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Note

Release of sialic acid from substrates by sialidase in the presence of $H_2[^{18}O]$

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Sialidases (E.C. 3.2.1.18) are found in viruses, bacteria, fungi, protozoa, and mammals[1–4], and those from *Clostridium perfringens, Vibrio cholerae,* and *Arthrobacter ureafaciens* are used in biological and structural studies of glycoproteins and glycolipids. They show a broad specificity in splitting off α-glycosidically bound sialic acid[4–6]. Although the effect of structural modifications in the sialic acid or aglycon parts of substrates on the rate of hydrolysis by sialidase has been studied[4,7–15], it is not known if glycosyl-(sialyl)–oxygen or oxygen–aglycon bond-cleavage occurs. Therefore, we have subjected suitable substrates, namely, *N*-acetyl-α-D-neuraminyl-(2→3)-lactose (sialyl-lactose), *N*-acetyl-α-D-neuraminyl-(2→3)-lactitol (sialyl-lactitol), and 2-*O*-(3-methoxyphenyl)-*N*-acetyl-α-D-neuraminic acid (MPN), to hydrolysis with sialidases from *Clostridium perfringens* (CPS) or *Vibrio cholerae* (VCS) in $H_2[^{18}O]$ buffer solutions, and have monitored the incorporation of the label.

In order to establish that non-enzymic transfer of label from $H_2[^{18}O]$ to the expected reaction products does not occur, *N*-acetyl-D-neuraminic acid (Neu5Ac) and lactose, the cleavage products of sialyl-lactose,were separately incubated in sodium/potassium phosphate buffer (95% $H_2[^{18}O]$, 0.1M, pH 5.4) for up to 48 h in the range 37–55°. G.l.c.–m.s., after lyophilisation and trimethylsilylation, showed that there was no introduction of ^{18}O into HO-1 of the glucose residue of lactose, or into HO-2 or the carboxyl group of Neu5Ac, when the incubation was carried out for 30 min at 37°. Neu5Ac turned out to be less prone to label incorporation than lactose.

In the ^{18}O-label-transfer studies, CPS or VCS was added to the substrate as a solution in $H_2[^{16}O]$, which required $^{18}O/^{16}O$-correction. This approach was necessary because lyophilisation of the protein solution partially inactivates the enzyme. Typically. a solution of CPS in $H_2[^{16}O]$ was incubated with a solution of sialyl-lactose in $H_2[^{18}O]$ buffer (see Experimental). After lyophilisation and trimethylsilylation, the mixture of cleavage products was analysed by g.l.c.–m.s. The

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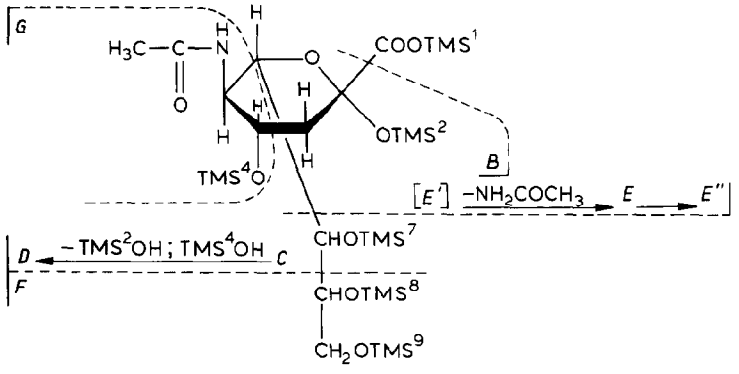
resulting, essential, mass-spectral data for Neu5Ac and lactose are given in Tables I and II, together with the main fragment ions[16,17]. The data for trimethylsilylated Neu5Ac (Table I) indicate the presence of ^{18}O at C-2. The fragment *D* originates from fragment *C* (see **1**), with loss of C-2-TMSOH and C-4-TMSOH and also the ^{18}O-label. Fragment *G*, which contains the TMSO group at C-4, does not contain ^{18}O. Since the *m/z* values of the fragments of standard lactose (Table II) were identical to those for the enzymic product, no ^{18}O-labelling had taken place.



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The incubation experiments using CPS or VCS were repeated with sialyl-lactitol and MPN; a longer incubation time was used since non-enzymic label-transfer to the aglycon is not possible. In these experiments, the ^{18}O-label was found exclusively at C-2 in Neu5Ac.

Thus, the sialidases from *Clostridium perfringens* and *Vibrio cholerae* cleaved the glycosidic bond of their substrates between the C-2 of Neu5Ac and the glycosidic oxygen. In contrast to CPS, VCS needs calcium for activity[18], but this does

TABLE I

MASS-SPECTRAL DATA FOR TRIMETHYLSILYLATED Neu5Ac (I) AND Neu5Ac (II) ENZYMICALLY RELEASED IN $H_2[^{18}O]$-CONTAINING BUFFER

m/z *(I)*	*Fragment*[a]	*Structure*[b]	m/z *(II)*
726	*A*	M – CH_3	728
624	*B*	M – $COOTMS^1$	626
536	*C*	M – $CHOTMS^8CH_2OTMS^9$	538
356	*D*	C – TMS^2OH – TMS^4OH	356
375	*E*	M – $CHOTMS^7CHOTMS^8CH_2OTMS^9$ – NH_2COCH_3	377
285	*E''*	E – TMS^2OH	285
205	*F*	$CHOTMS^8CH_2OTMS^9$	205
173	*G*	$CH_3CONHCHCHOTMS^4$	173

[a]See **1**. [b]TMS = Me_3Si.

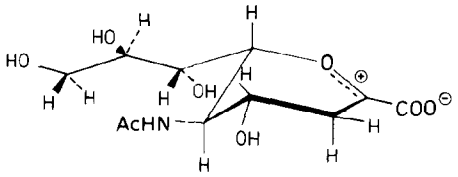
TABLE II

MASS-SPECTRAL DATA FOR TRIMETHYLSILYLATED LACTOSE (I) AND LACTOSE (II) ENZYMICALLY RELEASED IN $H_2[^{18}O]$-CONTAINING BUFFER

m/z *(I) and (II)*	*Fragment*[a]
723	M − CH_3 − TMSOH − TMSOH
683	TMSOCHC(OTMS)CHO–Glc
611	M − CH_2OTMS − TMSOCHCHOTMS
569	TMSOCHO–Glc (TMSO of Gal C-3)
521	611 − TMSOH
451	M − O-Glc
361	451 − TMSOH
204	TMSOCHCHOTMS
191	$CH(OTMS)_2$ (one TMSO of Glc C-1)

[a]Glc is the trimethylsilylated D-glucose residue; Gal is the trimethylsilylated D-galactosyl group; TMS = Me_3Si.

not change the mechanism of glycosidic bond cleavage. The type of cleavage found is consistent with that established for the hydrolysis of glycosides by acids[19,20] and by such other glycosidases as lysozyme[21], β-D-galactosidase[22], α- and β-D-glucosidase[23], β-D-glucuronidase[24], and invertase[25]. By analogy with β-D-galactosidase, an intermediate oxo-carbonium ion (**2**) is probably involved, which may be stabilised by non-covalent interactions with the enzyme or may react with, and become covalently bound to, the enzyme. The oxo-carbonium ion **2** possesses a distorted half-chair structure which resembles that of 2,3-dehydro-2-deoxy-Neu5Ac, and which may explain the strongly competitive inhibition of the latter in sialidase action[4]. The aglycon plays a minor role in the hydrolysis, which suggests that sialidase approaches its substrate from the glyconic site of the molecule. This view is supported by the observation that matrix-bound Neu5Ac can readily be cleaved by CPS[26], which emphasises the broad specificity of bacterial sialidase.



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Although a great deal is known about the substrate specificity of sialidases from micro-organisms and mammals[4], little has been reported on the identity of the amino acid residues in the active site and this aspect is now being investigated.

EXPERIMENTAL

Clostridium perfringens sialidase (0.6 U/mg of protein), purchased from

Boehringer, was purified further by preparative electrophoresis on polyacrylamide gel[15]. *Vibrio cholerae* sialidase (1 U/mL) was obtained from Behringwerke. 2-*O*-(3-Methoxyphenyl)-α-Neu5Ac was obtained from Boehringer. Purified α-Neu5Ac-(2→3)-lactose was a generous gift from Dr. G. Strecker, and α-Neu5Ac-(2→3)-lactitol was prepared[27] by reduction of the corresponding trisaccharide with $NaBH_4$.

The CPS incubation mixture consisted of 95 μL of sodium/potassium phosphate buffer (0.1M, pH 5.4) in 95% $H_2[^{18}O]$ and 5 μL of CPS (0.5 U) in $H_2[^{16}O]$. For the VCS incubations, a sodium acetate buffer (0.05M, pH 5.5) in $H_2[^{18}O]$ containing 0.154M NaCl and 9mM $CaCl_2$ was used. After addition of the substrate, each mixture was incubated for 30 min at 37° and lyophilised, and the residue was treated with hexamethyldisilazane–chlorotrimethylsilane–pyridine (1:1:5).

G.l.c. was carried out on a Varian Aerograph 2740, equipped with a dual flame-ionisation detector and glass columns (2.0 m × 4.0 mm i.d.) packed with 3.8% of SE-30 on Chromosorb W HP (80–100 mesh). The temperature programme was 210→280° at 2°/min. The nitrogen flow-rate was 40 mL/min. G.l.c.–m.s. was performed on a Carlo-Erba GC/Kratos MS80/Kratos DS55 System. Mass spectra (70 eV) were recorded using an ion-source temperature of 200°, an accelerating voltage of 3 kV, and an ionising current of 300 μA. The glass column (2.0 m × 4.0 mm i.d.) was packed with 3.8% of SE-30 on Chromosorb W HP (100–200 mesh). The temperature programme was 210→280° at 2°/min.

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