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Metal ion requirement of bifunctional UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase from rat liver

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The metal ion requirement for both enzymatic activities of the bifunctional UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (E.C. 5.1.3.14/ 2.7.1.60), the key enzyme of *N*-acetylneuraminic acid biosynthesis in rat liver, was investigated. UDP-*N*-acetylglucosamine 2-epimerase was active in imidazole/HCl buffer in the complete absence of any metal ion. 200 mM Na^+, K^+, Rb^+ and Cs^+ activated enzyme activity up to five-fold, whereas lower concentrations of these monovalent metal ions showed only a small effect on UDP-*N*-acetylglucosamine 2-epimerase activity. In sodium phosphate buffer the enzyme activity was increased by 0.5 mM Mg^{2+}, Sr^{2+}, Ba^{2+} and Mn^{2+}, while in the presence of 200 mM NaCl UDP-*N*-acetylglucosamine 2-epimerase activity showed a stronger activation by these divalent metal ions. In imidazole/HCl buffer, UDP-*N*-acetylglucosamine 2-epimerase activity was partially inhibited by 0.5 mM Be^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Sn^{2+} and Fe^{2+}, and completely inhibited by 0.5 mM Zn^{2+} and Cd^{2+}. Divalent metal ions were essential for *N*-acetylmannosamine kinase activity, the most effective being Mg^{2+}, followed by Mn^{2+} and Co^{2+}. The optimal concentration of these metal ions was 3 mM. Less effective were Ni^{2+} and Cd^{2+}, whereas Ca^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+} showed no effect on enzyme activity.

Keywords: *N*-Acetylmannosamine kinase, *N*-acetylneuraminic acid biosynthesis, metal ion requirement, UDP-*N*-acetylglucosamine 2-epimerase

Introduction

N-Acetylneuraminic acid (Neu5Ac) is an important component of the oligosaccharide residues of glycoconjugates. Neu5Ac is involved in a variety of biological processes including cellular adhesion, stabilization of the structure of glycoproteins, receptor functions and formation or masking of recognition determinants (for review see Schauer *et al.* 1995). Neu5Ac is biosynthesized in rat liver by four consecutive reactions (for review see Reutter *et al.* 1997). The first two reactions are catalysed by UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase, E.C. 5.1.3.14) and *N*-acetylmannosamine kinase (ManNAc kinase, E.C. 2.7.1.60). UDP-GlcNAc 2-epimerase and ManNAc kinase were recently purified from rat liver, and it was shown that both enzyme activities reside on the same polypeptide, which acts as a bifunctional enzyme during the biosynthesis of Neu5Ac (Hinderlich *et al.* 1997). The bifunctional enzyme binds two substrates with negatively charged functional groups, UDP-*N*-acetylglucosamine (UDP-GlcNAc) and ATP. Substrates with negatively charged groups often form complexes with metal ions in binding at the active sites of enzymes (Garfinkel & Garfinkel 1985). Whereas an earlier study focused on the inhibition of UDP-GlcNAc 2-epimerase and ManNAc kinase activities in rat liver homogenate by metal ions and selenium (Zeitler *et al.* 1992), the present study seeks to clarify the role of metal ions in the activity of purified UDP-GlcNAc 2-epimerase/ManNAc kinase.

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Materials and methods

Materials

[1–^{14}C]-*N*-acetylmannosamine and UDP-[U-^{14}C]-GlcNAc (free acid) were from ICN (Eschwege, Germany). All other chemicals were from Sigma (Deisenhofen, Germany).

Preparation of metal ion free substrates and enzyme: UDP (imidazole salt) and UDP-GlcNAc (imidazole salt) were prepared from UDP (sodium salt) and UDP-GlcNAc (sodium salt) by anion exchange on MonoQ HR 5/5 (Pharmacia, Freiburg, Germany). Substrates were dissolved in water, applied to the column and eluted with a linear gradient of 0 to 400 mM imidazole/HCl, pH 7.5. Substrate concentrations were determined from the absorption at 262 nm, using the sodium salts as standards.

UDP-GlcNAc 2-epimerase/ManNAc kinase was prepared from rat liver as described by Hinderlich *et al.* (1997). For UDP-GlcNAc 2-epimerase assays purified enzyme was applied to a gel filtration column (Superdex 200, Pharmacia) and eluted with 50 mM imidazole/HCl, pH 7.5, 0.1 mM EDTA (free acid), 0.1 mM dithiothreitol or with 20 mM NaH_2PO_4, pH 7.5, 0.1 mM EDTA (free acid), 0.1 mM dithiothreitol. For ManNAc kinase assays purified enzyme was eluted with 50 mM Hepes/NH_3, pH 8.1, 0.1 mM EDTA (free acid), 0.1 mM dithiothreitol. In order to stabilize the enzyme after chromatography UDP (imidazole salt) was added to a concentration of 0.1 mM to the respective fractions.

Enzyme assays: UDP-GlcNAc 2-epimerase assays contained 25 mM imidazole/HCl, pH 7.5, and 10 mM NaH_2PO_4, pH 7.5, respectively, 0.1 mM EDTA (free acid), 0.1 mM dithiothreitol, 0.5 mM UDP-GlcNAc (imidazole salt), 25 nCi [^{14}C]-UDP-GlcNAc (free acid), 0.2 μg UDP-GlcNAc 2-epimerase/ManNAc kinase and variable amounts of metal ions (chloride salts) in a total volume of 225 μl.

The ManNAc kinase assay was performed in a volume of 225 μl, containing 25 mM Hepes/NH_3, pH 8.1, 0.1 mM EDTA (free acid), 0.1 mM dithiothreitol, 10 mM ATP (Tris salt), 5 mM ManNAc, 50 nCi [1–^{14}C]-ManNAc, 0.2 μg UDP-GlcNAc 2-epimerase/ManNAc kinase and variable amounts of metal ions (chloride salts).

All assays were incubated for 30 min at 37 °C and stopped by addition of 350 μl ethanol. Radiolabelled substrates were separated by paper chromatography as described earlier (Zeitler *et al.* 1992). Radioactivity was determined in the presence of Ultima Gold XR (Packard, Groningen, the Netherlands) in a Tri-Carb 1900 CA liquid scintillation analyser (Packard).

Protein concentration was measured by the method of Bradford (1976), using BSA as standard. One unit (U) of enzyme activity was defined as the formation of 1 μmol of product per min at 37°C. Specific activity was expressed as U per mg of protein. All values were means of two independent experiments.

Results

UDP-GlcNAc 2-epimerase

UDP-GlcNAc 2-epimerase activity was tested in several metal ion-free buffer systems. The enzyme activity was totally inhibited in all buffers containing ammonium ions or primary amines (e.g. Hepes/NH_3, $NH_4H_2PO_4$, Tris/HCl; data not shown). In imidazole/HCl buffer, in the absence of any metal ions, UDP-GlcNAc 2-epimerase activity was 0.21 U mg^{-1}. In sodium phosphate buffer the specific enzyme activity was 4-fold higher than in imidazole/HCl. Therefore the influence of metal ions on UDP-GlcNAc 2-epimerase activity was investigated in both buffer systems.

The dependence of UDP-GlcNAc 2-epimerase activity on the concentration of Na^+ and K^+ showed similar profiles in imidazole/HCl and sodium phosphate buffer (Figure 1). Enzyme activity was

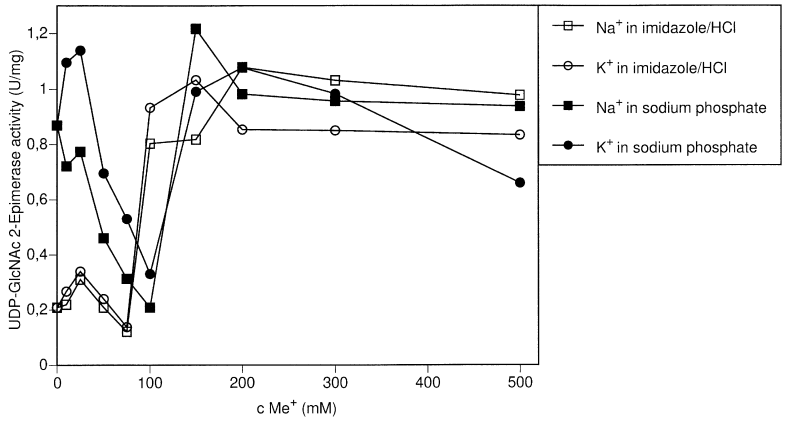

Figure 1. Effects of the concentrations of Na^+ and K^+ on the activity of UDP-GlcNAc 2-epimerase in 25 mM imidazole/HCl, pH 7.5, or 10 mM sodium phosphate, pH 7.5.

Table 1. Effects of monovalent metal ions on the activity of UDP-GlcNAc 2-epimerase in 25 mM imidazole/HCl, pH 7.5, or 10 mM sodium phosphate, pH 7.5

Metal ion	UDP-GlcNAc 2-epimerase activity (%)			
	Imidazole/HCl		Sodium phosphate	
	0.5 mM Me^+	200 mM Me^+	0.5 mM Me^+	200 mM Me^+
None	100	100	100	100
Li^+	106	116	114	48
Na^+	87	518	76	113
K^+	94	410	82	124
Rb^+	107	215	87	164
Cs^+	113	310	181	47

increased by Na^+ and K^+ concentrations up to 25 mM, inhibited by concentrations between 50 mM and 100 mM, then increased again at concentrations of 150 mM and higher. At Na^+ and K^+ concentrations up to 100 mM, the enzyme activity was different in the two buffers, but at concentrations between 150 mM and 500 mM no significant difference was detected between the activities in imidazole/HCl and sodium phosphate buffer. To determine whether the physiologically relevant Na^+ and K^+ ions exert a specific influence on UDP-GlcNAc 2-epimerase activity, the dependence of enzyme activity on metal ion concentration was investigated for the non-physiological metal ions, Li^+, Rb^+ and Cs^+, and the profiles compared with those for Na^+ and K^+ (Table 1). At a concentration of 0.5 mM, which was stoichiometric (1:1) with the UDP-GlcNAc concentration, very little or no effect was observed for all metal ions in imidazole/HCl and sodium phosphate buffer. However, at 200 mM enzyme activity was activated by Rb^+, as well as by Na^+ and K^+; in imidazole/HCl it was also activated by Cs^+. It is therefore concluded that the effect of monovalent cations on UDP-GlcNAc 2-epimerase activity is due to their ionic strength. Cs^+ in sodium phosphate showed anomalous behaviour; it activated at 0.5 mM but inhibited at 200 mM. The reason for this effect is unclear.

Several divalent metal ions were investigated for their influence on UDP-GlcNAc 2-epimerase activity (Table 2). Their effects were studied in the absence and presence of 200 mM NaCl to reveal any effect of ionic strength. In imidazole/HCl enzyme activity was inhibited by almost all the divalent metal ions investigated. Only Ca^{2+} and Sr^{2+} in the absence of NaCl had no inhibitory effect on enzyme activity. On the other hand, Zn^{2+} and Cd^{2+} inhibited the enzyme completely. In sodium phosphate buffer Mg^{2+} and Mn^{2+} slightly activated the enzyme, and Sr^{2+} and Ba^{2+} were most effective in enzyme activation. The other investigated metal ions (Be^{2+}, Ca^{2+},

Table 2. Effects of divalent metal ions on the activity of UDP-GlcNAc 2-epimerase in 25 mM imidazole/HCl, pH 7.5, or 10 mM sodium phosphate, pH 7.5

Metal ion	UDP-GlcNAc 2-epimerase activity (%)			
	Imidazole/HCl		Sodium phosphate	
	0.5 mM Me^{2+}	0.5 mM Me^{2+} +200 mM NaCl	0.5 mM Me^{2+}	0.5 mM Me^{2+} +200 mM NaCl
None	100	518	100	113
Be^{2+}	27	88	33	150
Mg^{2+}	55	94	118	150
Ca^{2+}	101	425	81	117
Sr^{2+}	105	321	168	178
Ba^{2+}	89	178	166	178
Mn^{2+}	41	77	132	139
Sn^{2+}	34	42	60	102
Fe^{2+}	11	15	28	79
Zn^{2+}	<1	<1	15	29
Cd^{2+}	<1	<1	8	12

Sn^{2+}, Fe^{2+}, Zn^{2+} and Cd^{2+}) inhibited the enzyme, but in the presence of 200 mM NaCl enzyme activity was partially protected against inhibition. In all the assays shown in Table 2 the divalent metal ions were present at concentrations of 0.5 mM. Higher concentrations of the ions inhibited UDP-GlcNAc 2-epimerase activity in all cases (data not shown). The activating effects of Mg^{2+}, Mn^{2+}, Sr^{2+} and Ba^{2+} were lost at concentrations higher than 3 mM, when these ions became inhibitory.

ManNAc kinase

ManNAc kinase was active in several buffer systems in the presence of 5 mM Mg^{2+}. Most effective was Hepes/NH_3, but also Tris/HCl, imidazole/HCl and sodium phosphate were also useful for the assay. Monovalent metal ions (Li^+, Na^+, K^+, Rb^+, Cs^+) could not replace Mg^{2+}; the enzyme was completely inactive in the presence of these ions, as well as in the absence of any metal ion. The effect of divalent metal ions was investigated in Hepes/NH_3.

Figure 2 shows the dependence of ManNAc kinase activity on Mg^{2+}, Mn^{2+} and Co^{2+}. The three ions showed similar influences on enzyme activity, the most effective ion in enzyme activation being Mg^{2+}. Between 1 mM and 5 mM Mg^{2+}, Mn^{2+} or Co^{2+}, ManNAc kinase activity was nearly constant. At higher concentrations the enzyme was partially inhibited by Mg^{2+} and strongly inhibited by Mn^{2+} and Co^{2+}. Further divalent metal ions were tested for their ability to replace Mg^{2+} in the kinase reaction (Table 3). Ni^{2+} and Cd^{2+} were found to stimulate ManNAc kinase activity at a concentration of 3 mM. Ca^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} and Sn^{2+} did not activate ManNAc kinase. ManNAc kinase activity was increased by the presence of 200 mM NaCl. In the presence of Mg^{2+} and NaCl enzyme activity was 25% higher than without NaCl. 200 mM NaCl produced very little or no stimulation (maximum 10% increased ManNAc kinase activity; data not shown) in the presence of the other tested divalent ions.

Discussion

We studied the role of metal ions in the activity of the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase, the key enzyme in the biosynthesis of Neu5Ac. The enzyme binds two substrates with negatively charged functional groups, UDP-GlcNAc and ATP, but these substrates have different characteristics. Whereas ATP contains a triphosphate group with a free acid function at the terminal position, UDP-GlcNAc has only a diphosphate bridge between two sugar residues. Because of the high negative charge of their common substrate, ATP-requiring enzymes, including the hexokinases, bind ATP as a Me^{2+}/ATP–complex (Garfinkel & Garfinkel 1985). Many UDP-GlcNAc-binding enzymes have been

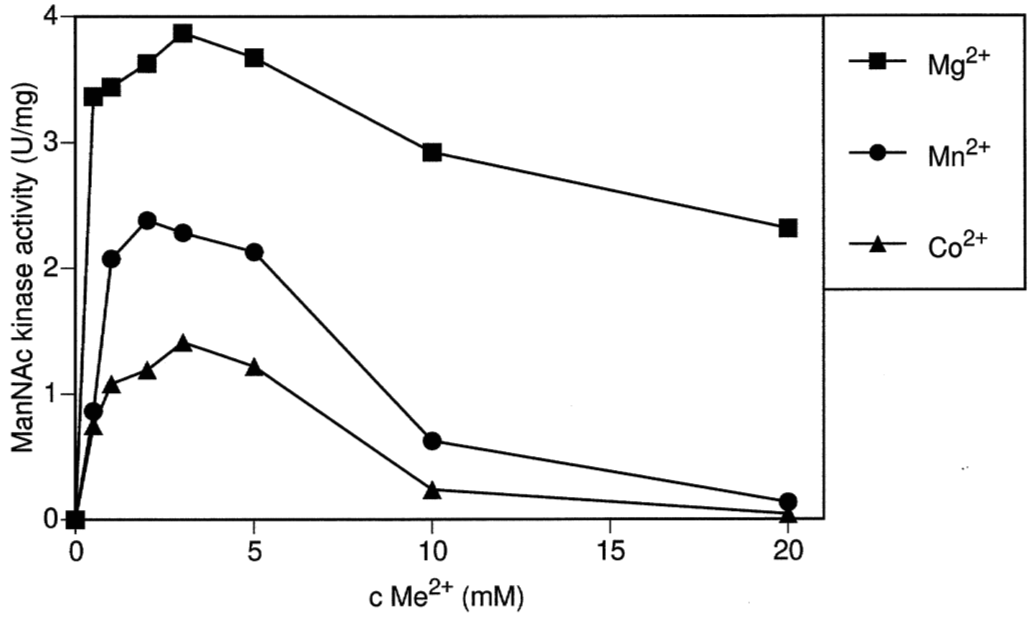

Figure 2. Effects of the concentrations of Mg^{2+}, Mn^{2+} and Co^{2+} on the activity of ManNAc kinase in 25 mM Hepes/NH_3, pH 8.1.

Table 3. Effects of divalent metal ions on the activity of ManNAc kinase in 25 mM Hepes/NH_3, pH 8.1. Me^{2+} concentration was 3 mM

Metal ion	ManNAc kinase activity (%)
Mg^{2+}	100
Mn^{2+}	59
Co^{2+}	34
Ni^{2+}	17
Cd^{2+}	11
Ca^{2+}	6
Ba^{2+}	5
Cu^{2+}	4
Fe^{2+}	4
Zn^{2+}	3
Sn^{2+}	<1
none	<1
Na^{+} (200 mM)	<1
Mg^{2+} + Na^{+} (200 mM)	125

tested for their metal ion requirement, and they behave more heterogeneously than the ATP-binding enzymes. Divalent metal ions are essential for the activity of several *N*-acetylglucosaminyltransferases (Kean 1983, Holmes 1988, Das & Gillin 1991, Tsuji *et al.* 1996) whereas the UDP-*N*-acetylglucosamine 2-epimerase from E. coli (Kawamura *et al.* 1978) and the β-1–6-*N*-acetylglucosaminyltransferase from tammar wallaby mammary gland (Urashima *et al.* 1992) are active in the absence of divalent metal ions.

The metal ion requirement of UDP-GlcNAc 2-epimerase/ManNAc kinase is comparable with that of the ATP- or UDP-GlcNAc-binding enzymes mentioned above. Divalent metal ions are essential for ManNAc kinase activity, the most effective ion being Mg^{2+}. The optimal Mg^{2+} concentration for ManNAc kinase activity is 3 mM, which is in the same order as the Mg^{2+} concentration in the cytosol (1.5 mM). It should be noted that ManNAc kinase activity does not depend on a 1:1 stoichiometry of Mg^{2+} and ATP (the ATP concentration in the assays was 10 mM). By contrast, a stoichiometric requirement has been reported, for example, for the related *N*-acetylglucosamine kinase from rat liver (Allen & Walker 1980). Mg^{2+} can be replaced by Mn^{2+} and Co^{2+} up to concentrations of 5 mM, but with a lower efficiency, as also reported for mammalian hexokinases (Darby & Trayer 1983, Lai & Blass 1985). At concentrations higher than 5 mM, Mn^{2+} and Co^{2+} inhibited ManNAc kinase activity more strongly than did comparable concentrations of Mg^{2+}. This inhibition may be due to the oxidation of cysteine residues in the active site of the enzyme (Zeitler *et al.* 1992). However, the inhibition of ManNAc kinase by nonphysiologically high Mg^{2+} concentrations cannot be due to a redox reaction. Inhibition by the redox-inactive metal ions Be^{2+}, Mg^{2+} and Ba^{2+} is also observed for UDP-GlcNAc 2-epimerase activity. These results reveal another inhibition mechanism, possibly an allosteric effect of the metal ions, which has already been reported, for example, for guinea pig hexokinase (Bachelard 1971). This suggestion is supported by a partial protection of UDP-GlcNAc 2-epimerase against inhibition by a high excess of sodium ions, which prevent the binding of the divalent metal ion at the potential allosteric site.

UDP-GlcNAc 2-epimerase was active in the absence of divalent metal ions. In this respect the enzyme resembles the prokaryotic UDP-GlcNAc 2-epimerase (Kawamura *et al.* 1978), and differs from most of the *N*-acetylglucosaminyltransferases investigated. It is therefore assumed that, in general, the binding of UDP-GlcNAc to an enzyme does not need divalent metal ions, but the metal ions can play a role in transferase activity. None of the studies mentioned above focused on the role of monovalent metal ions in the activities of the enzymes investigated. This study reveals no specific role of monovalent metal ions in the activity of UDP-GlcNAc 2-epimerase. However, the dependence of enzyme activity on ionic concentration shows that the ionic strength of the reaction medium, as generated by metal ions and their counter ions, plays a role in enzyme activity. The highest UDP-GlcNAc 2-epimerase activity was found in the presence of 150–200 mM of the metal salts. This concentration range agrees well with the salt concentration in cell cytosols (150 mM KCl). UDP-GlcNAc 2-epimerase/ManNAc kinase has a complex oligomeric structure. Six subunits form the active hexameric enzyme with a total molecular mass of 450 kD (Hinderlich *et al.*, 1997). Ionic strength influences UDP-GlcNAc 2-epimerase activity as well as ManNAc kinase activity. An appropriate ionic strength may therefore be important for the optimal conformation of this highly complex enzyme.

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References

Allen MB, Walker DG. 1980 Kinetic characterization of *N*-acetylglucosamine kinase from rat and liver kidney. *Biochem J* **185**, 577–582.

Bachelard HS. 1971 Allosteric activation of rat brain hexokinase by magnesium ions and by magnesium ion-adenosine triphosphate complex. *Biochem J* **125**, 249–252.

Bradford MM. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72** 248–254.

Darby MK, Trayer IP. 1983 Metal-nucleotide structure at the active sites of the mammalian hexokinases. *Eur J Biochem* **129**, 555–561.

Das S, Gillin FD. 1991 Chitin synthase in encysting Entamoeba invadens. *Biochem J* **280**, 641–647.

Garfinkel L, Garfinkel D. 1985 Magnesium regulation of the glycolytic pathway and the enzymes involved. *Magnesium* **4**, 60–72.

Hinderlich S, Stäsche R, Zeitler R, Reutter W. 1997 A bifunctional enzyme catalyzes the first two steps of *N*-acetylneuraminic acid biosynthesis in rat liver. Purification and characterization of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase. *J Biol Chem* **272**, 24313–24318.

Holmes, 1988 Characterisation of a beta *N*-acetylglucosaminyl transferase associated with synthesis of type 1 and type 2 lacto-series tumor-associated antigens from the human colonic adenocarcinoma cell line SW403. *Arch Biochem Biophys* **260**, 461–468.

Kawamura T, Kimura M, Yamamori S, Ito E. 1978 Enzymatic formation of uridine diphosphate N-acetyl-D-mannosamine. *J Biol Chem* **253**, 3595–3601.

Kean EL. 1983 Influence of metal ions on the biosynthesis of *N*-acetylglucosaminyl polyprenols by the retina. *Biochim Biophys Acta* **750**, 268–273.

Lai JC, Blass JP. 1985 Differences in the responses of brain cytosolic and mitochondrial hexokinases to three essential divalent metal ions. *Comp. Biochem. Physiol. C.* **80**, 285–290.

Reutter W, Stäsche R, Stehling P, Baum O. 1997 The biology of sialic acids: Insight into their structure, metabolism and function in particular during viral infection. In *Glycosciences. Status and Perspectives.* (Gabius, H.-J. and Gabius, G., Weinheim: Chapman & Hall), 245–259.

Schauer R, Kelm S, Reuter G, Roggentin P, Shaw L. 1995 Biochemistry and role of sialic acids. In *Biology of the sialic acids* Rosenberg, A. (ed), New York: Plenum Press), 7–67.

Tsuji Y, Urashima T, Matsuzawa T. 1996 The characterization of a UDP-*N*-acetylglucosamine:Gal beta1–4GlcNAc beta1–3 *N*-acetylglucosaminyltransferase in fluids from rat rete testis. *Biochim Biophys Acta* **1289**, 115–121.

Urashima T, Messer M, Bubb WA. 1992 Biosynthesis of marsupial milk oligosaccharides. II. Characterization of a beta 6-*N*-acetylglucosaminyltransferase in lactating mammary glands of the tammar wallaby, Macropus eugenii. *Biochim Biophys Acta* **1117**, 223–231.

Zeitler R, Banzer JP, Bauer C, Reutter W. 1992 Inhibition of the biosynthesis of *N*-acetylneuraminic acid by metal ions and selenium *in vitro*. *BioMetals* **5**, 103–109.