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Characterisation of the enzymatic 4-*O*-acetylation of sialic acids in microsomes from equine submandibular glands

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Microsomes prepared from equine submandibular glands and incubated with tritium-labelled AcCoA incorporated acid-insoluble radioactivity in a manner dependent on time, protein, membrane integrity and AcCoA concentration, with incorporation being optimal at 37°C and pH 6.6. Under the experimental conditions used a K_M of 32.1 μM for AcCoA and a V_{max} of 1.2 pmol/mg protein x min was obtained. The incorporation of acid-insoluble radioactivity was also inhibited by CoA in a competitive manner ($K_i = 240$ μM), as well as by *para*-chloromercuribenzoate, 3′-dephospho-CoA, 5′-IDP, 5′-ADP, ß-NAD and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate. We demonstrate here that this incorporation of radioactivity into endogenous sialic acid is due to the action of an AcCoA:sialate-4-*O*-acetyltransferase [EC 2.3.1.44]. Radio thin-layer chromatography analyses of propionic acid-released sialic acids showed that the incorporation of radioactivity correlated with the formation of a radiolabelled species that co-migrated with authentic Neu4,5Ac$_2$. Saponification experiments using NaOH, mouse hepatitis virus strain S and Influenza C/JJ/50 virus also showed that the transfer of [^{3}H]acetyl groups from [^{3}H]AcCoA to endogenous sialic acid acceptors was occurring exclusively at carbon 4 of the pyranose ring.

Keywords: **characterisation, equine submandibular gland, microsomes, sialate-4-*O*-acetyltransferase, sialic acid**

Abbreviations: **AcCoA, acetyl coenzyme A; BSM, bovine submandibular gland mucin; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; HPLC, high-performance liquid chromatography; HSM, horse submandibular gland mucin, ICV, Influenza C/JJ/50 Virus; MHV-S, Mouse Hepatitis Virus Strain S; Neu5Ac, *N*-acetylneuraminic acid; Neu4,5Ac$_2$, 5-*N*-acetyl-4-*O*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Neu4Ac5Gc, 4-*O*-acetyl-5-*N*-glycolylneuraminic acid; PCA, perchloric acid; *p*-CMB, *para*-chloromercuribenzoate; *p*PNA, *p*-nitrophenylacetate; R_{Neu5Ac}, relative retention time referred to Neu5Ac; TLC, thin-layer chromatography.**

Introduction

Sialic acids comprise a family of over 40 naturally occurring derivatives of the nine carbon sugar, commonly designated neuraminic acid (systematic name: 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranos-1-onic acid) [1,2]. The largest structural variation of naturally occurring sialic acids is at carbon 5, which can be substituted with either an *N*-acetyl, *N*-glycolyl or hydroxyl moiety to form *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) or deaminoneuraminic acid (Kdn), respectively [1,2]. Sialic acids can also undergo further modifications at any one of four hydroxyl groups, located at C-4, -7, -8 and -9. One such modification, the formation of *O*-acetyl esters, has been found to occur in some bacteria and a number of animal species including man [1–3]. One of the richest sources of *O*-acetylated sialic acids are the mucins from bovine and equine submandibular glands, with the latter possessing high levels of 4-*O*-acetylated sialic acids. Unlike *O*-acetylation at C-7 and C-9, the prevalence of 4-*O*-acetylated sialic acids appears to be restricted to only a handful of animal species, including the Japanese dace [4], the South American pit viper [5], the Australian monotreme Echidna [6], as well as guinea-pigs [7,8], horses [9] and indirect evidence of 4-*O*-acetylated sialic acids in mice [10].

The precise biological role of 4-*O*-acetylated sialic acids is as yet unclear, however they may serve to hinder the degradation of glycoconjugates, since sialic acids bearing this modification are resistant to the action of most sialidases, sialate lyases and sialate esterases [2,3,11]. However, the recent discovery that the mouse hepatitis virus strain S (MHV-S) possesses a sialate 4-*O*-acetylesterase [12] suggests that this

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modification may also play a role in the recognition and binding of MHV-S to its target cells. However, this is yet to be proven conclusively.

In general, sialate-*O*-acetylation results from the action of at least two distinct enzymes: AcCoA:sialate 7(9)-*O*-acetyltransferase [EC 2.3.1.45] and AcCoA:sialate 4-*O*-acetyltransferase [EC 2.3.1.44] [1,2,9,13]. In the early 1970's it was reported that the incubation of radiolabelled acetate with surviving slices from equine submandibular glands incorporated radiolabelled acetate at 4 the position of endogenous sialic acid [9]. Here we report on the use of enzymatic and chromatographic techniques to demonstrate that neo-*O*-acetylated sialic acids formed, in a cell-free system, by the AcCoA:sialate 4-*O*-acetyltransferase from equine submandibular gland are indeed 4-*O*-acetylated sialic acids. The characterisation of AcCoA:sialate 4-*O*-acetyltransferase activity in the fine membrane fraction from equine submandibular gland is also presented.

Materials and methods

Preparation of microsomal membranes

Microsomal membranes were prepared by a modification of the method described in [14]. Equine submandibular glands were obtained from the local slaughterhouse immediately following the death of the animal and either used fresh or frozen at −80°C until required. All procedures were performed at 4°C unless otherwise stated. Following the removal of fat and connective tissue the gland was finely sliced, washed in 5 L of 10 mM Tris/HCl, pH 7.2 and filtered through a sieve to remove the bulk of the mucin. The gland slices were then resuspended in 10 mL of homogenising buffer [100 mM Tris/HCl, pH 7.2 containing 2 mM Pefabloc (Boehringer Mannheim, Mannhein, Germany)] per gram of tissue and crudely homogenised with a Stabmixer. The suspension was then transferred to a 60 mL Potter-Elvehjem apparatus and homogenised with 20 strokes against a pestle rotating at the highest setting. The crude homogenate was then centrifuged at 10 000 rpm for 30 min (JA 17 rotor), with the resulting supernatant removed and placed on ice. The pellet was resuspended in homogenising buffer and rehomogenised in a Potter-Elvehjem apparatus with a further 20 strokes. Following centrifugation at 10 000 rpm for 30 min (JA 17 rotor) the supernatant was removed and combined with the previous post-10 000 rpm supernatant. The combined supernatant was then ultracentrifuged at 100 000× g for 1 h (Ti 45 rotor) and the upper, yellowish microsomal layer of the pellet resuspended in a minimum volume of homogenising buffer. Microsomal membranes were then collected in Eppendorf tubes by centrifugation at 100 000× g for 15 min and frozen at −80°C until required.

HPLC analysis of endogenous sialic acids

Endogenous equine submandibular gland sialic acids chemically released from the crude homogenate or microsomal membranes as described in [15], and purified as outlined in [16] were analysed by the methods described in [8,14,17].

Sialate-4-*O*-acetyltransferase assay involving endogenous acceptors

Sialate-4-*O*-acetyltransferase activity in equine submandibular microsomal membranes was determined using a modification of the assay described in [14]. The time, temperature, pH, as well as the protein, and inhibitor concentrations are indicated in the tables and figures under "Results". Assays were also carried out in the presence of 0.9% CHAPS (Sigma Chemicals, Deisenhofen, Germany), 0.03% Triton X-100 (Biomol, Hamburg, Germany), 0.02% Lubrol PX (Sigma Chemicals), 0.2% Zwittergent 3-12 (Fluka, Deisenhofen, Germany) and 2.25% n-octyl-ß-D-glucopyranoside (Sigma Chemicals).

Assays were typically performed as follows; 500 to 1000 µg of microsomal protein (approximately 0.05 µg sialic acid/µg protein) was incubated in a final volume of 100 µL of 50 mM potassium phosphate, pH 6.8 buffer containing 5.6 µM AcCoA [0.25–0.5 µCi [^{3}H]AcCoA (Moravek Biochemicals Inc., CA, U.S.A.)], 70 mM KCl, 4 mM Pefabloc and Complete^{TM} protease inhibitor cocktail (Boehringer Mannheim). Following incubation at 37°C for 30 min the reaction was stopped by the addition of 400 µL of 4% perchloric acid. The precipitate was collected by centrifugation at 100 000× g for 15 min and resuspended in 200 µL ice-cold water by sonication. A further 800 µL of water was added, the suspension vortexed and recentrifuged at 100 000 × g for 15 min. The resulting pellet was washed for a second time as described and resuspended in 200 µL ice-cold water by sonication. Thirty µL of the suspension was removed and dissolved in 200 µL of 1 M NaOH for 10 min at 80°C. Following neutralisation with 100 µL 2 M HCl the total incorporated radioactivity was measured by liquid scintillation counting.

The remaining 170 µL of washed and resuspended acid-insoluble pellet was hydrolysed for 4 h at 80°C in the presence of 2 M propionic acid. Following a 10 min incubation on ice the propionic acid was removed by lyophilisation and the sample resuspended in 20 µL ice-cold water. Fifteen µL of the supernatant obtained after a brief centrifugation at 14 000 rpm was then applied to either a 20 × 10 cm silica-TLC plate (Merck Eurolab, Darmstadt, Germany) and run for 3 h in methanol/chloroform/20 mM $CaCl_2$ (50:40:10 by vol.) or a 20 × 20 cm cellulose-TLC plate (Merck Eurolab) and run overnight in 1-butanol/1-propanol/0.1 M HCl (1:2:1 by vol.). Cellulose-TLC plates were pre-run twice in this solvent system prior to the application of sample. The transfer of [^{3}H]acetate from [^{3}H]AcCoA onto endogenous sialic acid acceptors was then quantified by radio-TLC using an automatic TLC-Linear Analyser LB 284 (Berthold, Wildbad, Germany). Samples analysed by radio-TLC were co-chromatographed with authentic Neu4,5Ac_2 purified from equine submandibular

gland. These standards were visualised by staining TLC plates with Fe^{3+}/orcinol/HCl-reagent as described in [16].

Sialate-4-*O*-acetyltransferase assay involving exogenous acceptors

Five hundred to one thousand micrograms of microsome or 300 to 500 μg cytosol (approximately 0.04 μg sialic acids/μg protein) isolated from equine submandibular glands was incubated in 50 mM potassium phosphate, pH 6.8 buffer containing 5.6 μM AcCoA [0.25–0.5 μCi [^{3}H]AcCoA], 70 mM KCl, 4 mM Pefabloc, Complete^{TM} protease inhibitor cocktail and either 70 μg de-*O*-acetylated bovine submandibular mucin (BSM) (0.14 μg sialic acid/μg protein), 200 μg de-*O*-acetylated horse submandibular mucin (HSM) (0.05 μg sialic acid/μg protein) or 2.0 mM Neu5Ac. Incubations carried out in the presence of de-*O*-acetylated BSM or de-*O*-acetylated HSM were performed as described above. Incubations carried out in the presence of Neu5Ac were performed as described in [18].

Incubations were also performed where de-*O*-acetylated HSM was added, as an exogenous acceptor, to assays carried out in the presence of various detergents.

Saponification of neo-*O*-acetylated sialic acids

In some experiments neo-*O*-acetylated sialic acids formed after the incubation of microsomal membranes with [^{3}H]AcCoA were saponified either in the presence of sodium hydroxide or ammonium vapour. The PCA-insoluble pellets were resuspended in water, hydrolysed and lyophilised as previously described. The lyophilised samples were then resuspended in 30 μL 0.1 M NaOH and incubated at 37°C for 30 min. Samples were then neutralised with 30 μL 0.1 M HCl and lyophilised. Following lyophilisation, samples were resuspended in 20 μL of ice-cold water and analysed by scintillation counting and radio-TLC as previously described. Saponification was also performed following radio-TLC analysis by incubating TLC plates overnight at room temperature in a tightly sealed chamber saturated with ammonium vapour. TLC plates treated in this manner were thoroughly dried under a stream of cool air and re-analysed using an automatic TLC-Linear Analyser.

Enzymatic de-*O*-acetylation of neo-*O*-acetylated sialic acids

Enzymatic de-*O*-acetylation of neo-*O*-acetylated sialic acids formed after the incubation of microsomal membranes with [^{3}H]AcCoA was performed using either MHV-S or ICV essentially as described in [12]. Briefly, PCA-insoluble pellets were resuspended in water, hydrolysed and lyophilised as previously described. The lyophilised samples were resuspended in 40 μL PBS and incubated at 37°C for 1 h with either 10 μL of MHV-S (1.1 mU sialate-4-*O*-acetylesterase) or 10 μL of ICV (2.75 mU sialate-9-*O*-acetylesterase). One unit is defined as the amount of esterase activity resulting in the cleavage of 1 μmol of *p*PNA per min. The reaction was stopped by heating at 95°C for 3 min. Following lyophilisation, samples were resuspended in 20 μL of ice cold water and analysed by scintillation counting and radio-TLC as previously described.

Kinetic analysis of sialate-4-*O*-acetyltransferase

The kinetic parameters, K_M and V_{max} values for AcCoA were determined using the assay described above. One thousand μg of microsomal membrane protein was incubated with AcCoA at 10 to 12 concentrations between 0.5 and 50 μM (constant specific activity of 0.25 μCi/nmol). Sialate-4-*O*-acetyltransferase activity was quantified by scintillation counting and radio-TLC and kinetic parameters calculated according to [19].

Results

HPLC analysis of endogenous sialic acids

Prior to investigating the nature of enzymatic 4-*O*-acetylation in equine submandibular glands, sialic acids isolated from the crude homogenate and microsomal fraction were analysed by fluorimetric HPLC. The two main forms of sialic acid detected in the crude homogenate were Neu4,5Ac$_2$ and Neu5Ac, with Neu4,5Ac$_2$ representing 16% of total sialic acids detected (Figure 1A). The identification of Neu4,5Ac$_2$ was afforded by comparing the retention time with that of a standard enriched in this sialic acid. A third sialic acid, representing 8% of total sialic acids detected, may be Neu4Ac5Gc, since the observed retention time is very similar to that reported by [17]. As well as these forms of sialic acid, a very small peak, with a retention time similar to Neu5Gc, was also observed. Three forms of sialic acid were found to exist in the microsomal fraction, two were identified as being Neu5Gc and Neu5Ac, the third, Neu4,5Ac$_2$, representing 12% of the total sialic acids detected (Figure 1B). No Neu4Ac5Gc was observed. A fourth peak could not be identified and is probably a DMB reagent peak.

Enzymatic 4-*O*-acetylation of endogenous sialic acid acceptors

The incubation of [^{3}H]AcCoA with microsomes isolated from equine submandibular glands resulted in an increase in the total incorporated acid-insoluble radioactivity in comparison to control incubations (Figure 2). The incubation of heat-treated microsomes, as well as microsomes in the presence of *p*-CMB, with [^{3}H]AcCoA resulted in low levels of radioactivity incorporated. Likewise, incubations in the absence of microsomes showed little or no incorporation. Taken together, this indicates that the incorporation of radioactivity onto endogenous acceptors is occurring enzymatically, probably *via* the action of the sialate-4-*O*-acetyltransferase, which has previously been postulated to *O*-acetylate endogenous sialic acid acceptors in equine submandibular glands [9]. Furthermore, this process is occurring specifically in the microsome, since no incorporation

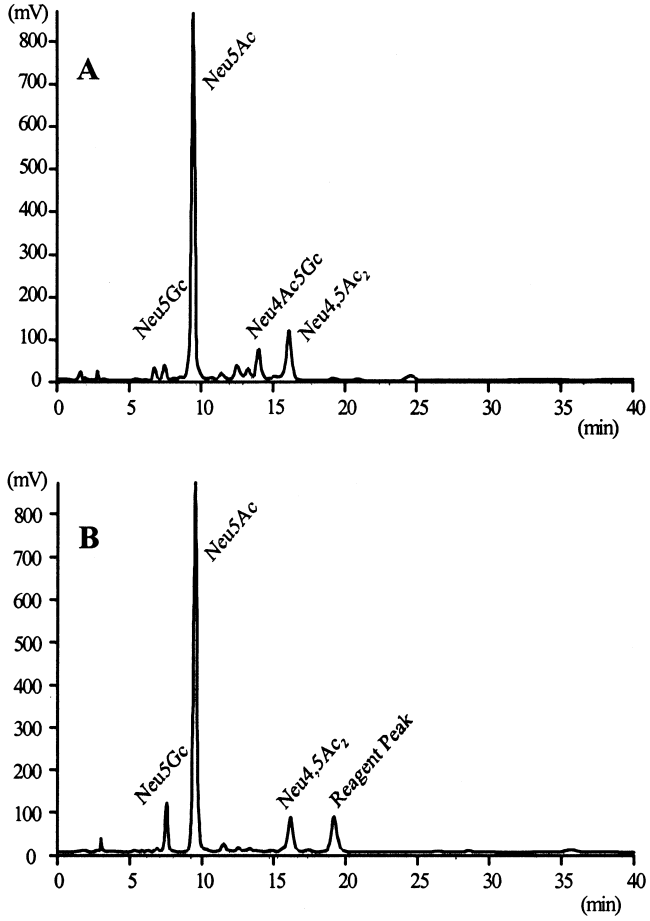

Figure 1. Fluorimetric detection of sialic acids isolated from the crude homogenate (A) and the microsomal membranes (B) of equine submandibular glands. For experimental details see Materials and methods. The R_{Neu5Ac} for Neu5Gc, Neu5Ac, Neu4Ac5Gc, Neu4,5Ac_2 and the reagent peak are 0.79, 1.00, 1.49, 1.71 and 2.02, respectively.

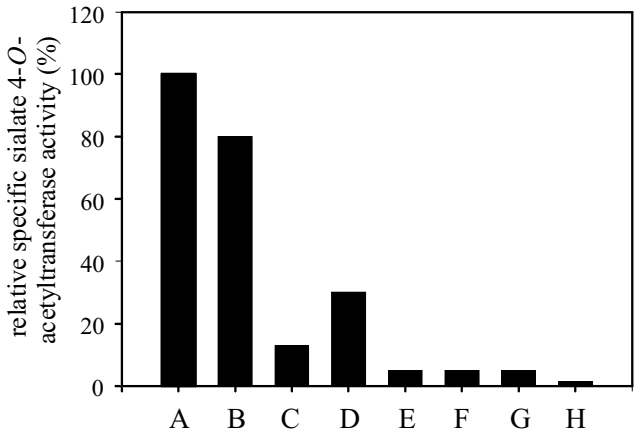

Figure 2. Relative specific sialate-4-*O*-acetyltransferase activities (%) in equine submandibular microsomes and cytosol against endogenous acceptors and de-*O*-acetylated BSM. Microsomal membrane fractions were incubated with [^{3}H]AcCoA in the absence (A) and presence (B) of de-*O*-acetylated BSM in 50 mM potassium phosphate buffer, pH 6.8 at 37°C for 30 min (optimal conditions, see Figure 5). Control incubations were performed in the presence of heat-treated microsomes (95°C for 5 min) (C) and 2 mM *p*-CMB (D). The cytosolic fraction isolated from equine submandibular gland was also incubated with [^{3}H]AcCoA in the absence (E) and presence (F) of de-*O*-acetylated BSM. Control assays were also performed in the presence of heat-treated cytosol (95°C for 5 min) (G), as well as in the absence of either microsomal or cytosolic fractions (H), For experimental details see Materials and methods.

of radioactivity was observed when [^{3}H]AcCoA was incubated with a cytosolic fraction in the presence and absence of the exogenous acceptor substrate de-*O*-acetylated BSM (Figure 2). Similar results were also obtained when incubations were performed in the presence of de-*O*-acetylated HSM and Neu5Ac. Free Neu5Ac has previously been shown to act as an acceptor for a cytosolic form of the 7(9)-*O*-acetyltransferase from bovine submandibular glands [18]. However, for the 4-*O*-acetyltransferase from equine submandibular glands this was found not to be the case.

Microsomal fractions were likewise incubated with de-*O*-acetylated BSM (Figure 2), de-*O*-acetylated HSM and Neu5Ac, however all failed to act as substrates for the 4-*O*-acetyltransferase. In the case of incubations in the presence of de-*O*-acetylated BSM this appeared to even slightly inhibit incorporation of radioactivity (Figure 2).

The incorporation of [^{3}H]acetate into microsomal fractions was also found to be dependent on membrane integrity, with the addition of detergents such as, CHAPS, Triton X-100, Lubrol PX, Zwittergent 3–12 and n-octyl-ß-D-glucopyranoside resulting in the total loss of transferase activity against endogenous acceptors. Experiments were also performed where de-*O*-acetylated HSM was added to assays along with the various detergents, however, supplementing these assays with an exogenous substrate failed to restore transferase activity.

To determine the nature of the radioactive product, released by propionic acid, following the incubation of [^{3}H]AcCoA with microsomes, radio-TLC analyses were performed on silica (Figure 3A and B) and cellulose (Figure 3C and D). These figures show that the increase in incorporated radioactivity (as shown in Figure 2) correlated with a peak of radioactivity that co-migrated with authentic Neu4,5Ac_2 (Figure 3A and C). Tritium-labelled sialic acids co-migrating with authentic Neu4,5Ac_2 were not observed in control incubations (data not shown). Following saponification of the radio-TLC plates in the presence of ammonium vapour the radioactive peak that co-migrated with Neu4,5Ac_2 disappeared (Figure 3B and D), indicating that radioactive ester groups had been incorporated into endogenous sialic acid acceptors upon incubation with [^{3}H]AcCoA.

In the method described here propionic acid-released tritium-labelled sialic acids were not purified by sequential ion exchange chromatography prior to radio-TLC analyses. The incorporation of [^{3}H]acetyl groups was found to be

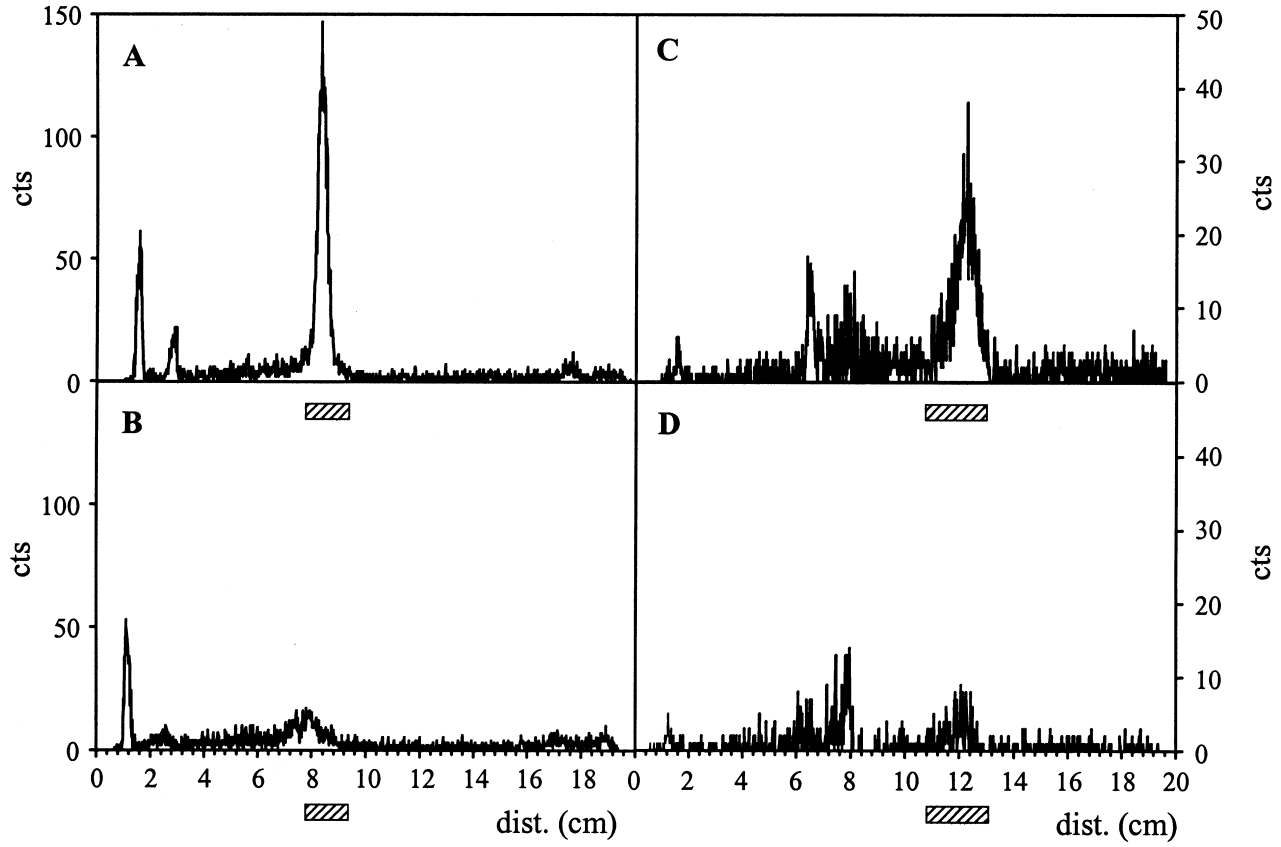

Figure 3. Radio-TLC on silica (A and B) and cellulose (C and D) plates of radiolabelled sialic acids isolated from equine submandibular microsomes following incubation with [^{3}H]AcCoA under optimal conditions. The only radiolabelled sialic acid observed co-migrated with authentic Neu4,5Ac$_2$ (A and C). Panels B and D show that the radiolabelled product could be saponified by an overnight incubation in the presence of ammonium vapour. The position of Neu4,5Ac$_2$ is indicated.

restricted to sialic acids, since radio-TLC analyses of acid-released sialic acids prior to and following purification showed no significant differences, neither quantitatively nor qualitatively (data not shown). The omission of sialic acid purification prior to TLC analyses provided a method of analysing neo-*O*-acetylated sialic acids which was significantly faster, therefore minimising the loss of unstable *O*-acetyl groups which can sometimes occur following sialic acid purification.

To further establish the nature of the neo-*O*-acetylated sialic acids, acid-released tritium labelled sialic acids were incubated with either MHV-S, ICV or 0.1 M NaOH. The 9-*O*-acetyl-specific esterase (sialate-9-*O*-acetylesterase) of the ICV was unable to saponify the neo-*O*-acetylated sialic acids (Figure 4D). However, saponification with NaOH was possible, with the complete loss of radioactive ester groups being observed (Figure 4E). Likewise, neo-*O*-acetylated sialic acids treated with the 4-*O*-acetyl-specific esterase (sialate-4-*O*-acetylesterase) from MHV-S and analysed by radio-TLC resulted in the complete hydrolysis (Figure 4B), compared to the non-treated sample (Figure 4A), of radiolabelled acetyl groups.

The ability and inability of MHV-S and ICV, respectively, to saponify neo-*O*-acetylated sialic acids confirms that *O*-acetylation is occurring exclusively at carbon 4 of the pyranose ring and not in the glycerol side chain.

The enzymatic 4-*O*-acetylation of endogenous, microsomal sialic acid acceptors was further characterised with respect to temperature, pH, time and protein. The properties of the membrane-bound sialate-4-*O*-acetyltransferase activity are shown in Figure 5. Under the experimental conditions used the 4-*O*-acetyltransferase possessed a temperature optimum of 37°C (Figure 5A) and a pH optimum of 6.6 (Figure 5B). This is consistent with that observed for the membrane-bound 4-*O*-acetyltransferase from guinea pig liver [8]. The microsomal fraction incorporated acid-insoluble radioactivity in a manner dependent on time (Figure 5C) and protein (Figure 5D), with incorporation of [^{3}H]acetate being linear for time points up to 30 min and protein up to 3.5 mg. The incorporation of acid-insoluble radioactivity was also shown to be dependent on the concentration of AcCoA (Figure 6), with the membrane-bound 4-*O*-acetyltransferase possessing a K_M of 32.1 μM and a V_{max} of 1.2 pmol/mg protein × min. A K_M and a V_{max} of 22.3 μM and 0.5 pmol/mg protein × min, respectively, was also calculated from the amount of isolated radiolabelled product, co-migrating with authentic Neu4,5Ac$_2$, detected by radio-TLC.

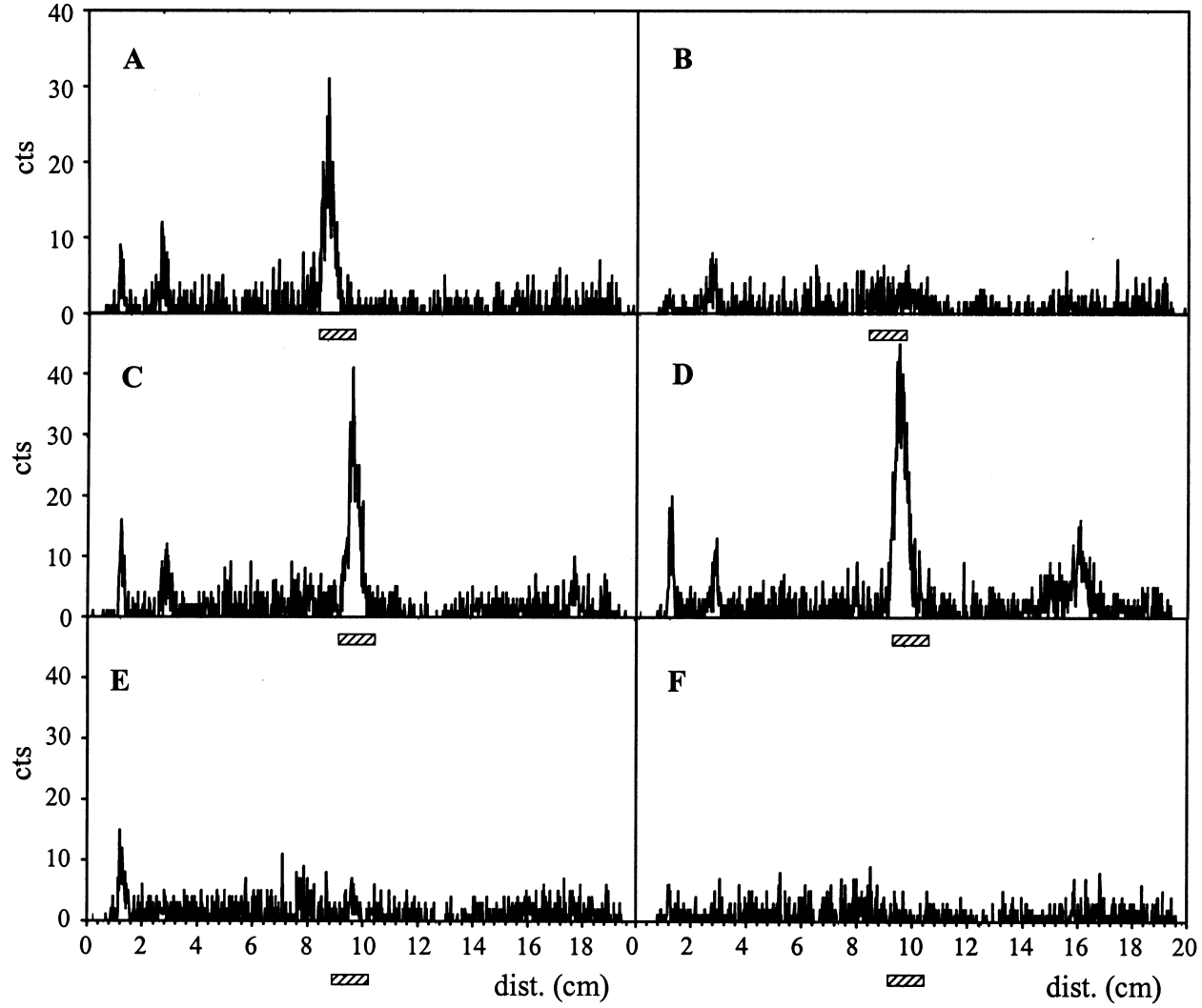

Figure 4. Radio-TLC on silica plates of radiolabelled sialic acids isolated from equine submandibular gland microsomes following incubation with [^{3}H]AcCoA under optimal conditions. The only radiolabelled sialic acid observed co-migrated with authentic Neu4,5Ac$_2$ (A and C). The radiolabelled product could be saponified with MHV-S (B) and NaOH (E), however it was resistant to the action of ICV (D). No radiolabelled sialic acids were observed when heat-denatured microsomes were incubated with [^{3}H]AcCoA (F). Radio-TLC chromatograms A and C represent the controls of incubations performed in the absence of MHV-S and ICV, respectively. The position of Neu4,5Ac$_2$ is indicated.

The transfer of [^{3}H]acetyl groups from [^{3}H]AcCoA to microsomal sialic acid acceptors was inhibited by CoA in a competitive manner, with a K_i of 240 μM. The competitive nature of inhibition by CoA was determined according to Lineweaver-Burk at four different concentrations of AcCoA and two different CoA concentrations under optimal conditions. Equine submandibular gland sialate 4-*O*-acetyltransferase activity was also inhibited by 3′-dephospho-CoA and DIDS, and to a lesser extent, by 5′-ADP, ß-NAD and 5′-IDP (Table 1). The nature of the inhibition exhibited by these compunds was not determined. As can be seen in Table 1, di-phosphorylated nucleotides were capable of inhibiting *O*-acetylation, whereas the corresponding mono-phosphorylated were not. However, ß-NADP, which is diphosphorylated at C-5 and mono-phosphorylated at C-2 of the ribose moiety was unable to inhibit transfer, even though ß-NAD was a moderate inhibitor.

Discussion

Early studies on the enzymatic 4-*O*-acetylation in equine submandibular glands were performed by incubating surviving slices with radioactive precursors, resulting in the synthesis of neo-4-*O*-acetylated sialic acids [9]. Here we report for the first time the characterisation of this enzymatic activity in a cell-free system.

Reversed phase HPLC analyses of sialic acids isolated and purified from equine submandibular glands showed that the *O*-acetylation of sialic acid in this tissue predominantly occurs

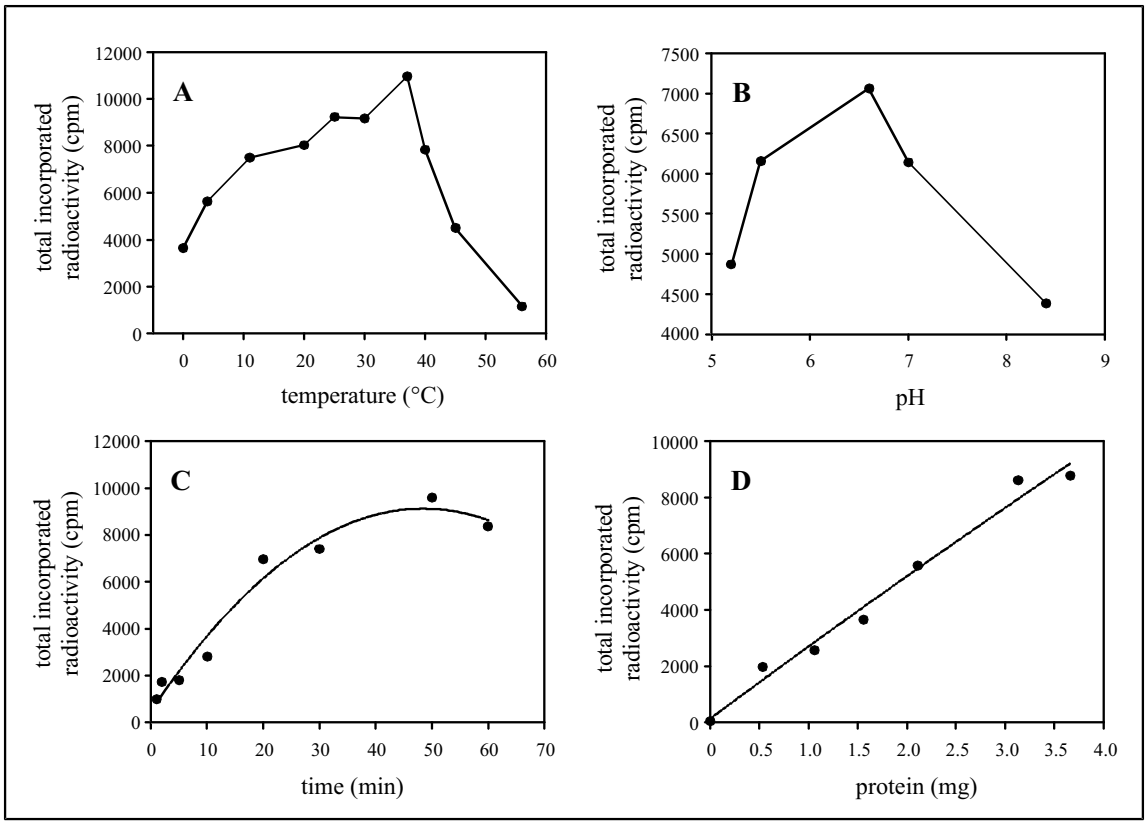

Figure 5. The incorporation of radioactivity from [^{3}H]AcCoA into endogenous microsomal sialic acid acceptors from equine submandibular glands depending on various parameters. The dependence of sialate-4-*O*-acetyltransferase activity on temperature (A), pH (B), time (C) and protein concentration (D) are shown. In (A) incubations were performed at pH 6.5 for 30 min. In (B) incubations were performed at 37°C for 30 min. In (C) and (D) incubations were performed at pH 6.5 and 37°C. Incubations (A–C) contained 400 to 1000 μg of microsomal protein. For further experimental details see Materials and methods.

at carbon 4, an observation that is in agreement with previous studies [20,21]. The isolation and identification of Neu4Ac5Gc, as well as Neu5Gc, has also been reported [20,21]. Due to the relatively high content of 4-*O*-acetylated sialic acids in equine submandibular glands, this tissue provides an excellent opportunity to investigate the underlying enzymatic reaction on a subcellular level.

The identification of the neo-*O*-acetylated product formed by the equine submandibular gland *O*-acetyltransferase, as well as the characterisation of this activity were carried out using endogenous acceptor substrates. Possible exogenous substrates, such as de-*O*-acetylated BSM, de-*O*-acetylated HSM and free Neu5Ac failed to act as substrates, with de-*O*-acetylated BSM actually being inhibitory. The inability of the equine submandibular gland *O*-acetyltransferase to utilise exogenous substrates is probably due to either the presence of relatively large quantities of endogenous substrates, probably mucins, or that substrates such as de-*O*-acetylated BSM and de-*O*-acetylated HSM, being very large molecules, may not be accessible to the enzyme which is likely to be embedded within the membrane. Endogenous acceptor substrates may, therefore, be preferentially *O*-acetylated not only because of their large number but also because of their location within the membrane.

This is further supported by the finding that the solubilisation of the *O*-acetyltransferase with various detergents resulted in the complete loss of transferase activity against endogenous acceptors. This has also been observed for the 7(9)-*O*-acetyltransferase from rat liver Golgi [23–25]. The transferase activity from equine submandibular gland microsomes solubilised by various detergents remained inactive even in the presence of exogenous de-*O*-acetylated HSM, suggesting that the enzyme, acceptor substrate, and possibly also an AcCoA transporter are closely associated, not only with each other but within the membrane. The search for appropriate solubilisation conditions, exogenous acceptor substrates, as well as the possible reconstitution of solubilised enzyme into artifical lipid bilayers, is ongoing.

The neo-*O*-acetylated product released, from endogenous substrates, by propionic acid, following the incubation of horse submandibular gland microsomes with radiolabelled AcCoA, co-migrated with authentic Neu4,5Ac$_2$ on both silica and cellulose TLC plates. The identification of Neu4,5Ac$_2$ as the neo-*O*-acetylated product was afforded by its susceptibility to both saponification by NaOH and the 4-*O*-acetyl-specific esterase (sialate-4-*O*-acetylesterase) from MHV-S. The identification of Neu4,5Ac$_2$ was further substantiated by control

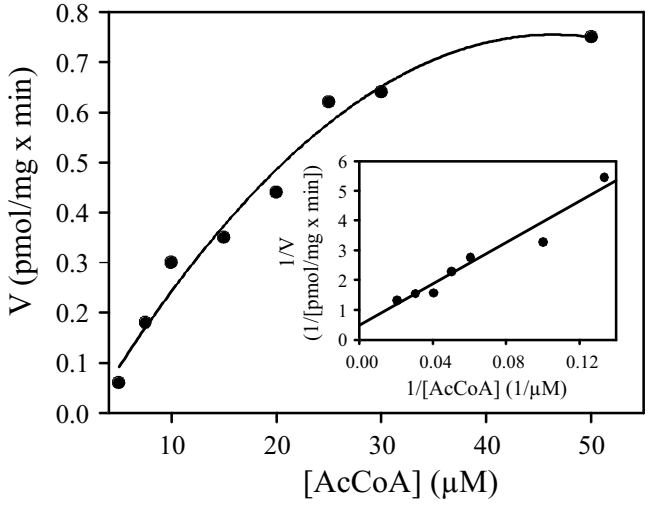

Figure 6. The saturation curve (V vs. [AcCoA]) and the Lineweaver-Burk plot (1/V vs. 1/[AcCoA]: insert) of the incorporation of [^{3}H]acetyl groups into endogenous sialic acid acceptors from equine submandibular glands. The K_M and V_{max} calculated for the incorporation of [^{3}H]acetate was 32.1 μM and 1.21 pmol/mg protein x min, respectively.

experiments where ICV was unable to de-*O*-acetylate the newly transferred acetyl groups. The discovery that the haemagglutinin-esterase proteins of MHV-S and ICV specifically recognise 4-*O* [12] and 9-*O*-acetylated sialic acids [26,27] respectively, provides extremely powerful tools for the detection and identification of sialic acids *O*-acetylated at either C-4 or C-9. Not only can these viruses be used for the identification of neo-*O*-acetylated sialic acids, as was shown in this study, but also, in the case of the IVC, they have been used to detect *O*-acetylated sialic acids in isolated immobilised sialoglycoconjugates [28], immobilised gangliosides from human melanomas [29], as well as in tissue sections [30].

The characterisation of the membrane bound 4-*O*-acetyltransferase showed, with respect to temperature, pH, time and protein, similarities to those described for the corresponding guinea pig liver enzyme [8]. However, the K_M-value of 32.1 μM for AcCoA is significantly higher than that observed for the 4-*O*-acetyltransferase from guinea pig liver Golgi (K_M: 0.6 μM) [8] and the 9-*O*-acetyltransferase from rat liver Golgi (K_M: 0.6 μM) [22]. This discrepancy may be due to the relatively large quantity of endogenous acceptor substrates (mucin) present within the microsomes isolated from equine submandibular gland in comparison to that from guinea pig and rat liver. This is further substantiated by the finding that the membrane-bound 9-*O*-acetyltransferase from bovine submandibular gland microsomes was, likewise, only saturable at relatively high AcCoA concentrations (10 μM) [14].

Table 1. The inhibition of [^{3}H]acetate incorporation into endogenous sialic acid from bovine submandibular gland microsomes.

Inhibitors (1 mM final concentration unless otherwise stated)	*% Inhibition*
0.1 mM CoA	40
CoA	100
3′-dephospho-CoA	79
5′-IMP	–
5′-IDP	17
5′-AMP	–
5′-ADP	36
ß-NAD	25
ß-NADP	–
DIDS	95

As was the case with the K_M for AcCoA, the K_i value determined for CoA (240 μM) was found to be significantly higher than that observed for the sialate *O*-acetyltransferases from guinea pig liver (K_i: 4.2 μM) [8] and rat liver (K_i: 26.3 μM) [23]. Inhibition of *O*-acetylation was also observed for a number of other nucleotides. Information obtained from these inhibition studies suggest that di-phosphorylation at C-5 is required for substrate binding. Similar findings were also observed with the sialate-4-*O*-acetyltransferase from guinea-pig liver (Iwersen *et al*., unpublished data). Interestingly, ß-NADP was unable to inhibit *O*-acetylation, suggesting that substrate binding is not only influenced by the degree of phosphorylation, but also the position. That is, a phosphate group at C-2 of the ribose moiety (ß-NADP) resulted in the complete loss of inhibitory activity, whereas its absence, in the case of ß-NAD, resulted in moderate inhibition. However, the presence of a phosphate group at C-3 of the ribose moiety appears to be important, with CoA being a better inhibitor of *O*-acetylation that 3′-dephospho-CoA.

The non-penetrating probe DIDS was also capable of significantly inhibiting the incorporation of [^{3}H]acetate into endogenous sialic acid acceptors. DIDS has been shown to inhibit the utilisation of the sugar nucleotides, UDP-glucose, and UDP-*N*-acetylglucosamine, which are required for the formation of the oligosaccharide-lipid involved in *N*-glycosylation, by interacting with the cytoplasmically oriented anion binding sites of the corresponding glycosyltransferases [31]. A previous report has also shown that DIDS inhibits the incorporation of radiolabelled acetate into acid-insoluble sialic acid acceptors upon incubation of [^{3}H]AcCoA with intact rat liver Golgi vesicles [22]. Whether the inhibition of *O*-acetylation by DIDS is due to the non-specific inhibition of an AcCoA transporter, as has been reported for the adenosine 3′-phosphate 5′-phosphosulfate and CMP-Neu5Ac transporters [32,33], is as yet unclear. It may be possible that DIDS inhibits the putative first step, the utilisation of exogenous AcCoA to form an acetyl intermediate, in a transmembrane reaction similar to that described by [23].

The exact mechanism by which enzymatic sialate *O*-acetylation occurs in equine and bovine submandibular glands, as well as in guinea pig liver, is still open to debate. To date there is insufficient data available to decide whether

a transmembrane enzyme complex similar to that postulated for rat liver [23] also occurs in other systems. Only by experiments utilising the purified protein(s) involved in sialate *O*-acetylation will the mechanism of the reaction be elucidated. Thus far, all attempts to purify one of the sialate-*O*-acetyltransferases to homogeneity have failed. The isolation of the cDNA encoding the 9-*O*-acetyltransferase by expression cloning has also proved difficult. A number of groups have recently isolated cDNAs by expression cloning of human melanoma libraries in either CHO [34] or COS [35] cells expressing the ganglioside substrate G_{D3}. The clones isolated encoded proteins with sequence similarities to the milk fat globule membrane glycoprotein [34] and a fusion protein between Tet^r and a sequence reported to be part of the P3 plasmid [35], respectively. In another approach a clone encoding a protein identical to the Vitamin D binding protein was isolated by expression cloning of rat liver cDNA library in COS cells expressing terminal α2,6-linked sialic acids [35]. Kanamori *et al*. (1997) [36] have also isolated the cDNA of the putative AcCoA transporter from COS-1 cells. All the clones isolated by expression cloning were capable of inducing *O*-acetylation at the sialic acid side chain, but clearly do not encode one of the sialate-*O*-acetyltransferases in itself. It is hoped that in the not too distant future one of the sialate-*O*-acetyltransferases will be isolated either by conventional protein purification or by expression cloning.

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