when the corresponding sialates of fetuin were applied, although the total amount of radioactivity that was transferred by sialyltransferase was reduced (about 1/3) and could only incompletely be removed by the sialidase (not shown).

4. DISCUSSION

9-Fluoro-*N*-[^{14}C]acetylneuraminic acid is readily prepared from 6-fluoro-*N*-acetylmannosamine and radioactive pyruvic acid in the presence of *C. perfringens* acylneuraminate pyruvate-lyase. These results confirm the findings in [11] that the 'tail' portions of *N*-acetylneuraminic acid (carbon 9) and of *N*-acetylmannosamine (carbon 6), respectively, are not essential for enzyme activity. The 8-carbon analogue of *N*-acetylneuraminic acid was obtained from pyruvate and 2-acetamido-2-deoxy-D-lyxose with the bacterial enzyme. Similarly the 9-azido-9-deoxy-*N*-acetylneuraminic acid was synthesized from 6-azido-6-deoxy-*N*-acetylmannosamine and phospho*enol*pyruvate with the *Neisseria meningitidis* *N*-acetylneuraminic acid synthase [12].

As the reaction rates of all enzymes involved in *N*-acetylneuraminic acid metabolism is decreased with 9-fluoro-*N*-acetylneuraminic acid as a substrate in the herein described in vitro system, we speculate that the 9-fluoro derivative may represent a tool for the investigations of *N*-acetylneuraminic acid metabolism in whole cells. The uptake of *N*-acetylneuraminic acid and subsequent incorporation into glycoproteins by mammalian cells has been demonstrated [9].

The 9-fluoro-*N*-acetylneuraminic acid may interfere in vivo with sialic acid metabolism at the level of:

(i) the pool size of CMP-*N*-acetylneuraminic acid;
(ii) sugar transfer onto asialoglycoproteins and glycolipids;
(iii) insertion of glycolipids and sialoglycoproteins into cell membranes or secretion of sialoglycoproteins;
(iv) catabolism of sialoglycoconjugates.

Investigations to elucidate such a probable role of 9-fluoro-*N*-acetylneuraminic acid will be greatly facilitated by this radiolabelled sugar derivative. This work is now in progress.

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