Activity of influenza C virus *O*-acetyl-esterase with *O*-acetyl-containing compounds

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Influenza C virus (strain C/Johannesburg/1/66) was grown, harvested, purified and used as source for the enzyme *O*-acetyl-esterase (*N*-acyl-*O*-acetylneuraminate *O*-acetylhydrolase; EC 3.1.1.53). This activity was studied and characterized with regard to some new substrates. The pH optimum of the enzyme is around 7.6, its stability at different pH values shows a result similar to that of the pH optimum, and its activity is well maintained in the pH range from 7.0 to 8.5 (all these tests were performed with 4-nitrophenyl acetate as substrate). Remarkable differences were found in the values of both K_m and V_{max} with the synthetic substrates 4-nitrophenyl acetate, 2-nitrophenyl acetate, 4-methylumbelliferyl acetate, 1-naphthyl acetate and fluorescein diacetate. The use of 4-nitrophenyl acetate, 4-methylumbelliferyl acetate or 1-naphthyl acetate as substrate seems to be convenient for routine work, but it is better to carry out the measurements in parallel with those on bovine submandibular gland mucin (the latter is a natural and commercially available substrate). It was found that 4-acetoxybenzoic acid, as well as the methyl ester of 2-acetoxybenzoic acid, but not 2-acetoxybenzoic acid itself, are cleaved by this enzyme. Triacetin, di-*O*-acetyladenosine, tri-*O*-acetyladenosine, and di-*O*-acetyl-*N*-acetyladenosine phosphate, hitherto unreported as substrates for this viral esterase, are hydrolysed at different rates by this enzyme. We conclude that the *O*-acetyl-esterase from influenza C virus has a broad specificity towards both synthetic and natural non-sialic acid-containing substrates. Zn^{2+} , Mn^{2+} and Pb^{2+} (as their chloride salts), *N*-acetylneuraminic acid, 4-methylumbelliferone and 2-acetoxybenzoic acid (acetylsalicylic acid) did not act as inhibitors.

INTRODUCTION

Influenza C virus was discovered in human respiratory tract infections by Taylor (1949), who described it as being antigenically unrelated to the influenza A and B viruses. Francis *et al.* (1950) isolated a strain which was similar to that studied by Taylor (1949). As late as 1977, very little was known about the molecular biology of influenza C virus in comparison with the type A and B viruses (Compans *et al.*, 1977), in part due to the greater importance of the type A and B viruses as human pathogens, and also due to the availability of better systems for the propagation of these viruses in cell culture. However, it was known that both the receptor for and the receptor-destroying activity of influenza C virus were different from those of influenza A and B viruses (Ohuchi *et al.*, 1978). Information concerning the glycoproteins of influenza C virus was published (Nakamura *et al.*, 1979; Herrler *et al.*, 1979, 1981; Sugawara *et al.*, 1981). It has also been reported (Ohuchi *et al.*, 1982) that the envelope of this virus is able to fuse with the host cell membrane at acid pH, a very important phenomenon for viral infectivity.

Nevertheless, the nature of the receptor for influenza C virus was not known until 1985. In that year, Herrler *et al.* (1985a) observed that, contrary to the previously accepted idea, sialic acid could very well be an essential component of the receptor for influenza C virus. Soon after, Herrler *et al.* (1985b) reported that the receptor-destroying enzyme (RDE) demonstrated by Hirst (1950), occurring on the influenza C virus, is a sialate *O*-acetyl-esterase (*N*-acyl-*O*-acetylneuraminate *O*-acetylhydrolase; EC 3.1.1.53). This enzyme was comparable with neuraminidase, the RDE for the influenza A and B viruses. Among the different *O*-acetyl-*N*-acetylneuraminic acids, influenza C virus uses 9-*O*-

acetyl-*N*-acetylneuraminic acid (Neu5,9Ac₂) as a high-affinity receptor for attachment to cells (Rogers *et al.*, 1986; Herrler *et al.*, 1987). Additionally, gangliosides containing this type of sialic acid are potential receptors for influenza C virus (Herrler & Klenk, 1987). Influenza C virus glycoprotein HE exhibits both haemagglutinin (H) and esterase (E) (equivalent to the RDE) activities (Vlasak *et al.*, 1987). However, it was shown (Herrler *et al.*, 1988) that the influenza C glycoprotein is the only myxovirus glycoprotein with three different activities: haemagglutinin (H), esterase (E) (precisely an *O*-acetyl-esterase), and a fusion factor (F); accordingly, it should be designated HEF and not as HE. Studies on the variations in influenza C virus have also been carried out (Chakraverty, 1978; Palese & Young, 1982; Buonagurio *et al.*, 1986; Kawamura *et al.*, 1986; Sugawara *et al.*, 1986; Yamashita *et al.*, 1988a), along with other studies on its glycoproteins (Hongo *et al.*, 1986; Sugawara *et al.*, 1988) and proteins (Yamashita *et al.*, 1988b, 1989).

Several papers concerning the study of the activity of the *O*-acetyl-esterase from influenza C virus have appeared (Vlasak *et al.*, 1987; Muchmore & Varki, 1987; Schauer *et al.*, 1988a,b). It has been reported that the esterase and the haemagglutinating activities, contained in the same glycoprotein and recognizing the same sialic acid (Neu5,9Ac₂), do not share the same binding site (Muchmore & Varki, 1987), and that the catalytic site seems to be 'antigenically silent', although the two postulated sites are probably very close to each other (Hachinohe *et al.*, 1989). However, certain peculiarities and the biological significance of this acetyl-esterase are yet barely understood.

In the present study we describe results of studies on the activity and kinetic characteristics of this enzyme towards several synthetic and natural *O*-acetyl-containing compounds which are

Abbreviations used: BSM, bovine submandibular gland mucin; DFP, di-isopropyl fluorophosphate; HEF, haemagglutinating, esterase and fusion factor; 4-MUAc, 4-methylumbelliferyl acetate; Neu5,9Ac₂, *N*-acetyl-9-*O*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PSM, porcine submandibular gland mucin; RDE, receptor-destroying enzyme.

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not sialic acids. These results show that the specificity of this enzyme towards these hitherto unreported substrates is relatively broad. The significance of this is discussed.

MATERIALS AND METHODS

Virus

The strain of virus used was C/Johannesburg/1/66 (briefly designated as C/JHB/1/66). It was kindly provided by G. Herrler (University of Marburg, Germany) and selected for its relatively good growth capacity. The virions were grown in 8-day-old embryonated eggs, harvested after 3 days, and concentrated and purified as follows. The clarified fluid was centrifuged at 25000 *g* (Beckman J-21B centrifuge, JA-14 rotor) for 150 min. The pellets were resuspended in 10 mM-Tris/HCl/100 mM-NaCl/1 mM-EDTA buffer (pH 7.4), then gently sonicated (30 s), deposited on the top of a continuous potassium tartrate gradient (10–50%, w/v) in the Tris/HCl/EDTA buffer and centrifuged (Beckman L50 centrifuge, SW41 rotor) at 200000 *g* for 5 h. The bands containing the viral fractions were harvested using a peristaltic pump and the pool was dialysed overnight against 10 mM-potassium phosphate buffer (pH 7.4)/100 mM-NaCl at 4 °C.

Control of the viral preparation

The purity and concentration of virus stocks were checked by: (i) haemagglutination and haemagglutination-inhibition tests (Kendal, 1975) with sera from rabbits inoculated with influenza C virus; (ii) electron microscopy using phosphotungstic acid for negative staining; and (iii) SDS/PAGE under reducing conditions (Laemmli, 1970; O'Farrell, 1975), blotting on to nitrocellulose sheets and colouring with colloidal gold (Aurodyne; Janssen, Piscataway, NJ, U.S.A.). A purified sample of virus was employed as the source of *O*-acetyltransferase activity.

Substrates and chemical reagents

Authentic Neu5,9Ac₂ and porcine submandibular gland mucin (PSM) has been previously provided by R. Schauer (University of Kiel, Germany) and L. Warren (Wistar Institute, Philadelphia, PA, U.S.A.) respectively. 4-Nitrophenol, 2-nitrophenol, 1-naphthol, fluorescein, 4-methylumbelliferone, 4-nitrophenyl acetate, 2-nitrophenyl acetate, 1-naphthyl acetate, fluorescein diacetate, 4-methylumbelliferyl acetate (4-MUAc), triacetin (tri-*O*-acetylated glycerol), 5-acetoxyamyl chloride, *O*-acetylserine, 2-acetoxybenzoic (acetylsalicylic) acid, *O*-acetylmandelic acid, 2',3'-di-*O*-acetyladenosine, 2',3',5'-tri-*O*-acetyladenosine, N⁶,2'-*O*,5'-*O*-triacetyladenosine 3'-phosphate, bovine submandibular gland mucin (BSM), glycophorin A, fetuin, BSA, *N*-acetylneuraminic acid (NeuAc) and diisopropyl fluorophosphate (DFP) were all obtained from Sigma (St. Louis, MO, U.S.A.). 4-Acetoxybenzoic acid was purchased from Aldrich (Steinheim, Germany). General laboratory chemicals were obtained from Probus (Barcelona, Spain). Methyl esters of 2-hydroxybenzoic acid, 2-acetoxybenzoic acid, *O*-acetylserine and *O*-acetylmandelic acid were synthesized by esterification with diazomethane; the purity of these esters was checked by i.r. spectroscopy. All commercially available substrates used in this work were pure, except for triacetin (99%), N⁶,2'-*O*,5'-*O*-triacetyladenosine 3'-phosphate (95%) and 4-acetoxybenzoic acid (98%). The purities of BSM, glycophorin A and fetuin were not provided by the manufacturer, but the sample used was of the highest purity available.

Enzyme assays

Influenza C/JHB/1/66 virus was incubated in 100 mM-pot-

assium phosphate buffer, pH 7.6, with the substrate at variable concentrations depending on the sensitivity of the assays (see below). Hydrolysis of the substrates was monitored as described below.

(i) The esterase activity towards glycoproteins was measured as follows. A 10 μ l sample of a virus suspension containing 0.1 mg of protein/ml was added to 250 μ l of a glycoprotein solution (1 mg/ml or 10 mg/ml) in the reaction buffer and incubated for 30 min, 60 min or 12 h at 37 °C. The reaction was stopped by heating for 2 min at 100 °C. Free acetate released by the enzyme was determined with a commercial test kit from Boehringer (Mannheim, Germany; cat. no. 148 261) using a Cary 219 spectrophotometer at 340 nm.

(ii) The free acetate concentration was measured using triacetin, 5-acetoxyamyl chloride, *O*-acetylserine, *O*-acetylserine methyl ester, 4-acetoxybenzoic acid, 2-acetoxybenzoic acid, *O*-acetylmandelic acid, *O*-acetylmandelic methyl ester, 2',3'-di-*O*-acetyladenosine, N⁶,2'-*O*,5'-*O*-triacetyladenosine 3'-phosphate, 2',3',5'-tri-*O*-acetyladenosine and Neu5,9Ac₂ as substrates. A 25 μ l sample from the virus suspension containing 0.1 mg of protein/ml was added to 250 μ l of 1 mM (or 10 mM) substrate in the reaction buffer, incubated for 30 min at 37 °C, and the enzymic reaction was stopped by heating for 2 min at 100 °C, except when N⁶,2'-*O*,5'-*O*-triacetyladenosine 3'-phosphate (free acetate being measured directly without stopping the reaction because all of the substrate is hydrolysed by heating) or 2',3',5'-tri-*O*-acetyladenosine (only assayed at 1 mM, because a 10 mM concentration could not be reached without substrate precipitation) were used as substrates.

(iii) Direct measurement of the increase in absorbance at either 400 nm (4-nitrophenol) or 420 nm (2-nitrophenol) using a Cary 219 spectrophotometer, was used when the enzymic hydrolysis of 4-nitrophenyl acetate or 2-nitrophenyl acetate respectively was measured. The substrates were solubilized in acetonitrile at a concentration of 80 mM. A 25 μ l sample of this solution was added to 2 ml of buffered solution at 25 °C and the spontaneous substrate hydrolysis was measured. Then 25 μ l of a virus suspension (0.05 mg of protein/ml) was added and the enzymic activity was monitored. A reaction temperature of 37 °C is not recommended because of the high degree of spontaneous substrate hydrolysis; thus incubations were carried out at 25 °C.

(iv) The release of 1-naphthol was measured directly at 321 nm in a Cary 219 spectrophotometer. 1-Naphthyl acetate was solubilized in ethanol at a concentration of 100 mM for use as substrate. Then 10 μ l of the ethanolic solution and 10 μ l of a virus suspension (0.1 mg of protein/ml) were added to 1 ml of buffered solution at 37 °C. Spontaneous hydrolysis of the substrate in these conditions was practically negligible.

(v) The salicylate or salicylate methyl ester concentration, produced by esterase activity, was measured at 300 nm in a Cary 219 spectrophotometer when a 1–10 mM-acetylsalicylic acid (2-acetoxybenzoic acid) or its methyl ester solution were used with the buffer, the reaction being carried out at 25 °C using 1–10 μ l of virus suspension (0.1 mg of protein/ml).

(vi) The relative fluorescence of 4-methylumbelliferone or fluorescein released by the enzyme was measured using a Hitachi F4010 spectrofluorimeter, with excitation wavelength at 365 or 490 nm and emission at 450 or 520 nm respectively, when 4-MUAc or fluorescein diacetate were employed. Aliquots of 2 μ l of 5 mM-4-MUAc or 1 mM-fluorescein diacetate solubilized in acetone, 10 μ l of virus suspension (0.01 mg of protein/ml) and 90 μ l of buffer reaction were mixed and incubated for 10 min at 37 °C, and the reaction was stopped by adding 200 μ l of ethanol.

All assays were performed with blanks of heat-denatured enzyme. Protein concentrations were determined by the method of Markwell *et al.* (1978), with BSA as standard. One unit of

enzyme activity was defined as the amount of enzyme which releases 1 μmol of product/min under the assay conditions.

Characterization of the esterase activity

pH optimum. The pH optimum of the enzyme was determined with 4-nitrophenyl acetate as substrate in the pH range from 3.0 to 10.0. 4-Nitrophenol was measured at 330 nm at pH values below 7, and at 400 nm above pH 7.

pH stability. The stability of the enzyme at different pH values was determined by preincubating the enzyme for 30 min at 37 °C at different pH values, ranging from 2.5 to 9.5. The pH was then adjusted to 7.6 by the addition of the appropriate amount of 100 mM-potassium phosphate buffer. Finally, enzyme activity was assayed using 4-nitrophenyl acetate as substrate (see the 'Enzyme assays' section).

Thermal stability. Thermal stability was determined after preincubation for 15–90 min at different temperatures. 4-Nitrophenyl acetate was used as substrate.

Kinetic parameters. Michaelis constants (K_m) and maximal velocity (V_{max}) were determined for several substrates. The enzyme (purified virus) concentration used was carefully chosen in order to perform the assays within the limits of linearity. This had to be established because the different substrates employed in this study have different solubilities and each assay shows a different sensitivity in the product detection. Each preparation was incubated in 100 mM-phosphate buffer, pH 7.6, with the following substrates and concentrations: 4-nitrophenyl acetate (0.05–1 mM), 2-nitrophenyl acetate (0.05–2 mM), 4-MUAc (0.01–0.1 mM), 1-naphthyl acetate (0.1–1 mM) and fluorescein diacetate (0.002–0.02 mM), incubations being carried out under the conditions indicated above. With all substrates the maximum concentration assayed was limited by their very low solubility, preventing concentrations above the calculated K_m being used, except for fluorescein diacetate.

The kinetic parameters were obtained by fitting the experimental data (in a Robust Weighting scheme) to the Michaelis-Menten equation with a non-linear regression least-squares fit computer program (Leatherbarrow, 1987). This computer program, marketed as 'Enzfitter' by Elsevier Biosoft, uses the Marquardt algorithm as an iterative technique. Tolerance in fitting was always achieved after two interactions, thus confirming the accuracy of our data. Each value of velocity versus substrate concentration was the mean of five assays.

Inhibitors. The eventual inhibitory effects of some substances were tested in 100 mM-phosphate buffer, pH 7.6, with 4-nitrophenyl acetate and 4-MUAc as substrates and 12 munits of enzyme in 2 ml or 0.5 munits in 0.1 ml respectively, after a 15 min preincubation at 25 °C with the following inorganic salts: Zn^{2+} (1 mM), Mn^{2+} (1 mM) and Pb^{2+} (1 mM), as their chloride salts. The organic compounds NeuAc (1 mM), 4-methylumbelliferone (0.2 mM) and 2-acetoxybenzoic acid (acetylsalicylic acid, aspirin) (2 mM) were also assayed, as well as DFP (1 mM) which has been described as an inhibitor for this enzyme.

RESULTS

Purity of the virus preparation and its proteins

The high degree of purity of the influenza C virus preparation and the protein fractions separated by SDS/PAGE are shown in Fig. 1. All expected polypeptides were found to be present, without significant contaminating components. Isolation of HEF

Fig. 1. Photomicrograph of the influenza C virus (C/JHB/1/66 strain)

Phosphotungstic acid was used for negative staining. On the bottom are crystals of tartrate. Inset: typical protein separation of purified virus stock revealed by SDS/PAGE. NP, nucleoprotein; M, matrix protein.

Fig. 2. Optimum pH profile of *O*-acetyltransferase from influenza C virus (C/JHB/1/66 strain)

The assay was carried out with 4-nitrophenyl acetate as substrate.

glycoprotein caused a loss in the esterase activity (results not shown).

pH optimum, pH stability and thermal stability

The pH optimum of the enzyme was found to be around 7.6 (Fig. 2) with 4-nitrophenyl acetate as substrate. This is similar to the value found by other authors (Schauer *et al.*, 1988b) using Neu5,9Ac₂ as substrate. Activity was strongly decreased at pH values below 6 and above 9.

Enzyme stability at various pH values was highest at a pH similar to the optimum pH (Fig. 3).

Enzyme activity (without preincubation) was highest at 40 °C (results not shown). The activity was relatively well maintained in the temperature range from 34 to 40 °C. This activity decreased when incubation was carried out for 15 min or longer at 40 °C; greater decreases were produced at 50 °C and 60 °C (Fig. 4). Nevertheless, the enzyme maintained more than 20% of its activity after 5 min of preincubation at 64 °C. Thus the esterase from influenza C virus showed a great stability at high temperatures in comparison with neuraminidase from human influenza A and B viruses (Cabezas *et al.*, 1989; Fiszson *et al.*,

Fig. 3. Effect of pH on stability of *O*-acetylsterase from influenza C virus (C/JHB/1/66 strain)

The enzyme was preincubated at the pH values indicated for 30 min. The assay of enzyme activity was carried out at pH 7.6, with 4-nitrophenyl acetate as substrate.

Fig. 4. Thermostability of the *O*-acetylsterase from influenza C virus (C/JHB/1/66 strain)

The enzyme was preincubated at the temperature indicated for 90 min. Assays were carried out at 40 °C (□), 50 °C (○) and 60 °C (△), with 4-nitrophenyl acetate as substrate.

Table 1. Activity and some kinetic characteristics of the *O*-acetylsterase from influenza C virus

The enzyme activity (units/mg) with the substrates indicated, the Michaelis constants (K_m) and the maximal velocities (V_{max}) were determined under the conditions described in the text. The values for fluorescein diacetate are approximate, since the solubility of the substrate is so low. N.D., not determined.

Substrate	Activity (units/mg)	K_m (mM)	V_{max} (units/mg of protein)	V_{max}/K_m
Neu5,9Ac ₂ :	2.5	N.D.	N.D.	N.D.
4-Nitrophenyl acetate:	10.0	1.1 ± 0.1	22 ± 2	20
2-Nitrophenyl acetate:	1.1	7.0 ± 2.8	11 ± 4	1.5
4-MUAc:	5.6	0.17 ± 0.05	16 ± 3	94
1-Naphthyl acetate:	8.9	2.7 ± 0.6	33 ± 6	12
Fluorescein diacetate:	1.3	0.009 ± 0.003	1.5 ± 0.3	166

Table 2. Activity of the *O*-acetylsterase from influenza C virus towards several non-sialic acid-containing compounds: cyclic *O*-acetyl derivatives

Substrate	Activity (% hydrolysis)
4-Acetoxybenzoic acid	100
2-Acetoxybenzoic acid (acetylsalicylic acid)	0
2-Acetoxybenzoic acid methyl ester*	10
<i>O</i> -Acetylmandelic acid	0
<i>O</i> -Acetylmandelic methyl ester*	5
2',3'-Di- <i>O</i> -acetyladenosine	38
2',3',5'-Tri- <i>O</i> -acetyladenosine	100
<i>N</i> ⁶ ,2'- <i>O</i> ,5'- <i>O</i> -Triacetyladenosine 3'-phosphate	≤ 22†

* Compound obtained by esterification.

† Approximate value, since it was difficult to stop the enzymic reaction.

Table 3. Activity of the *O*-acetylsterase from influenza C virus towards several non-sialic acid-containing compounds: acyclic *O*-acetyl derivatives

Substrate	Activity (% hydrolysis)
Triacetin (tri- <i>O</i> -acetylated glycerol)	44
5-Acetoxyamyl chloride	0
<i>O</i> -Acetylserine	0
<i>O</i> -Acetylserine methyl ester*	0

* Compound obtained by esterification.

1989). Freezing and thawing (three cycles over a period of 4 months) had a negligible influence on the activity of the enzyme. However, when a dilute solution of the esterase was kept at 4 °C for few days, a remarkable loss of activity was observed.

Activity and kinetic characteristics of the enzyme

The activity, K_m , V_{max} , and the V_{max}/K_m ratio towards several substrates are summarized in Table 1. Remarkable differences were found in the values of K_m and V_{max} for the substrates assayed.

The Neu5,9Ac₂ contained in the BSM was cleaved. By contrast, neither PSM [which contains *N*-glycolylneuraminic acid, (NeuGc) as the predominant sialic acid, and NeuAc] nor fetuin (which predominantly contains NeuAc, and also NeuGc) or glycophorin A (which contains NeuAc) were hydrolysed (results not shown).

Table 2 shows that the methyl esters of both 2-acetoxybenzoic acid and *O*-acetylmandelic acid were hydrolysed (at a low rate) by the enzyme, but 2-acetoxybenzoic acid or *O*-acetylmandelic acid themselves. However, the 4-acetoxybenzoic acid was 100 % cleaved. The di- and tri-*O*-acetyladenosine derivatives assayed were hydrolysed, although at different rates.

Triacetin, but not other acyclic *O*-acetyl-containing compounds (Table 3), was also hydrolysed.

Effects of inorganic and organic compounds on enzyme activity

None of the inorganic salts assayed (Zn, Mn and Pb chlorides) were effective inhibitors. NeuAc, 4-methylumbelliferone and acetylsalicylic acid, also tested as possible inhibitors, did not have this effect. As expected, DFP was an inhibitor.

DISCUSSION

The results shown in Tables 1–3 demonstrate that *O*-acetylsterase from influenza C virus hydrolyses *O*-acetyl-containing substrates of very different compositions, although with very different efficiencies.

Since the natural substrate Neu5,9Ac₂ is a non-commercially-

available substrate and the enzyme works very well with 4-nitrophenyl acetate (although it is not its natural substrate), it seems that the latter compound could be employed for routine assays. However, for some experiments it seems convenient to also employ BSM as a natural (and commercially available) substrate, although its hydrolysis rate is low.

Other synthetic substrates readily available and convenient for this measurement might be 4-MUAc and 1-naphthyl acetate. By contrast, fluorescein diacetate, although exhibiting a high $V_{\max}/K_m$ ratio, is limited by its low degree of solubility. However, all of these substrates are labile at high pH values.

PSM, fetuin and glycoporphin A, which have not been reported to contain Neu5,9Ac₂ (Graham, 1972; Gottschalk & Bhargava, 1972; Blumenfeld & Adamany, 1978) were not hydrolysed by this enzyme. This result could be the expected one; however, it is not in agreement with the possibility suggested by Nishimura *et al.* (1988) 'that most influenza C virus receptors on human erythrocytes, if not all, reside on glycoporphin A'.

The specificity of the *O*-acetyl esterase from influenza C virus seem to be a complicated matter. On the one hand, it has been reported (Schauer *et al.*, 1988b) that the specificity is very strict towards sialic acids; thus the enzyme hydrolyses its physiological substrate Neu5,9Ac₂, and also acts (but more slowly) on *N*-glycoloyl-9-*O*-acetylneuraminic acid and (much more slowly) on *N*-acetyl-4-*O*-acetylneuraminic acid, but does not act on *N*-acetyl-7-*O*-acetylneuraminic acid (Schauer *et al.*, 1988b). Accordingly, the following question arises. Is the latter sialic acid not hydrolysed because of the relatively hidden position of the 7-*O*-acetyl residue in the molecule, which could hinder its accessibility with respect to the active site of the enzyme?

On the other hand, very different synthetic and natural substrates (see Tables 1–3), which only have in common a composition of esterified acetyl groups and which differ in the other part of their molecule, are hydrolysed by the enzyme, as shown in the present paper. Our results seem to be in agreement with those of Nishimura *et al.* (1988), who have proposed 'the possibility that the activity of influenza C virus *O*-acetyl esterase may not be restricted to neuraminate-9-acetyl esters'. Furthermore, Luther *et al.* (1988) have also suggested 'that the receptor specificity of influenza C viruses should be extended to include acetyl groups in the 'N' position of galactosamine'. However, the broad specificity of the enzyme for some compounds should not be generalized; for instance, linear acyclic *O*-acetyl-containing compounds (*O*-acetylserine and its methyl ester), and cyclic compounds such as acetylsalicylic and *O*-acetylmandelic acids, are not cleaved by this enzyme, as shown in Table 3. Moreover, according to Schauer *et al.* (1988a,b), acetyl-CoA and acetylthiocholine iodide are not hydrolysed by this esterase; nevertheless, the two latter compounds are thioesters, not esters.

Recently it has been shown that there is a serine residue in the active site of the *O*-acetyl esterase (Vlasak *et al.*, 1989). From the present work we have deduced that acetylsalicylic acid and *O*-acetylmandelic acid are not hydrolysed by the enzyme. These compounds are ionized under the reaction conditions, exposing a strong electronegative group ($-\text{CO}_2^-$) near their *O*-acetyl group. However, the corresponding methyl esters are hydrolysed by the enzyme. It could be suggested that the replacement of the $-\text{CO}_2^-$ from the former compounds by a $-\text{CO}_2\text{-CH}_3$ group in these derivatives permits hydrolysis. Since it seems that there is a very similar conformation for acetylsalicylic acid and its methyl ester (as well as for *O*-acetylmandelic acid and its methyl ester), the different behaviour of the enzyme towards these acids in comparison with their esters can hardly be attributable to steric hindrance in the active site of the enzyme. The hydrolysis of these methyl esters might be due to their lower polarity (in comparison

with that of the respective acid) which could: (i) decrease the electronic repulsion of the amino acid residues of the active site or its proximity, and (ii) facilitate the participation of the $-\text{OH}$ of the serine residue component of the active site. Moreover, the absence of inhibition of the esterase activity by acetylsalicylic acid suggests that the latter is probably unable to reach the active site due to electronic repulsions.

This result is in agreement with the observation that other substances with electronegative groups near the *O*-acetyl ester, such as *O*-acetylserine or 2-nitrophenyl acetate, either were not hydrolysed by the esterase or were hydrolysed with lower efficiency respectively than similar compounds with polar groups distant from the acetyl ester group, such as 4-acetoxybenzoic acid and 4-nitrophenyl acetate; thus the enzyme showed no activity towards 2-acetoxybenzoic acid, but a high activity towards 4-acetoxybenzoic acid (Table 2). Furthermore, its activity with 4-nitrophenyl acetate as substrate was 9-fold higher than with 2-nitrophenyl acetate under the same conditions (Table 1).

The absence of esterase activity using *O*-acetylserine methyl ester as substrate (Table 3) could be explained by the location of other polar groups near the *O*-acetyl group.

Additionally, the three acetyladenosine derivatives that we assayed (Table 2) were hydrolysed by this acetyl esterase, although at different rates, probably depending on factors such as (i) their respective *O*-acetyl content (e.g. tri-*O*-acetyladenosine at a higher rate than di-*O*-acetyladenosine); (ii) their polarity (di-*O*-acetyl-*N*-acetyladenosine phosphate, which has the highest polarity, was cleaved at the lowest hydrolysis rate); and (iii) the type of linkage [*N*-acetyl residue not cleaved according to Schauer *et al.* (1988b)].

Finally, the following facts seem to confirm the convenience of developing complementary research in this field: (i) the singularity of this viral *O*-acetyl esterase in being localized in a peculiar glycoprotein, the only one known with three activities; (ii) the possibility that this enzyme might facilitate or enable the action of sialidases (Schauer & Reuter, 1988); and (iii) its broad specificity towards very different *O*-acetyl-containing compounds, as deduced from the present paper, a property which could be related to the infective capacity of influenza C virus.

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REFERENCES

- Blumenfeld, O. O. & Adamany, A. M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2727–2731
- Buonagurio, D. A., Nakada, S., Fitch, W. M. & Palese, P. (1986) *Virology* **153**, 12–21
- Cabezas, J. A., Milicua, M., Bernal, C. S., Villar, E., Pérez, N. & Hannoun, C. (1989) *Glycoconjugate J.* **6**, 219–227
- Chakraverty, P. (1978) *Arch. Virol.* **58**, 341–348
- Compans, R. W., Bishop, D. H. L. & Meier-Ewert, H. (1977) *J. Virol.* **21**, 658–665
- Fiszon, B., Hannoun, C., García-Sastre, A., Villar, E. & Cabezas, J. A. (1989) *Res. Virol.* **140**, 395–404
- Francis, T., Jr., Quilligan, J. J., Jr. & Minuse, E. (1950) *Science* **112**, 395–397

- Gottschalk, A. & Bhargava, A. S. (1972) in *Glycoproteins: Their Composition, Structure and Function* (Gottschalk, A., ed.), vol. 5B, pp. 823–829, Elsevier, Amsterdam
- Graham, E. R. B. (1972) in *Glycoproteins: Their Composition, Structure and Function* (Gottschalk, A., ed.), vol. 5A, pp. 722–731, Elsevier, Amsterdam
- Hachinohe, S., Sugawara, K., Nishimura, H., Kitame, F. & Nakamura, K. (1989) *J. Gen. Virol.* **70**, 1287–1292
- Herrler, G. & Klenk, H. D. (1987) *Virology* **159**, 102–108
- Herrler, G., Compans, R. W. & Meier-Ewert, H. (1979) *Virology* **99**, 49–56
- Herrler, G., Nagele, A., Meier-Ewert, H., Bhowan, A. S. & Compans, R. W. (1981) *Virology* **113**, 439–451
- Herrler, G., Rott, R. & Klenk, H. D. (1985a) *Virology* **141**, 144–147
- Herrler, G., Rott, R., Klenk, H. D., Müller, H. P., Shukla, A. K. & Schauer, R. (1985b) *EMBO J.* **4**, 1503–1506
- Herrler, G., Reuter, G., Rott, R., Klenk, H. D. & Schauer, R. (1987) *Biol. Chem. Hoppe-Seyler* **368**, 451–454
- Herrler, G., Dürkop, I., Becht, H. & Klenk, H. D. (1988) *J. Gen. Virol.* **69**, 839–846
- Hirst, G. K. (1950) *J. Exp. Med.* **91**, 177–184
- Hongo, S., Sugawara, K., Homma, M. & Nakamura, K. (1986) *Arch. Virol.* **89**, 189–201
- Kawamura, H., Tashiro, M., Kitame, F., Homma, M. & Nakamura, K. (1986) *Virus Res.* **4**, 275–288
- Kendal, A. P. (1975) *Virology* **65**, 87–99
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leatherbarrow, R. J. (1987) *Enzfitter Manual*, Elsevier Biosoft, Amsterdam, The Netherlands
- Luther, P., Cushley, W., Hölzer, C., Desselberger, U. & Oxford, J. S. (1988) *Arch. Virol.* **101**, 247–254
- Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–210
- Muchmore, E. A. & Varki, A. (1987) *Science* **236**, 1293–1295
- Nakamura, K., Herrler, G., Petri, T., Meier-Ewert, H. & Compans, R. W. (1979) *J. Virol.* **29**, 997–1005
- Nishimura, H., Sugawara, K., Kitame, F. & Nakamura, K. (1988) *J. Gen. Virol.* **69**, 2545–2553
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Ohuchi, M., Homma, M., Muramatsu, M. & Ohyama, S. (1978) *Microbiol. Immunol.* **22**, 197–203
- Ohuchi, M., Ohuchi, R., Mifune, K. (1982) *J. Virol.* **42**, 1076–1079
- Palese, P. & Young, J. F. (1982) *Science* **215**, 1468–1474
- Rogers, G., Herrler, G., Paulson, J. C. & Klenk, H. D. (1986) *J. Biol. Chem.* **261**, 5947–5951
- Schauer, R. & Reuter, G. (1988) *Proc. Jpn.-German. Symp. Sialic Acids* 164–165
- Schauer, R., Reuter, G. & Stoll, S. (1988a) *Biochimie* **70**, 1511–1519
- Schauer, R., Reuter, G., Stoll, S., Posadas del Río, F., Herrler, G. & Klenk, H. D. (1988b) *Biol. Chem. Hoppe-Seyler* **369**, 1121–1130
- Sugawara, K., Ohuchi, M., Nakamura, K. & Homma, M. (1981) *Arch. Virol.* **68**, 147–151
- Sugawara, K., Nishimura, H., Kitame, F. & Nakamura, K. (1986) *Virus Res.* **6**, 27–32
- Sugawara, K., Kitame, F., Nishimura, H. & Nakamura, K. (1988) *J. Gen. Virol.* **69**, 537–547
- Taylor, R. M. (1949) *Am. J. Public Health* **39**, 171–178
- Vlasak, R., Krystal, M., Nacht, M. & Palese, P. (1987) *Virology* **160**, 419–425
- Vlasak, R., Muster, T., Lauro, A. M., Powers, J. C. & Palese, P. (1989) *J. Virol.* **63**, 2056–2062
- Yamashita, M., Krystal, M., Fitch, W. M. & Palese, P. (1988a) *Virology* **163**, 112–122
- Yamashita, M., Krystal, M. & Palese, P. (1988b) *J. Virol.* **62**, 3348–3355
- Yamashita, M., Krystal, M. & Palese, P. (1989) *Virology* **171**, 458–466

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