

Sialic acids in human gastric aspirates: detection of 9-O-lactyl- and 9-O-acetyl-N-acetylneuraminic acids and a decrease in total sialic acid concentration with age

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1. The total sialic acid content of human gastric aspirates was measured using a colorimetric assay. Care was taken to optimize the assay and to eliminate interference.

2. The sialic acid content of gastric aspirates collected under resting conditions from 77 patients with non-ulcer dyspepsia was found to decrease with age from >100 µg/ml at 25 years and younger to <20 µg/ml above 70 years of age.

3. Analysis of the sialic acids by gas chromatography, mass spectrometry and thin-layer chromatography showed the presence of N-acetylneuraminic acid and two O-acylated derivatives, 9-O-acetyl- and 9-O-lactyl-N-acetylneuraminic acids. These forms were predominantly glycosidically bound.

4. Thin-layer chromatographic analysis of individual aspirate samples showed that the O-acetylated sialic acids were present in all samples, with a maximum of 25% and a minimum of 5% of the total sialic acids.

INTRODUCTION

The mucus glycoproteins secreted by the gastric mucosa, including the surface epithelium and the glands, form a characteristic gel layer which protects the mucosa [1-3]. This gel layer is in dynamic equilibrium with soluble mucus, and breakdown products are formed as a result of the acid environment and pepsin attack [1, 4]. Most of the complex carbohydrates found in the gastric juice are derived from the degradation of the gastric and salivary mucins. Many studies have been carried out to characterize human gastric mucus glycoproteins and have consistently indicated a low level of sialic acid in the purified mucins [5-7] in agreement with histochemical analyses of 'neutral' mucins [8]. The mucus glycoproteins require careful purification before a study of their chemical composition can be

made [1, 7], and this prerequisite has limited the number of individual samples which can be studied, particularly in man.

The sialic acids are a family of acidic N- and O-acyl monosaccharides widespread in nature [9] and are associated with a variety of biological functions in sialoglycoconjugates [10]. The nature of the sialic acids found in human gastric mucins or gastric aspirates has not been studied and the present work was initiated to assess the total sialic acid complement of gastric secretions. However, histochemical staining of gastric mucosa for sialic acids has shown that in gastric disease the appearance of intestinal metaplasia has been correlated with the presence of O-acyl sialic acids, typical for the normal colonic mucosa [8, 11]. The ready access to gastric aspirates obtained at endoscopy has resulted in a search for the presence of markers for gastric function. The action of factors such as pepsin and acid leads to continuous mucus degradation [1, 2, 4], possibly generating such markers. In addition, the level of degradation itself may reflect disease, and measurement of eroded glycoprotein fragments has been used to demonstrate disease [12-16]. These studies have indicated that the levels of sialic acid present in the aspirate under controlled conditions of collection may be valuable as an indicator of mucin degradation [15, 16] or simply as a means of identifying patients with gastroduodenal disease [12]. Carbohydrate analysis of gastric aspirates in normal subjects before and after pentagastrin stimulation revealed little difference in sialic acid concentrations either in non-fractionated aspirate or purified mucin [6] and this contrasted with results reported for gastric secretory studies determining the output of sialic acid in normal and diseased subjects [15]. The age of the normal group in these two reports was different and the nature of the sialic acids was not assessed; neither of these factors was considered in the final analysis of results [6, 15].

Key words: O-acylation, ageing, gastric aspirates, sialic acids.

Abbreviations: NSAID, non-steroid anti-inflammatory drug; sialic acids are abbreviated according to Schauer and co-workers [9, 10]: Neu5Ac, N-acetyl-D-neuraminic acid; Neu5,9Ac₂, 9-O-acetyl-N-acetyl-D-neuraminic acid; Neu5Ac9Lc, 9-O-lactyl-N-acetyl-D-neuraminic acid.

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The present work was undertaken to evaluate the nature and amount of sialic acids present in human gastric aspirates in order to assess their value as markers for gastric function and to assess age-related change.

Part of this work was presented at the 621st Meeting of the Biochemical Society, London, 17–19 December 1986 [16a].

MATERIALS AND METHODS

Materials

The following chemicals were used: *N*-acetylneuraminic acid and periodic acid from Sigma Chemical Co., Poole, Dorset, U.K., thiobarbituric acid and cyclohexanone from Aldrich Chemicals, Gillingham, Dorset, U.K., Dowex 50W, 50–100 mesh and Dowex 2 × 8, 200–400 mesh from BioRad, Watford, Herts., U.K., Sepharose CL 4B from Pharmacia-LKB, Hounslow, Middx, U.K., and plastic-backed cellulose thin-layer plates (0.2 mm thickness) from E. Merck, Darmstadt, Germany.

Bovine submandibular gland glycoprotein containing 0.7 μmol of sialic acid/mg dry weight and 2.1 mol of *O*-acetyl ester/mol of sialic acid was prepared as described previously [17]. α₁-Acid glycoprotein was obtained from the Scottish National Blood Transfusion Centre, Edinburgh, Scotland, U.K.

N-Acetyl-[4, 5, 6, 7, 8, 9-¹⁴C]neuraminic acid (8.95 GBq/mmol) was purchased from Amersham International plc, Amersham, Bucks, U.K.

Patients

Seventy-seven patients referred to the Medical Day Unit of the Bristol Royal Infirmary with dyspepsia were included in the study. They had normal endoscopy and histology. After overnight fasting, gastric juice was aspirated during endoscopy under vision. This was carried out using a trap in the suction line with prior washout to minimize salivary contamination. Two biopsies were taken from each of the antrum and body of the stomach for histological examination and *Helicobacter pylori* identification.

Gastric aspirates

Gastric aspirates were obtained from patients within 30 min. Any solid material was removed by centrifugation and the volume and pH were measured. Aliquots of 100 μl were taken in duplicate for sialic acid determination. The remaining aspirate was frozen at -20°C until processed. Selected samples were analysed immediately for the composition of sialic acids to allow comparison after storage of these samples at -20°C.

Total sialic acid measurement

Total sialic acid was measured by a modification of the methods of Schauer and co-workers [18, 19] using H₂SO₄. Aspirate samples (50 μl) were saponified for 60 min at 4°C in a final concentration of 50 mmol/l NaOH and then acidified with 25 μl of 0.7 mol/l H₂SO₄ in a final volume of 100 μl and incubated at 80°C for 60 min. The solution was cooled and centrifuged at 12 000 *g* for 5 min, the supernatant was removed and the remaining pellet hydrolysed again in 100 μl of 100 mmol/l H₂SO₄. The hydrolysate was cooled on ice, centrifuged at 12 000 *g* and the two supernatants combined and extracted three times with 1 ml volumes of diethyl ether [19]. The aqueous layer was retained and aliquots were taken for sialic acid determination by a microadaptation of the periodate-thiobarbituric acid method as described previously [18, 20]. The extracted chromogen was measured at both 549 and 532 nm and corrected for interference as detailed by Warren [20]. Initial studies showed that this was the optimum condition for release of total sialic acids from gastric aspirates. Recovery of sialic acids was tested in this method using gastric aspirate samples spiked with 5 μg of authentic *N*-acetylneuraminic acid. The influence of pH on the content of free sialic acid was measured in selected aspirate samples and with pure sialoglycoproteins (bovine submandibular gland glycoprotein and α₁-acid glycoprotein).

Isolation of different sialic acids

The nature of different sialic acids in all gastric aspirates was studied with volumes of gastric aspirate containing a minimum of 100–120 μg of total sialic acids, using previously described methods [18, 21]. A total of 10 randomly selected samples were dialysed against three changes of 5 litres of distilled water and analysed in the same way. The samples were adjusted to 2 mol/l with respect to acetic acid and incubated for 4 h at 80°C in capped tubes [21]; the optimum time for hydrolysis was determined in preliminary experiments. The samples were cooled on ice, dialysed against 50 ml of distilled water at 4°C overnight and passed through a column (5 ml) of Dowex 50W H⁺ form [21]. The effluent and washings were loaded on to Dowex 2 × 8 formate form, washed with distilled water and eluted with 10 ml of 2 mol/l formic acid. Formic acid was removed by rotary evaporation at 30°C, the residue dissolved in 2.5 ml of water and extracted with 5 ml of diethyl ether three times. The aqueous fraction was passed through Dowex 50W H⁺ and concentrated by rotary evaporation. An aliquot containing approximately 50 μg of total sialic acids was applied to prewashed cellulose thin-layer plates and developed with butan-1-ol/propan-1-ol/0.1 mol/l HCl (1:2:1, by vol.). The sialic acids were visualized using the orcinol/ferric chloride spray reagent [18].

The proportion of sialic acids in the different bands was determined by scraping the cellulose from the plates and extracting the chromophore in 1 ml of isoamyl alcohol. The absorbance was measured at 572 nm against blanks from sprayed and scraped cellulose extracts in lanes with no sample.

Recovery of sialic acids by this procedure was assessed by the inclusion of tracer amounts of radioactive *N*-acetylneuraminic acid, measured as described previously [22]. The recovery of *O*-acetyl groups in sialic acids was measured with hydroxylamine reagent as described previously [18] for representative aspirate samples and for bovine submandibular gland glycoprotein as a standard.

G.l.c. and m.s.

Two representative dialysed gastric aspirate samples were used to prepare 300–400 µg of sialic acids as described above. These were analysed by t.l.c. and subjected to g.l.c.–m.s. For g.l.c.–m.s., sialic acids were converted into methyl ester, trimethylsilyl ether derivatives as described in [23]. Combined g.l.c.–electron impact m.s. was carried out on a Carlo Erba GC/Kratos MS80/Kratos DS 55 system: electron energy, 70 eV; accelerating voltage, 2.7 kV; ionizing current, 100 µA; ion-source temperature, 225°C; BP-1 capillary column (25 m × 0.33 mm; SGE); oven temperature, 210°C for 2 min followed by 210–300°C at 4°C/min.

Statistical analysis

Non-parametric data were analysed for statistical significance using the Mann–Whitney *U*-test and Spearman correlation analysis. The C-stat statistical package was used on a mainframe computer.

RESULTS

Patients

Forty-two patients under 60 years of age (younger group) and 35 patients over 60 years of age (elder group) were studied. The mean age was 52 (range 19–93) years. The male/female ratio was 6:7. Eight of the elder group were on non-steroid anti-inflammatory drugs (NSAIDs). *H. pylori* infection was identified in 13 patients.

Total sialic acid content

The optimum conditions for the release and measurement of total sialic acids in aspirates tested on six randomly chosen samples showed that maximum release of sialic acids, as judged by the periodate–thiobarbiturate method, occurred after 70–80 min of hydrolysis at 80°C with sulphuric acid. Two sequential hydrolyses of 60 min, removing the supernatant and rehydrolysing the pelleted material after centrifugation, gave a larger yield (18 ± 4%, *n* = 6) com-

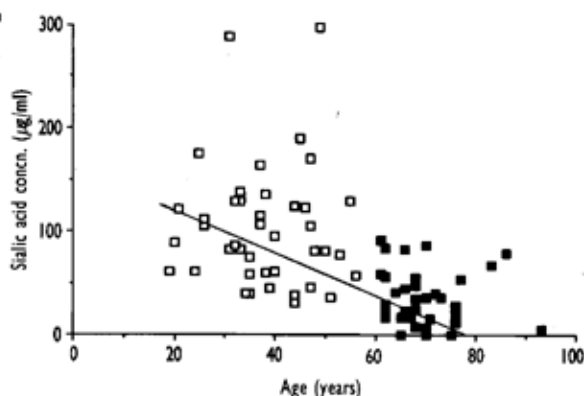


Fig. 1. Variation of gastric aspirate sialic acid content with age. The total sialic acid content of gastric aspirates was measured as described in the Methods section and is plotted against patient age. □, Younger group (under 60 years old); ■, elder group (over 60 years old). The line was determined by regression analysis and the coefficient of correlation for sialic acid with age was -0.64 .

pared with continuous hydrolysis for 80 min. The occurrence of significant chromogen in the ether extract after acid hydrolysis could not be predicted from any parameter relating to the patient or the aspirate itself and for this reason ether extraction was included routinely in the complete procedure. The recovery of free *N*-[14 C]acetylneuraminic acid added to the aspirates (*n* = 12) was $86 \pm 8\%$ (mean ± SD) for the complete procedure.

The content of free sialic acid in samples varied between 5 and 20% of total sialic acids. The samples with higher pH (average pH = 7.5) showed lower contents of free monosaccharide ($8 \pm 3\%$, *n* = 4) compared with those with an average pH of 2.1, which had $15 \pm 6\%$ free sialic acid. However, this difference was not statistically significant. Experiments with dialysed gastric aspirates showed that incubation at 37°C resulted in release of sialic acid. Similar results were obtained with dialysed aspirates heat-treated to destroy sialidase activity and also with purified glycoprotein samples under the same conditions. No differences were observed at the two pH values tested.

Variation of aspirate total sialic acid concentration with age

The sialic acid content of gastric aspirates from patients with non-ulcer dyspepsia were plotted as a function of age. In a sample of 77 normal patients a significant decrease with age was found (Fig. 1). A correlation coefficient of -0.64 was calculated ($P < 0.001$, Spearman rank correlation). The gastric aspirate total sialic acid concentrations in both the younger and elder groups are shown in Table 1. There was a significant difference between the two groups ($Z = -5.77$, $P < 0.001$, Mann–Whitney *U*-test). The sialic acid level in the elder group for *H. pylori*-positive and -negative patients is shown in

Table 1. Gastric aspirate sialic acid levels in younger and elder groups. The total sialic acid in gastric aspirates of patients with normal histology is shown. The median, upper and lower quartiles (range) are given for each group. Statistical significance (Mann-Whitney *U*-test): **P* < 0.001 compared with the younger group.

	Total sialic acid ($\mu\text{g/ml}$)	<i>n</i>
Younger group (under 60 years)	88 (61-130)	42
Elder group (60 years and over)	29 (16-55)*	35

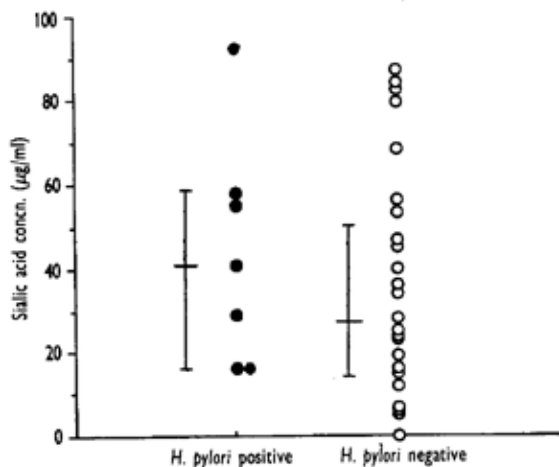


Fig. 2. Gastric aspirate sialic acid concentrations and *H. pylori* infection in patients over 60 years old. Sialic acid concentrations in the gastric aspirates of patients over 60 years old (elder group) with (●, *n* = 7) and without (○, *n* = 28) *H. pylori* infection were compared. No significant difference was found when tested by the Mann-Whitney *U*-test ($Z = -0.86$, $P = 0.38$). Bars show the median, upper and lower quartiles.

Fig. 2. There was no significant difference between the positive and negative groups ($Z = -0.8626$, $P = 0.38$). Also in the elder group, no difference in sialic acid concentrations was found between those patients who were on NSAIDs and those who were not.

Nature of sialic acids present in gastric aspirates

Total aspirate sialic acids (100–120 μg) were prepared for analysis after acetic acid hydrolysis. Recovery of total sialic acids using this technique, as judged by recovery of radioactive sialic acid added to the initial aspirate sample, was $83 \pm 6\%$ (mean \pm SD, $n = 10$). Recovery of *O*-acyl groups by direct measurement of aspirate samples was not possible due to the presence of interfering compounds. Bovine submandibular gland glycoprotein containing about 2 mol of *O*-acetyl ester/mol of sialic acid was therefore used as a standard and showed recovery of $67 \pm 5\%$ ($n = 3$) in the complete procedure.

All aspirates were successfully analysed by t.l.c. A

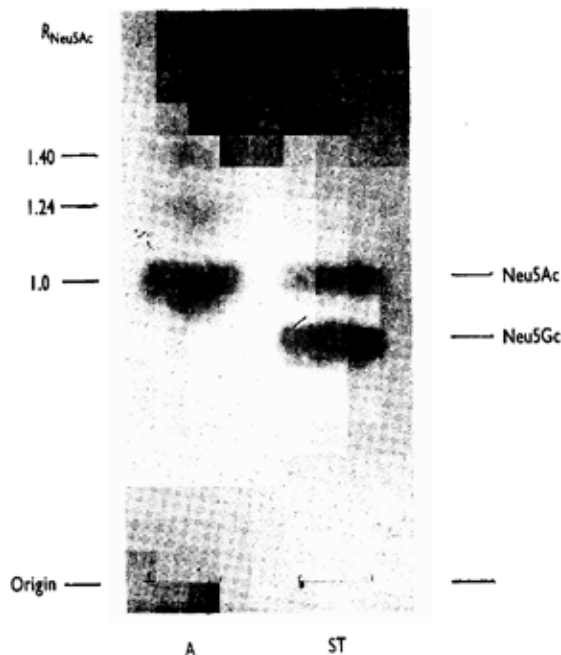


Fig. 3. Detection of sialic acids from gastric aspirates on cellulose t.l.c. The plates were sprayed with orcinol/ferric chloride and heated at 80°C. The standards (ST) are Neu5Ac and *N*-glycolyl-*D*-neuraminic acid (Neu5Gc). The migration of human gastric aspirate sialic acids (A) is shown relative to *N*-acetylneuraminic acid, R_{Neu5Ac} 1.0. The bands at R_{Neu5Ac} 1.24 and R_{Neu5Ac} 1.40 are Neu5Ac9Lt and Neu5,9Ac₂, respectively.

representative chromatogram is shown in Fig. 3. A major band was found at R_{Neu5Ac} 1.0 and two bands of *O*-acylated sialic acids with R_{Neu5Ac} values of 1.24 and 1.40 were seen. A range of 5–25% *O*-acylation relative to sialic acid on a molar basis was found with a mean \pm SD of $14 \pm 8\%$ ($n = 33$). The inclusion of dialysis resulted in a non-significant reduction in the amount of total *O*-acylated sialic acids to a mean \pm SD of $11 \pm 5\%$ ($n = 10$). The 9-*O*-lactyl derivative was detected as 2–15% of total sialic acids with a mean \pm SD of $8 \pm 5\%$ ($n = 33$). No differences between the two age groups could be detected.

G.l.c.-m.s.

A typical total-ion current chromatogram of one of the two samples analysed by g.l.c.-m.s. is depicted in Fig. 4. The peaks 1a/b, 2 and 3 correspond to the methyl ester, trimethylsilyl ether derivatives of *N*-acetyl-*D*-neuraminic acid β/α (Neu5Ac β/α), 9-*O*-acetyl-*N*-acetyl-*D*-neuraminic acid (Neu5,9Ac₂) and 9-*O*-lactyl-*N*-acetyl-*D*-neuraminic acid (Neu5Ac9Lt), respectively, as deduced from their related mass spectra. In each mass spectrum the typical fragmentation pattern is observed, indicative of the *N*, *O*-acyl substitution pattern of neuraminic acid [23, 25].

DISCUSSION

The sialic acids detected in gastric aspirates are largely derived from the mucus glycoproteins, the

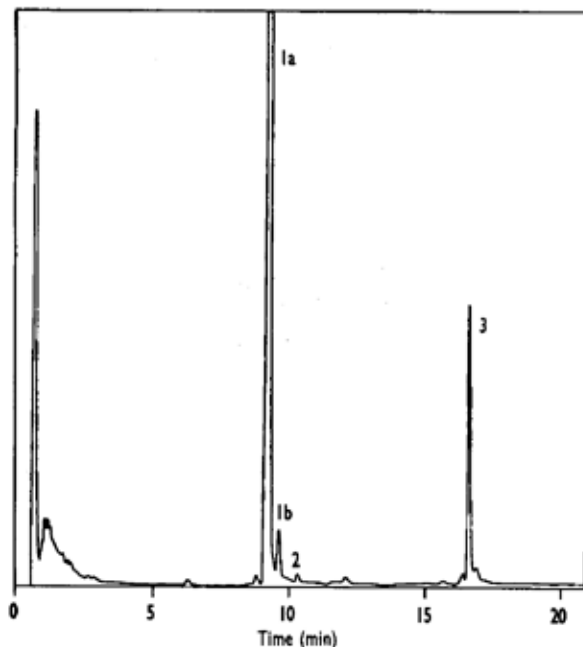


Fig. 4. G.l.c.-m.s. Total-ion current chromatogram and fragment ions for sialic acids isolated from human gastric aspirate. The peaks 1a/b, 2 and 3 correspond to the methyl ester, trimethylsilyl ether derivatives of Neu5Ac β (α , Neu5,9Ac₂ and Neu5Ac9Lt, respectively [21, 25].

Sialic acid	m/e values						
	A	B	C	D	E	F	G
Neu5Ac	668	624	478	298	317	205	173
Neu5,9Ac ₂	638	ND	478	298	317	175	173
Neu5Ac9Lt	740	696	478	298	317	277	173

where ND is not detected, as this fragment ion is not formed.

major secretory products of the gastric mucosa [1, 2]. The majority of this sialic acid was found in a glycosidically bound form. A small part of the total sialic acid may be derived from saliva where free *O*-acylated sialic acids have been described [25]. The presence of saliva in the collected gastric aspirates was kept to a minimum by careful clinical management during collection, and free sialic acids were removed by dialysis in control samples. Thus, the glycosidically bound *O*-acylated sialic acids detected are likely to be derived from gastric mucosal products.

The analysis of the sialic acid in the aspirate samples was carefully controlled for recoveries and interference in the periodate-thiobarbituric acid assay. Saponification as an initial step eliminates underestimation of total sialic acid concentrations by removing the *O*-acyl groups which would hinder the periodic oxidation step in the assay [18, 21, 25]. The gastric aspirate contains many different components, such as lipid peroxides, which result in falsely high values in the colorimetric assay or others, e.g., acidity, which may result in degradation of the sialic acid [18, 19]. In addition, acid hydrolysis generates compounds known to interfere with the assay [19].

The present work describes a new finding, that the total aspirate sialic acid concentration decreased with age in patients with non-ulcer dyspepsia (Fig. 1). The phenomenon indicates an age-related variation in gastric mucosal secretion and may be due to qualitative changes in mucus glycoproteins or subtle atrophy of the mucus cell layer in the elderly. These changes are independent of *H. pylori* infection and ingestion of NSAIDs. The basis for the observation remains unexplained. However, it is relevant to the possible use of the sialic acid index as a diagnostic test. The correlation of these measurements with other parameters, such as mucin, requires separation of aspirate components and assessment of individual sialic acid compositions. Previous assay of individual sialic components (e.g. [7]) has shown that this is time-consuming, requires more sample and limits the number of samples which can be examined. This remains, however, an important aim for future studies.

The value of aspirate sialic acid levels as a measure of mucus glycoprotein secretion and possible change in gastric disease has been both supported [12, 15, 16] and refuted [24]. A dramatic increase in the rate of sialic acid secretion in response to pentagastrin stimulation has been reported [15, 16] using the same colorimetric method employed in the current work, but with fewer controls to eliminate interference in the assay due to lipids or other aspirate components. This result is in contrast to work by Ene and Clamp [6], where gas chromatographic analysis of the total carbohydrate composition of purified mucin, including sialic acid, showed no difference between basal (unstimulated) and pentagastrin-stimulated sialic acid levels. The subjects used in this work had an average age of 21 years [6], which would be expected to represent the highest level of total sialic acid in the aspirate (Fig. 1), whereas the average age of patients in the other study [15] was approximately 42 years and underlines the need for age correction. The results thus suggest that sialic acid levels in gastric aspirate may be a useful parameter with which to monitor age-related normal gastric function.

The phenomenon of age-related changes in glycoconjugate sialic acid concentration is not limited to the stomach. Decreases have been found in human brain ganglioside [26] and increases were reported for total sialic acid in human serum [27] and in free, lipid-bound and total sialic acid in rat serum [28].

The discovery of different types of sialic acids, including 9-*O*-acetyl- and 9-*O*-lactyl-*N*-acetylneuraminic acid, by t.l.c. was confirmed using m.s. It is striking that the presence of these two *O*-acylated sialic acids could be detected in all 33 aspirates tested. The *O*-acyl sialic acids found in these studies were covalently bound (non-dialysable) components of glycoproteins, in contrast to the monosaccharide forms reported in saliva [25]. This suggests that

they are products of the gastric mucosa and not contamination from saliva. No evidence was found for the presence of *N*-glycolylneuraminic acid in any of the samples analysed, in keeping with the normal occurrence of *N*-acetylneuraminic acid in man [9]. The significance of these modified sialic acids in gastric secretions is not known at present, although they have been reported in gastric intestinal metaplasia [8, 11]. Histochemical detection of *O*-acylated sialic acids has been improved and correlated with biochemical detection [29, 30]. *O*-Acetylated sialic acids are known to act as receptors for several viruses, including influenza C virus [31], retrovirus [32], haemagglutinating encephalomyelitis virus and bovine coronavirus [33]. The significance of viral binding to *O*-acylated sialic acids in the gastric mucosa has not been studied. Furthermore, the gastric bacterium *H. pylori* associated with gastritis possesses haemagglutinins which bind sialic acids. Binding can be inhibited by sialoglycoproteins and in particular bovine salivary glycoprotein rich in *O*-acetylated sialic acids. A selective regional colonization of the gastric mucosa by *H. pylori* is known to occur [34]. The presence of *H. pylori* infection had no influence on the aspirate total sialic acid levels found in the elder group (Fig. 2). The detection of *O*-acylated sialic acids in gastric aspirate gives no indication of any regional distribution in the gastric mucosa and whether this may relate to *H. pylori* distribution. In addition, colonization of areas of gastric intestinal metaplasia rich in *O*-acylated sialic acids has not been reported and deserves attention in the light of this study.

Finally, future analysis is required to assess the relationship between the decreases in sialic acid levels with increasing age reported here and the concentrations of individual aspirate sialoglycoproteins.

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