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GLYCOSYLTRANSFERASES OF THE HUMAN CERVICAL EPITHELIUM

II. CHARACTERIZATION OF A CMP-*N*-ACETYLNEURAMINATE: GALACTOSYL-GLYCOPROTEIN SIALYLTRANSFERASE

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GMP-*N*-Acetylneuraminate: galactosyl-glycoprotein sialyltransferase (CMP-*N*-acetylneuraminate: D-galactosyl-glycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) activity was identified in the human cervical epithelium. The enzyme has a pH optimum of 6.0, a temperature optimum of 28°C, and demonstrates a partial requirement for Triton X-100. Michaelis constants for asialofetuin and CMP-*N*-acetyl[^{14}C]neuraminic acid are $0.64 \cdot 10^{-5}$ M (expressed as the concentration of terminal galactose residues) and $2.05 \cdot 10^{-5}$ M, respectively. Sialyltransferase demonstrated minimal affinity for the low molecular weight acceptors tested, and may have a requirement for a glycoprotein acceptor having a terminal *N*-acetyllactosamine (Galβ(1 → 4)GlcNAc) type structure. Cytidine nucleotides are potent inhibitors of the sialyltransferase reaction; CMP acts as a competitive inhibitor.

Introduction

A reciprocal relation between levels of sialic acid and L-fucose has been recognised in many epithelial glycoproteins (mucins) [1], which may be due to the mutually exclusive addition of these carbohydrates during oligosaccharide synthesis [2]. During the menstrual cycle, the glycoprotein responsible for the visco-elastic properties of human cervical mucus demonstrates a cyclic variation in its proportion of *N*-acetylneuraminic acid and L-fucose. Early work suggested that levels of human cervical mucin *N*-acetylneuraminic acid were maximal during the luteal phase, while levels of L-fucose were at a minimum [3]. The charge present on *N*-acetylneuraminic acid in mucin at physiological pH was considered to be a factor controlling the rheological properties of cervical mucus [4]. However, Chantler and Debruyne [5] and Hatcher et al. [6] have reported that cervical mucin sialic acid levels are maximal during the periovulatory, and not the late luteal phase, while Von Kooij et al. [7] observed no cyclic variation in the carbohydrate composition of human cervical mucin.

The incorporation of *N*-acetylneuraminic acid into the mucin molecule is accomplished by a group of enzymes, the CMP-*N*-acetylneuraminate: galactosyl-glycoprotein sialyltransferases. Sialic acid is terminally located in mucin type glycoproteins and is commonly found linked $\alpha(2 \rightarrow 3)$ with a sub-terminal galactose residue and $\alpha(2 \rightarrow 6)$ with an *N*-acetylgalactosamine residue that is *O*-glycosidically linked with a serine residue of the protein core [8]. Both of these structures have been identified in the mucin isolated from bonnet monkey (*Macaca radiata*) cervical mucus [9]. Sialyltransferases specific for the synthesis of these structures have been purified from porcine submaxillary gland and fully characterised [10–12].

As part of a study of the control of *N*-acetyl-

neuraminic acid and L-fucose metabolism in human cervical mucus glycoprotein, an investigation of the occurrence and properties of sialyltransferases of the cervical epithelium was undertaken. This paper describes the characterization of a cervical CMP-*N*-acetylneuraminate: galactosyl-glycoprotein sialyltransferase.

Materials and Methods

CMP-*N*-Acetyl [4,5,6,7,8,9-^{14}C]neuraminic acid (11.2 GBq · mmol^{-1}) and potassium boro[^{3}H]-hydride (37.5 GBq · mmol^{-1}) were purchased from the Radiochemical Centre, Amersham, U.K. Fetal calf fetuin, monosaccharides, nucleotides and commonly used reagents were from Sigma. Human α_1 acid glycoprotein was supplied by Miles Laboratories. *N*-Acetyllactosamine was a gift from Dr. A. Gauhe.

Preparation of glycoprotein acceptors. Asialofetuin and asialo α_1 acid glycoprotein were prepared as previously described [13] and contained 0.24 and 0.36 μmol terminal galactose residues/mg glycoprotein, respectively, (calculated assuming that each mol glycoprotein galactose is linked sub-terminally to 1 mol *N*-acetylneuraminic acid). Agalacto-asialofetuin was prepared by Smith degradation of asialofetuin [14] and contained 0.16 μmol terminal *N*-acetylglucosamine residues/mg.

Preparation of tissue homogenate. Cervical tissue, obtained at hysterectomy, was homogenised in 0.25 M sucrose, pH 7.0 [13].

Sialyltransferase assay. The standard incubation mixture contained asialofetuin, CMP-*N*-acetyl[^{14}C]-neuraminic acid, buffer, Triton X-100 and tissue homogenate in a final volume of 60 μl as described in Table I. Duplicate assays were incubated at 37°C for 20 min. Control incubations which lacked asialofetuin were included for all assays. The reaction was terminated by the addition of 10 μl aqueous $Na_2B_4O_7$ (30 g · l^{-1}) and an aliquot of the reaction mixture applied to Whatman 3 MM chromatography paper and subjected to high voltage electrophoresis for 1 h at 30 V · cm^{-1} in aqueous $Na_2B_4O_7$(10 g · l^{-1}), pH 9.2. After electrophoresis the paper was dried, cut into strips, added to 15 ml Permablend III scintillant (Packard) and counted.

Preparation of [^{3}H]sialic acid-fetuin. The sialic acid residues of fetuin were specifically labelled with tritium by oxidation with a low concentration of periodate, followed by reduction with potassium boro[^{3}H]hydride [15]. The tritium-labelled fetuin had a specific activity of 4.3 · 10^7 Bq · μmol^{-1} sialic acid.

Protein. Total protein was measured by the method of Lowry et al. [16] using bovine serum albumin as a standard.

Results

The rate of incorporation of *N*-acetyl[^{14}C]-neuraminic acid into asialofetuin demonstrated a distinct biphasic curve. Incorporation was constant for up to 30 min, above which the reaction rate decreased by almost 90% and then remained constant for at least 210 min (Fig. 1). The sialyltransferase reaction rate was proportional to the homogenate protein concentration to an assay concentration of at least 1 g · l^{-1}. Fig. 2 shows the pH profile of sialyltransferase activity which demonstrated a well defined pH optimum of 6.0. The optimum temperature for the incorporation of *N*-acetyl[^{14}C]-neuraminic acid into asialofetuin was 28°C. Addition of Triton X-100 caused a marked stimulation of enzyme activity, omission of detergent resulted in a 70% loss of sialyltransferase activity (Table I). Addition of manganese or magnesium had no stimulatory effect on sialyltransferase activity. At manganese concentrations above 1 mM there was slight inhibi-

TABLE I

Requirements for the assay of cervical sialyltransferase. Complete assay mixture contained in a final volume of 60 μl: 0.33 nmol CMP-*N*-acetyl[^{14}C]neuraminic acid (220 000 cpm)/5.0 ml · l^{-1} Triton X-100/1.0 mg asialofetuin/20 μl cervical tissue homogenate (containing up to 3.0 g · l^{-1} protein/3.2 μmol cacodylate-HCl buffer, pH 6.0.

Incubation mixture	^{14}C-labelled product (nmol · h^{-1} · mg^{-1} protein)
Complete assay mixture	20.0
Minus asialofetuin	1.0
Minus Triton X-100	5.6
Plus Triton X-100 (10 ml · l^{-1})	20.4
Plus 0.3 μmol $MnCl_2$	18.8
Plus 0.6 μmol $MnCl_2$	17.0
Plus 0.6 μmol EDTA	16.0

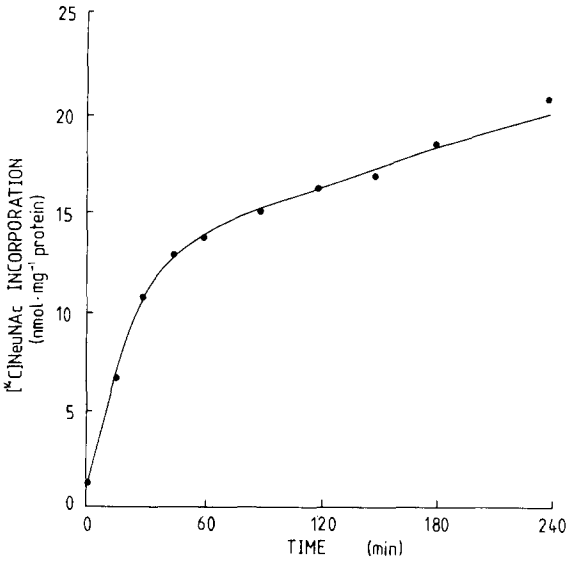

Fig. 1. Effect of incubation time on the incorporation of *N*-acetyl[^{14}C]neuraminic acid into asialofetuin. Assay conditions are as described in Table I.

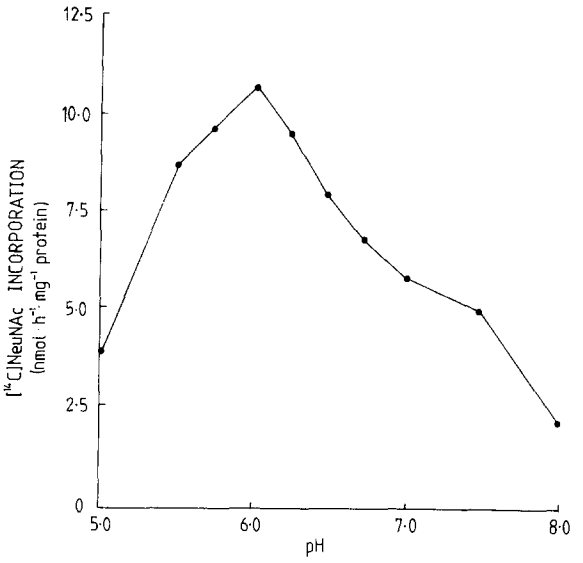

Fig. 2. Effect of pH on the incorporation of *N*-acetyl[^{14}C]-neuraminic acid into asialofetuin. Assay conditions are as described in Table I. Other buffers used, each at 3.2 μmol per assay, were as follows: pH 5.0–7.0, cacodylate-HCl; and pH 7.5–8.0, Tris-HCl.

tion of enzyme activity with 15% inhibition at 10 mM. A minimal cation requirement may be indicated by the inhibitory effect of EDTA. At concentrations above 2.5 mM EDTA there was graded inhibition of sialyltransferase activity with 65% of the control reaction rate remaining at a concentration of 20 mM.

Stability. The sialyltransferase activity present in the crude tissue homogenate was not affected by repeated freezing and thawing and was stable to freezing at –20°C for at least 3 months.

Substrate concentration. Fig. 3 shows the effect on enzyme activity of varying the concentration of the acceptor substrate, asialofetuin. Maximal velocity was seen at an asialofetuin concentration of 25 g $\cdot$ l^{-1} which, expressed as terminal galactose acceptor sites, is 4.6 mM. A plot of [S] against [S]/v was linear ($P < 0.001$) with a K_m of 0.64 $\cdot$ 10^{-5} M (expressed as the concentration of terminal galactose residues) and V of 1.7 nmol $\cdot$ h^{-1} $\cdot$ mg^{-1} protein. A plot of 1/v against 1/[S] for the donor substrate. CMP-*N*-acetyl-[^{14}C]neuraminic acid, was also linear ($P < 0.001$) and gave a K_m for the nucleotide sugar of 2.05 $\cdot$ 10^{-5} M and V of 3.6 nmol $\cdot$ h^{-1} $\cdot$ mg^{-1} protein.

Product identification. Separation of the reaction products by high voltage electrophoresis revealed only one ^{14}C-labelled product which was located at or near the origin. Free *N*-acetyl[^{14}C]neuraminic acid was not detected indicating that there was negligible hydrolysis of both the nucleotide sugar and *N*-acetyl[^{14}C]neuraminic acid-fetuin. The absence of significant neuraminidase activity under the conditions of the sialyltransferase assay was confirmed by incubating 400 μg [^{3}H]sialic acid-fetuin (92 000 dpm) with the standard assay mixture scaled up 2-times, when there was no detectable release of free [^{3}H]sialic acid for at least 120 min. In the absence of asialofetuin *N*-acetyl[^{14}C]neuraminic acid was incorporated into an endogenous acceptor having an electrophoretic mobility similar to the product of enzymic transfer of *N*-acetyl[^{14}C]neuraminic acid to asialofetuin.

Fig. 4 shows the results of chromatography of the reaction products on Sepharose CL-4B. Two major ^{14}C-labelled products were identified which eluted between the characteristic V_e of fetuin and asialofetuin. When the control assay lacking asialofetuin was chromatographed a single product, which was totally excluded from Sepharose CL-4B, was identi-

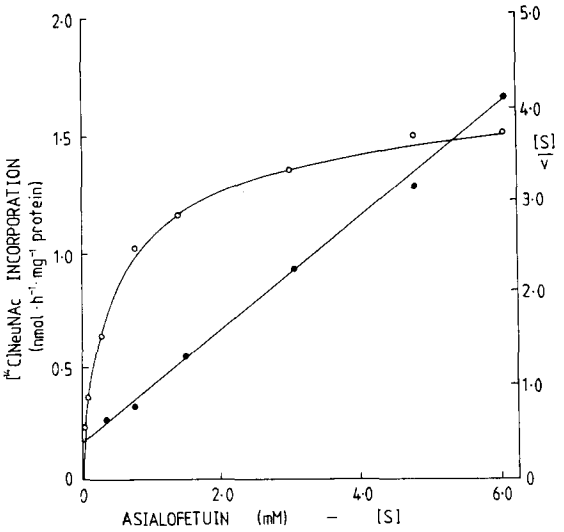

Fig. 3. Effect of substrate (asialofetuin) concentration, [S], on the activity, v, of sialyltransferase (o———o). The concentration of asialofetuin is expressed as the concentration of terminal galactose residues (mM). Also shown is a Hanes-Woolf plot of $[S]/v$ against [S] (•———•). Assay conditions are as described in Table I.

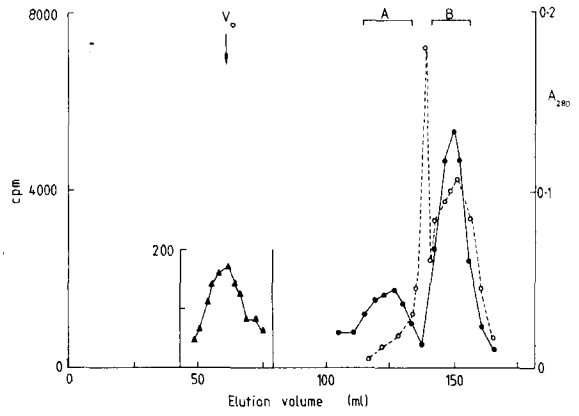

Fig. 4. Column chromatography of the sialyltransferase reaction products. The reaction mixture described in Table I was diluted with 1 ml 50 mM phosphate buffer, pH 7.4, applied to a column (1.5 X 100 cm) of Sepharose CL-4B equilibrated with the same buffer and developed at a flow rate of 8 ml · h^{-1} collecting fractions of 2.2 ml. Products of *N*-acetyl[^{14}C]-neuraminic acid incorporation (o - - - - - - o) were measured by liquid scintillation counting. A control assay lacking asialofetuin was treated similarly, and *N*-acetyl[^{14}C]neuraminic acid incorporation into endogenous acceptor (▲———▲) measured as above. Reference standards of fetuin (peak A), asialofetuin (peak B) and Dextran 2000 (V_0) were also chromatographed and monitored at 280 nm (•———•).

field indicating incorporation of *N*-acetyl[^{14}C]neuraminic acid into an endogenous high molecular weight glycoprotein acceptor. In the control incubation, incorporation of *N*-acetyl[^{14}C]neuraminic acid into endogenous acceptor was typically 5% of that into asialofetuin.

Substrate specificity. The acceptor specificity of cervical sialyltransferase activity was studied by comparing the incorporation of *N*-acetyl[^{14}C]-neuraminic acid into fetuin and some of its derivatives, asialo α_1 acid glycoprotein and several low molecular weight carbohydrates.

The highest sialyltransferase activity was seen towards asialofetuin. Fetuin also served as an acceptor (relative incorporation of 34%) but no incorporation of *N*-acetyl[^{14}C]neuraminic acid into agalacto-asialofetuin could be detected. Human asialo α_1 acid glycoprotein also acted as a substrate, though at half the efficiency of asialofetuin. Galactose, *N*-acetylgalactosamine and *N*-acetylglucosamine (2.7 μmol per assay) would not act as substrates for cervical sialyltransferase, but low activity (4% of that with asialofetuin as the acceptor) towards the disaccharide, *N*-acetyllactosamine (Galβ(1 → 4)GlcNAc), was observed.

Inhibition studies. A number of nucleotides were tested for their effect on cervical sialyltransferase activity. Cytidine nucleotides were potent inhibitors of the enzyme reaction. CDP and CTP were more effective than CMP and completely inhibited incorporation of *N*-acetyl[^{14}C]neuraminic acid into asialofetuin at a concentration of 0.4 mM. CMP inhibited the reaction at concentrations above 0.05 mM, with total inhibition of sialyltransferase activity at 1.5 mM. The nature of this inhibition was investigated between 0.14 and 0.7 mM CMP, at two fixed concentrations of CMP-*N*-acetyl[^{14}C]neuraminic acid. Graphical evaluation of the data using the method described by Dixon [17] revealed that CMP was behaving as a competitive inhibitor with a K_i of 0.18 mM.

Preliminary data indicate that the addition of UTP to the standard assay may have a dual effect on sialyltransferase activity. Incorporation of *N*-acetyl[^{14}C]-neuraminic acid into asialofetuin was almost completely inhibited (95%) by 0.25 mM UTP, but above this concentration inhibition progressively diminished with 35% of the original enzyme activity being seen

at 2.5 mM UTP. Copper (Cu^{2+}) inhibited sialyltransferase activity at concentrations above 20 μM, with 60% inhibition at a copper concentration of 0.8 mM. Cervical sialyltransferase was relatively insensitive to the presence of *N*-ethylmaleimide, and at a concentration of 10 mM *N*-ethylmaleimide more than 90% of the original enzyme activity remained.

Discussion

This study demonstrates the existence of a glycoprotein sialyltransferase in the human cervical epithelium which has a high affinity for the acceptor substrate, asialofetuin, and is probably therefore of significance in glycoprotein synthesis in vivo. Loss of activity of asialofetuin, after removal of terminal galactose residues by Smith degradation, indicates that asialofetuin is a substrate for an epithelial β-galactoside sialyltransferase but the presence of an epithelial α-*N*-acetylgalactosaminide sialyltransferase [2] catalysing the incorporation of sialic acid into Galα(1 → 3)GalNAc structures of asialofetuin [18] cannot be excluded. The almost 100% increase in sialyltransferase activity towards asialofetuin compared with asialo α_1 acid glycoprotein may be due to the higher concentration of terminal Galβ(1 → 4)-GlcNAc structures in asialofetuin (a proportion of the terminal structures of asialo α_1 acid glycoprotein take the form Galβ(1 → 6)GlcNAc [19]) and may reflect the cervical enzyme's high specificity for this type of structure. The near absolute requirement of cervical sialyltransferase for a high molecular weight, glycoprotein acceptor is demonstrated by its extremely low activity towards the disaccharide, *N*-acetyllactosamine. This requirement is not shared by all sialyltransferases, for example the enzyme from porcine submaxillary gland has a similar affinity for low and high molecular weight acceptors [12], while human serum sialyltransferase shows a preference for low molecular weight acceptors [20].

In common with many other sialyltransferases, the cervical enzyme does not require the presence of manganese or magnesium for maximal activity [21], but a minimal cation requirement may be indicated by the inhibitory effect of EDTA. The pH optimum of the sialyltransferase reaction (pH 6.0) resembles that of ovine submaxillary gland sialyltransferase [22] but is lower than human serum asialofetuin sialyltransferase which has a pH optimum of 6.8 [20].

Biphasic rate curves similar to that described for the cervical enzyme-catalysed incorporation of *N*-acetyl[^{14}C]neuraminic acid into asialofetuin have been reported for the incorporation of sialic acid into asialotransferrin and asialo α_1 acid glycoprotein by bovine colostrum β-galactoside α(2 → 6) sialyltransferase [2]. This type of curve may represent the activity of more than one cervical sialyltransferase or the preferential sialylation of one of the antennary structures.

The affinity of the cervical enzyme for the donor substrate, CMP-*N*-acetyl[^{14}C]neuraminic acid (K_m $2.05 \cdot 10^{-5}$ M), is similar to that described for rat liver asialofetuin sialyltransferase [23] but an order of magnitude lower than that reported for porcine liver glycoprotein sialyltransferase [24], bovine colostrum sialyltransferase [25] and ovine submaxillary gland glycoprotein sialyltransferase [22]. The affinity of cervical sialyltransferase for the acceptor substrate, asialofetuin (K_m $0.64 \cdot 10^{-5}$ M) closely resembles that for CMP-*N*-acetyl[^{14}C]neuraminic acid and is unusual since glycosyltransferases commonly demonstrate a relatively high K_m for their acceptor substrates [26].

During the assay of cervical sialyltransferase, incorporation of *N*-acetyl[^{14}C]neuraminic acid into endogenous acceptor could only be demonstrated in the absence of asialofetuin, hence it is not normally necessary to perform a control assay lacking asialofetuin as a correction for *N*-acetyl[^{14}C]neuraminic acid incorporation into endogenous acceptor. Neither is the assay complicated by the presence of endogenous glycoprotein neuraminidase activity which might otherwise cause hydrolysis of the enzyme product.

Inhibition studies demonstrated that cytidine nucleotides were potent inhibitors of cervical sialyltransferase activity. CMP, a product of the sialyltransferase reaction, was found to be a competitive inhibitor (K_i 0.18 mM). Both CDP and CTP were more effective inhibitors of enzyme activity than CMP. The extent of nucleotide inhibition of human serum sialyltransferase increases with increasing number of phosphate groups in the nucleotide [28], but the reverse is true for cellular, membrane-bound sialyltransferases which demonstrate greater inhibi-

tion in the presence of CMP [23,27]. Cytidine nucleotide inhibition of cervical sialyltransferase thus resembles that of serum sialyltransferase, but differences were shown to exist between these two enzymes in response to EDTA and *N*-ethylmaleimide. Serum asialofetuin sialyltransferase activity is stimulated by almost 100% in the presence of 8 mM EDTA [29], but at the same concentration, EDTA inhibited the cervical enzyme by 10%, and no stimulatory effect could be demonstrated in the presence of this compound. In contrast to the serum enzyme, which is inhibited by *N*-ethylmaleimide at concentrations above 1 mM [28], cervical sialyltransferase is relatively insensitive to the presence of this thiol-blocking reagent, and cystine is probably therefore not involved at the active site of the cervical enzyme. The effect of UTP on the activity of cervical sialyltransferase is unusual and represents almost complete inhibition of enzyme activity at low UTP concentrations (below 0.25 mM), with diminished inhibition, or reactivation, at higher concentrations of nucleotide. A similar effect has not previously been described, although UTP can act as either an activator or stimulator of sialyltransferase depending on the enzyme source. Shah and Ragupathy [29] have shown that 1 mM UTP almost totally inhibits serum asialofetuin sialyltransferase, while Bernacki [23] reported an almost 100% stimulation of rat liver asialofetuin sialyltransferase by 1.15 mM UTP, and showed that this, and other uridine nucleotides, behaved as allosteric activators. The possibility of UTP having a dual effect on cervical sialyltransferase must be studied over a wider concentration range of UTP, but since cellular uridine nucleotide levels have been reported to be in the millimolar range [30] it seems possible that UTP may regulate sialylation of cervical mucin in vivo.

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