Identification of *N*-Acetyl-4-*O*-acetylneuraminyl-lactose in Echidna Milk

By MICHAEL MESSER*

Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006, Australia

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The identity of a novel form of sialyl-lactose found in milk of the echidna (*Tachyglossus aculeatus*) was investigated. The sialyl-lactose yielded equimolar amounts of *N*-acetylneuraminic acid and lactose during mild acid hydrolysis but was resistant to the action of a bacterial neuraminidase. A viral neuraminidase hydrolysed it to lactose plus a form of sialic acid that reacted positively with thiobarbituric acid reagent but whose chromatographic mobility was greater than that of *N*-acetylneuraminic acid. Treatment with alkali converted the sialyl-lactose into a substance with the same chromatographic mobility as *N*-acetylneuraminyl-(2→3)-lactose and made it susceptible to the action of bacterial neuraminidase. The sialyl-lactose contained one mol of ester (identified as acetyl), and released one mol of formaldehyde during periodate oxidation, per mol of sialic acid. It did not contain *N*-glycolylneuraminic acid. These results indicate that the sialyl-lactose is *N*-acetyl-4-*O*-acetylneuraminyl-(2→3)-lactose. Echidna milk contained, in addition, a small amount of *N*-acetylneuraminyl-(2→3)-lactose.

Recent work in this laboratory has shown that the milk of monotremes (egg-laying mammals) is unusual in its carbohydrate composition. Whereas the principal free carbohydrate of the milk of placental mammals is lactose (Jenness & Sloan, 1970), that of a sample of milk of the platypus (*Ornithorhynchus anatinus*) was found to be difucosyl-lactose, and that of three samples of echidna milk was sialyl-lactose (Messer & Kerry, 1973). The sialyl-lactose of the echidna milk constituted, on average, 50% of the total free milk carbohydrate (w/w, lactose used as standard), the other components being fucosyl-lactose (26%), difucosyl-lactose (13%) and lactose (8%).

Although sialyl-lactose was thus the principal carbohydrate of echidna milk, its structure remained unknown. This paper describes experiments indicating that it is a form of *N*-acetylneuraminyl-lactose in which the *N*-acetylneuraminic acid residue has an *O*-acetyl substituent at position C-4.

Experimental

Chemicals and enzymes

N-Acetylneuraminic acid (synthetic, type IV), *N*-glycolylneuraminic acid, *N*-acetylneuraminyl-lactose (bovine colostrum, type II), glucose pentaacetate, bacterial neuraminidase (EC 3.2.1.18; type V, from *Clostridium perfringens*), β -galactosidase (EC

3.2.1.23; grade IV, from *Escherichia coli*) and peroxidase (EC 1.11.1.7; type I) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. The *N*-acetylneuraminyl-lactose was labelled as containing approx. 85% of the 2→3 isomer and 15% of the 2→6 isomer. Viral neuraminidase (from influenza A2 virus) was kindly given by Dr. W. G. Laver, Australian National University, Canberra, A.C.T.; 0.1 ml of this enzyme solution liberated 0.02 μ mol of *N*-acetylneuraminic acid from bovine *N*-acetylneuraminyl-lactose/min at pH 7.0 at 37°C. Glucose oxidase (EC 1.1.3.4; highly purified) was purchased from Nagase and Co., Ohama, Amagasaki, Japan.

Methods

Paper chromatography. This was done with the following solvent systems: A, pyridine-ethyl acetate-acetic acid-water (5:5:1:3, by vol.) (Kuhn & Brossmer, 1956); B, butan-1-ol-acetic acid-water (4:1:5, by vol.), upper layer (Thompson, 1951); C, butan-1-ol-propan-1-ol-acetic acid-water (1:2:1:1, by vol.). Whatman no. 2 paper was used for solvent A and no. 1 paper for systems B and C. Carbohydrates were detected with alkaline AgNO₃ (Trevelyan *et al.*, 1950), sialic acids by the thiobarbituric acid method (Warren, 1960) and acetylhydrazamate with 5% (w/v) FeCl₃·6H₂O in methanol-acetone (4:3, v/v) (Neuberger & Ratcliffe, 1972).

Lactose. This was determined by measuring the amount of glucose released by an excess of β -galactosidase. The test solution (0.5 ml), containing up to

* Present address: Department of Experimental Pathology, University College Hospital Medical School, University St., London WC1E 6JJ, U.K.

0.1 μ mol of lactose, was mixed with 0.5 ml of 0.2 M sodium-potassium phosphate buffer, pH 7.0, containing 0.01% (w/v) *o*-dianisidine and 0.001% (w/v) peroxidase, followed by 0.05 ml of β -galactosidase (0.25 mg/ml) and 0.05 ml of glucose oxidase (2 mg/ml). The mixture was incubated at 37°C for 1 h; 0.5 ml of 50% (v/v) H_2SO_4 was then added (Messer & Dahlqvist, 1966) and the E_{530} of the solution measured. Lactose was used for preparation of the standard curve.

Sialic acid. This was determined by the thiobarbituric acid method of Aminoff (1961), synthetic *N*-acetylneuraminic acid being used as the standard. In experiments in which sialic acid was determined after mild acid hydrolysis in 0.05 M- H_2SO_4 at 85°C for 1 h the standard solutions of *N*-acetylneuraminic acid were heated with 0.05 M- H_2SO_4 under the same conditions to allow for destruction of sialic acid.

Acetyl hydroxamate. This was formed by treating about 2 mg of sialyl-lactose or 0.2 mg of glucose penta-acetate with 0.02 ml of 0.09 M-hydroxylamine hydrochloride in 50% (v/v) NH_3 solution for 1 h at room temperature. The solution was then chromatographed in solvent system B.

Ester content. This was determined by the method of Hestrin (1949), with glucose penta-acetate as the standard; all quantities were decreased by a factor of 5.

Alkaline hydrolysis before paper chromatography. This was done by treating about 0.5 mg of sialyl-lactose with 0.1 ml of 50% NH_3 solution at room temperature for 1 h; samples of 0.01 or 0.02 ml were used for chromatography. In experiments to determine the effect of prior alkaline treatment of sialyl-lactose on its susceptibility to the action of neuraminidase, the sialyl-lactose (about 0.04 mg in 0.02 ml of water) was mixed with 0.02 ml of 0.76 M-NaOH; after 30 min at room temperature (Neuberger & Ratcliffe, 1972) the solution was neutralized with 0.04 ml of 0.38 M-HCl and diluted to 0.15 ml with water.

Release of sialic acid by neuraminidase. In experiments to detect the products of the action of neuraminidase by paper chromatography, about 0.25 mg of sialyl-lactose in 0.1 ml of water was treated with 0.02 ml of bacterial neuraminidase (10 mg/ml) or viral neuraminidase solution (undiluted); the solution was adjusted to pH 5-6 with dilute acetic acid and then incubated at 37°C for 24 h. Samples (0.02 or 0.04 ml) were taken for paper chromatography, with solvent system C (Table 1).

For quantitative experiments, sialyl-lactose (about 0.04 mg in 0.15 ml) was mixed with 0.2 ml of buffer solution and 0.05 ml of enzyme solution and incubated at 37°C for a measured time. The reaction was stopped by the addition of 0.1 ml of 0.5 M- H_2SO_4 , and the sialic acid released was determined. For experiments with bacterial neuraminidase the buffer solution was

0.1 M-potassium acetate, pH 5.5, containing 0.9% NaCl and 0.1% $CaCl_2$; the enzyme solution (neuraminidase from *Cl. perfringens*) contained 0.75 mg/ml. For experiments with viral neuraminidase the buffer solution was 0.1 M-sodium-potassium phosphate, pH 7.0; the enzyme solution was a 1:10 dilution of a solution of neuraminidase from influenza A2 virus.

Release of formaldehyde by periodate oxidation. About 0.1 mg of sialyl-lactose in 0.25 ml of water was mixed with 0.25 ml of 20 mM-sodium periodate; after incubation at 0°C in the dark for either 10 or 20 min (Neuberger & Ratcliffe, 1972), 0.1 ml of 0.5 M-KI and then 0.1 ml of 0.5 M- $Na_2S_2O_3$ was added and the mixture diluted to 1.0 ml. The formaldehyde produced was determined by the method of Nash (1953). Solutions of *N*-acetylneuraminic acid (containing up to 0.2 μ mol) that had been taken through the same procedure were used as standards. The amount of formaldehyde released from *N*-acetylneuraminic acid was the same after 10 min of oxidation as after 20 min.

Glycollic acid. This was determined by the method of Gibbons (1962); all quantities were halved.

Isolation of sialyl-lactose from echidna milk. The milk used (kindly given by Dr. M. Griffiths, Division of Wildlife Research, CSIRO, Canberra, A.C.T.) consisted of three 4 ml samples obtained from two different animals (Messer & Kerry, 1973). Milk fat was removed with chloroform-methanol, and milk protein with ice-cold 10% (w/v) trichloroacetic acid, as described previously (Messer & Kerry, 1973). An extract from 2 ml of milk was filtered through a column (2.2 cm $\times$ 180 cm) of Sephadex G-15 with water as the eluent. Fractions (4-5 ml) were collected and analysed for carbohydrate with an anthrone reagent (Brin, 1966) and for sialic acid (after mild acid hydrolysis) as described above. The fractions containing sialic acid, which were eluted at a ratio of elution volume to void volume of between 1.1 and 1.3 (see Fig. 1 of Messer & Kerry, 1973), were combined and freeze-dried. The dried material (about 8 mg) was stored in a desiccator at -15°C. Sialyl-lactose from each of the three samples of echidna milk was combined for the experiments described below.

Results

Mild acid hydrolysis

When examined by paper chromatography in three different solvent systems the sialic acid-containing free carbohydrate of echidna milk showed a minor component with the same chromatographic mobility as *N*-acetylneuraminyl-(2 $\rightarrow$ 3)-lactose and a major component whose mobility was considerably greater (Table 1). After mild acid hydrolysis in 0.05 M- H_2SO_4 at 85°C for 1 h the carbohydrate showed two major spots which co-chromatographed

Table 1. *Paper chromatography of sialic acids and sialyl-lactoses*

All results are expressed as $R_{Lactose}$, i.e. the mobility relative to that of lactose. Abbreviations: 3'-AcNeu-Lac, *N*-acetylneuraminyl-(2→3)-lactose, the major component of bovine *N*-acetylneuraminyl-lactose; 6'-AcNeu-Lac, *N*-acetylneuraminyl-(2→6)-lactose, the minor component of bovine *N*-acetylneuraminyl-lactose; AcNeu and GcNeu, *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid respectively; echidna sialic acid, presumptive *N*-acetyl-4-*O*-acetylneuraminic acid liberated from the major component of the echidna milk sialyl-lactose by viral neuraminidase. For solvent systems see the Experimental section.

Solvent system	3'-AcNeu-Lac	6'-AcNeu-Lac	AcNeu	GcNeu	Echidna sialyl-lactose (major component)	Echidna sialic acid
A	0.45	0.25	0.74	0.64	0.87	1.47
B	0.41	0.41	1.70	1.41	0.73	2.29
C	0.63	0.45	2.30	1.93	1.18	2.76

with lactose and *N*-acetylneuraminic acid respectively. The latter stained with thiobarbituric acid reagent as well as with alkaline AgNO_3 . No *N*-glycolylneuraminic acid was detected. Treatment of the acid hydrolysate with β -galactosidase resulted in disappearance of the spot migrating as lactose and the appearance of spots with the mobilities of galactose and glucose.

Quantitative measurement of the amounts of lactose and sialic acid present in the acid hydrolysate gave a molar ratio of lactose to sialic acid of 1.05.

These results indicated that the sialic acid-containing echidna milk carbohydrate consisted of two forms of sialyl-lactose; a major unknown form and a minor one which was probably *N*-acetylneuraminyl-(2→3)-lactose.

O-Acetyl content

The chromatographic behaviour of the major component suggested that this might be an *O*-acetylated form of sialyl-lactose, such as has been found in bovine colostrum (Kuhn & Brossmer, 1956). The sialyl-lactose was therefore treated with alkaline hydroxylamine and the product examined chromatographically in solvent system B, which separates the hydroxamates of formic acid, acetic acid and propionic acid (Thompson, 1951; Neuberger & Ratcliffe, 1972). A sample of glucose penta-acetate was similarly treated to provide a reference standard. Acetylhydroxamate ($R_F = 0.51$) was readily detected in both cases.

Quantitative measurement of the ester content of the echidna sialyl-lactose gave a molar ratio of ester groups to sialic acid of 0.87.

These results indicated that 87% of the echidna sialyl-lactose contained one *O*-acetyl group per molecule.

Alkaline hydrolysis

After treatment of the echidna sialyl-lactose with 50% NH_3 at room temperature for 1 h, paper chro-

matography showed that most of the major component had disappeared, the only prominent spot being one migrating as *N*-acetylneuraminyl-(2→3)-lactose.

Since *O*-acetyl groups of sialic acid are readily removed by alkali (Tuppy & Gottschalk, 1972), this result suggested that the major component of the sialyl-lactose was an *O*-acetyl derivative of *N*-acetylneuraminyl-(2→3)-lactose.

Action of bacterial and viral neuraminidases

In *O*-acetylated sialoglycoproteins the location of the *O*-acetyl group on the sialic acid residue is known to affect the susceptibility of the α -ketosidic *N*-acetylneuraminosyl linkage toward the action of neuraminidases. Both bacterial and viral neuraminidases can release *N*-acetyl-7-*O*-acetyl- or *N*-acetyl-8-*O*-acetyl-neuraminic acid from bovine submaxillary mucin (Schauer & Faillard, 1968), but only the viral enzyme splits *N*-acetyl-4-*O*-acetylneuraminic acid from horse submaxillary mucin (Schauer & Faillard, 1968) or horse serum α_2 -macroglobulin (Pepper, 1968). Prior removal of the 4-*O*-acetyl group with alkali makes the ketosidic bond susceptible to the action of both neuraminidases. Another difference between bacterial and viral neuraminidases is that the bacterial enzyme catalyses the hydrolysis of the ketosidic linkage of both *N*-acetylneuraminyl-(2→3)- and *N*-acetylneuraminyl-(2→6)-lactose, whereas viral neuraminidases have little or no action on the latter (Cassidy *et al.*, 1965; Schneir & Rafelson, 1966; Drzeniek, 1967). Hence comparisons of the actions of bacterial and viral neuraminidases on the echidna sialyl-lactose, both before and after treatment with alkali, could provide information on the location of the *O*-acetyl group of its major component, as well as on the type of ketosidic linkage in both components.

After incubation of the sialyl-lactose with an excess of a bacterial neuraminidase (from *Cl. perfringens*), paper chromatography showed that the

minor component had disappeared and was replaced by small amounts of lactose and *N*-acetylneuraminic acid, but the major component had remained unchanged. Quantitative studies showed that only 9% of the total sialic acid content of the sialyl-lactose was liberated by the bacterial neuraminidase (Fig. 1). Prior treatment of the echidna sialyl-lactose with alkali greatly increased its susceptibility to the bacterial neuraminidase, 88% of the total sialic acid being released in 24h.

Similar experiments with neuraminidase from influenza A2 virus showed that this enzyme hydrolysed the echidna sialyl-lactose to a small amount of *N*-acetylneuraminic acid and larger amounts of lactose plus a substance which stained with the thiobarbituric acid reagent for sialic acids, but whose chromatographic mobility was considerably greater than that of *N*-acetylneuraminic acid (Table 1). Quantitative studies confirmed that the echidna sialyl-lactose was hydrolysed by viral neuraminidase, but at a relatively slow rate (Fig. 2). Prior treatment of the sialyl-lactose with alkali greatly increased its susceptibility to this enzyme, 88% of its total sialic acid again being released during 24h.

Paper-chromatographic studies with bovine *N*-acetylneuraminyl-lactose, which was a mixture of *N*-acetylneuraminyl-(2→3)-lactose (major component) and *N*-acetylneuraminyl-(2→6)-lactose, showed that the bacterial neuraminidase hydrolysed both isomers, whereas the viral enzyme hydrolysed the

former only. This was confirmed by quantitative studies which showed that the bacterial enzyme released almost 100% of the total sialic acid of the bovine material (Fig. 1), whereas the viral enzyme released only 78% (Fig. 2). It appears that the bovine *N*-acetylneuraminyl-lactose contained 78% of the 2→3 isomer and 22% of the 2→6 isomer.

The observation that the major component of echidna sialyl-lactose was resistant to the action of the bacterial neuraminidase indicated that the *O*-acetyl group of its sialic acid moiety was not located on either C-7 or C-8. Further, the sialic acid released from it by viral neuraminidase (presumably *N*-acetyl-*O*-acetylneuraminic acid) reacted with the thiobarbituric acid reagent; this eliminated the possibility of acetylation at C-7, since *N*-acetyl-7-*O*-acetylneuraminic acid does not react with this reagent (Aminoff, 1961; Paerels & Schut, 1965).

Since the viral neuraminidase had no action on bovine *N*-acetylneuraminyl-(2→6)-lactose but readily hydrolysed both the minor component of echidna sialyl-lactose, and the major component after alkaline treatment, the ketosidic linkage of neither component could be 2→6. Although the apparent degree of hydrolysis of the alkali-treated sialyl-lactose by the viral neuraminidase was only 88%, this did not necessarily imply the presence of a 2→6 linkage, since the same incomplete hydrolysis was observed with the bacterial enzyme. The failure to obtain 100% hydrolysis in both cases may have been due to incomplete de-*O*-acetylation by alkali, or the formation of non-specific products. The observation that after alkaline treatment the initial rate of hydrolysis of the echidna sialyl-lactose by viral neuraminidase

Fig. 1. Release of sialic acid from sialyl-lactoses by neuraminidase from *Cl. perfringens*

Free and total sialic acid were determined by the thiobarbituric acid method, the latter after mild acid hydrolysis (see the Experimental section): ○, bovine *N*-acetylneuraminyl-lactose; ●, echidna milk sialyl-lactose; ■, echidna milk sialyl-lactose after treatment with 0.38M-NaOH for 30min at room temperature.

Fig. 2. Release of sialic acid from sialyl-lactoses by neuraminidase from influenza A2 virus

For details and explanation of symbols, see Fig. 1.

was identical with that of bovine *N*-acetylneuraminyl-lactose (i.e. its 2→3 isomer) supported previous chromatographic evidence that the ketosidic linkage of the major component of the echidna sialyl-lactose was 2→3.

These results therefore indicated that the major component of echidna sialyl-lactose was *N*-acetyl-*O*-acetylneuraminyl-(2→3)-lactose, the *O*-acetyl group being located on either C-4 or C-9 of the sialic acid moiety.

Release of formaldehyde during periodate oxidation

When sialic acid or sialyl-lactose is oxidized with periodate, any formaldehyde that is formed can be derived only from C-9 of the sialic acid, provided that both the galactose and glucose of the sialyl-lactose are in the pyranose-ring form (Neuberger & Ratcliffe, 1972). Treatment of the echidna sialyl-lactose with periodate at 0°C resulted in the formation of 1.04 mol of formaldehyde per mol of sialic acid after 10 min of oxidation, and 1.07 mol after 20 min. Hence the *O*-acetyl group of the major component of the echidna sialyl-lactose could not be located on C-8 or C-9 of the sialic acid, since in either case formaldehyde would have been released only from the minor component, i.e. about 0.1 mol per mol of sialic acid. The slightly high values observed may be due to the fact that the glucose residue of the sialyl-lactose, having a free reducing group, is able to exist in open-chain form (in, however, small amount) in equilibrium with the pyranose form: this would give rise to a slow release of formaldehyde with periodate (E. R. B. Graham, personal communication; cf. also Ohman & Hygstedt, 1968).

Glycollic acid content

No glycollic acid could be detected after acid hydrolysis in 0.25M-H₂SO₄ at 100°C for 16 h of the echidna sialyl-lactose, although the expected amount was obtained after similar hydrolysis of *N*-glycollyl-neuraminic acid. This confirmed the previous chromatographic results showing the absence of *N*-glycollyl-neuraminic acid from echidna sialyl-lactose after mild acid hydrolysis.

Discussion

The results indicate that echidna milk sialyl-lactose consists of a major component, *N*-acetyl-4-*O*-acetylneuraminyl-(2→3)-lactose (Fig. 3), and a minor component (comprising about 10% of the total) identified as *N*-acetylneuraminyl-(2→3)-lactose. The *O*-acetyl group of the major component was localized to position C-4 of the sialic acid moiety by elimination of positions C-7, C-8 and C-9 as alternative possibilities. The α-ketosidic *N*-acetylneuraminyl linkage was found to be 2→3 on the basis of chromatographic evidence and its rate of hydrolysis by viral neuraminidase after treatment of the sialyl-lactose with alkali. Although a 2→6 linkage was ruled out, other linkages cannot entirely be excluded; however, a 2→4 linkage is unlikely on the basis of the known substrate specificities of viral neuraminidases (Huang & Orlich, 1972), whereas a 2→2 linkage has not so far been reported for sialyl oligosaccharides or sialoglycoproteins.

N - Acetyl - *O* - acetylneuraminyl - lactose has previously been found in mammals only in bovine colostrum (Kuhn & Brossmer, 1956) and rat urine

Fig. 3. Structure of *N*-acetyl-4-*O*-acetylneuraminyl-(2→3)-lactose

(Maury, 1971). The material from both these sources was hydrolysed by bacterial neuraminidase and therefore differs from the echidna sialyl-lactose. The *O*-acetyl group of the bovine *N*-acetyl-*O*-acetylneuraminyl-lactose is probably located at position C-7 or C-8, since the sialic acids of bovine submaxillary mucins are acetylated at either or both of these positions (Blix & Lindberg, 1960).

N-Acetyl-4-*O*-acetylneuraminic acid has been positively identified in mammals only in equine glycoproteins (see Tuppy & Gottschalk, 1972). It would therefore be of interest to examine mare's milk for the presence of *N*-acetyl-4-*O*-acetylneuraminyl-lactose.

Non-*O*-acetylated sialyl-lactoses have been found in rat mammary gland and milk, bovine milk and colostrum, human and canine milk (Carubelli *et al.*, 1964; Kuhn, 1972) as well as in rat and human urine (Maury, 1971). In view of the lability of the *O*-acetyl group it is not certain to what extent these might be degradation products of *O*-acetylated sialyl-lactoses (Kuhn & Brossmer, 1956; Maury, 1971). The small amount of non-*O*-acetylated *N*-acetylneuraminyl-lactose found in the present work might similarly be due to partial degradation of the 4-*O*-acetylated form during isolation of the material.

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