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# The presence of *N*-acetylneuraminic acid in Malpighian tubules of larvae of the cicada *Philaenus spumarius*

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**Sialic acid-containing glycoconjugates are generally considered to be unique to the deuterostomes, a lineage of the animal kingdom which includes animals from the echinoderms up to the vertebrates. There are, however, two isolated reports of sialic acid occurring in the insect species *Drosophila melanogaster* and *Galleria mellonella*. Since insects are classified as protostomes, these findings call previous assumption on the phylogenetic distribution and thus on the evolution of sialic acids into question. Here, we report the occurrence of *N*-acetylneuraminic acid (Neu5Ac) in larvae of the cicada *Philaenus spumarius*. Cytochemical analysis of larval sections with lectins from *Sambucus nigra* and *Limax flavus* suggested the presence of sialic acids in the concrement vacuoles of the Malpighian tubules. The monoclonal antibody MAb 735, which is specific for polysialic acid, labelled the same structures. A chemical analysis performed by HPLC of fluorescent derivatives of sialic acids and by GLC-MS provided sound evidence for the presence of Neu5Ac in the *Philaenus spumarius* larvae. These data suggest that in this cicada Neu5Ac occurs in α2,8-linked polysialic acid structures and in α2,6-linkages. The results provide further evidence for the existence of sialic acids in insects and in linkages known to occur in glycoconjugates of deuterostomate origin.**

***Keywords:*** **N-acetylneuraminic acid, polysialic acid, insects, lectins, Malpighian tubules**

***Abbreviations:*** **AEC, 3-amino-9-ethylcarbazole; DMB, 1,2-diamino-4,5-methylene dioxybenzene; Endo-N, endo-*N*-acyl-neuraminidase; GLC-MS, gas–liquid chromatography combined with mass spectrometry; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; LFA, *Limax flavus* agglutinin; MAA, *Maackia amurensis* agglutinin; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; PSA, polysialic acid; SNA, *Sambucus nigra* agglutinin; TLC, thin-layer chromatography; VCS, *Vibrio cholerae* sialidase.**

## Introduction

Sialic acids comprise a family of more than 40 structurally related derivatives of *N*-acetylneuraminic acid (Neu5Ac). They generally occupy terminal positions in the oligosaccharide chains of glycoconjugates where they can be bound by a variety of glycosidic linkages to the sub-terminal sugar residues. The total amount and type of sialic acid, as well as the nature of the glycosidic linkage are strictly regulated and vary considerably according to cell type, organism and the stage in development. The sialic acid residues of glycoconjugates fulfil a variety of biological functions. Due to their negative charge, they can influence the physicochemical properties of individual glycoconjugates and cell surfaces. They also serve as ligands in receptor-mediated cell–cell interactions of inflammation, the immune response, and a number of adhesion processes [1,2].

[†]This manuscript has been written in remembrance of our friend and collaborator Dr. Elena B. Lapina, Moscow

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Sialic acid-containing glycoconjugates were considered to be unique to the deuterostomate lineage of the animal kingdom, where they have been detected in echinoderms, hemichordates, cephalochordates and vertebrates [3,4]. Interestingly, an increasing number of pathogenic and commensal bacteria, viruses, protozoa and fungi have also been found to contain sialic acids [4,5]. However, their repertoire of sialylated glycoconjugates and sialic acid structures is very limited in comparison to the above-mentioned animal types. Moreover, it has been suggested that bacteria acquired the capability to synthesise sialic acids at a later stage in evolution, possibly as an adaptation to interactions with their hosts [6]. The homologies found at the molecular level among microbial sialidases, enzymes of sialoglycoconjugate catabolism, support the idea of the sialidase genes originating in higher animals with subsequent transfer, for example via phages, among bacteria [7].

For a long time, it was generally accepted that, in common with other organisms of the protostomate lineage, members of the phylum insecta do not possess sialic acids [3,4]. Moreover, viral and recombinant mammalian glycoproteins expressed in baculovirus-insect cell systems do not, as a rule, possess complex forms of *N*- and *O*-linked oligosaccharides with terminal sialic acids [8–11]. Nevertheless, some evidence for the presence of sialic acids in insects has appeared during the last decade. The occurrence of sialic acids was reported in the vitellogenin receptor of *Locusta migratoria* [12], and in a parasitism-specific protein of *Anastrepha suspensa* [13]. Since these investigations were not primarily concerned with identifying sialic acids, the analytical procedures in these studies were limited to the sialidase treatment of glycoproteins and lectin-binding studies, and no chemical structural analyses were undertaken. However, using a combination of cytochemical detection, immunoblot analysis and mass spectrometric identification procedures, Roth *et al*. [14] demonstrated the presence of Neu5Ac and homopolymers of α2,8-linked Neu5Ac (PSA) in *Drosophila melanogaster*. While Neu5Ac was detected throughout the development of *Drosophila* from the blastoderm to the third larval instar, the expression of PSA was more strictly regulated and appeared only in embryos. Recently, Neu5Gc7,9$Ac_2$ was reported to occur in the prothoracic glands in the last larval instar of *Galleria mellonella* [15] using mass spectrometry, histochemistry and spectrophotometry. However, the electron-ionisation mass spectrometry of trifluoroacetylated sialic acids, performed in this work, is not a commonly used method, and the omission of the mass-spectra for authentic standards call the above results into question.

In the present work, we report the occurrence of glycoconjugates, containing α2,6-linked Neu5Ac and homopolymers of α2,8-linked Neu5Ac in the concrement vacuoles of the Malpighian tubules of *Philaenus spumarius* larvae. Histochemical staining of larval sections with sialic acid-specific lectins and an antibody, was supported by chemical analyses of extracted sialic acids by HPLC of their fluorescent derivatives as well as by a combination of gas–liquid chromatography and mass spectrometry.

## Materials and methods

### Insects

*Philaenus spumarius* larvae were collected from fields surrounding Kiel in the period from the end of May until the beginning of June in 1997 and 1998. The animals were washed in phosphate-buffered saline, pH 7.4 (PBS) and immediately fixed in 4% paraformaldehyde or Bouin fixation solution for histochemical investigations, or frozen and stored at −20°C for the chemical analyses.

### Lectins and antibody

For immunohistochemical analyses the mouse monoclonal antibody MAb735 [16] and the biotinylated lectins from *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA), both from Oxford GlycoSystems (Abingdon, UK) and *Limax flavus* (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) were used. *Limax flavus* agglutinin (LFA) was biotinylated using succinimidyl-6-[biotinamido]hexanoate (Sigma Chemicals Ltd, Deisenhofen, Germany). 500 µg LFA were mixed with 50 µg succinimidyl-6-[biotinamido]hexanoate in 0.1 M sodium carbonate buffer pH 8.3 (final reaction volume 1 ml) for 2 h at room temperature, and the reaction mixture was dialysed overnight against PBS.

### Light microscopy

The immunostaining of paraformaldehyde-fixed and paraffin-embedded sections (10 µm) of whole larvae was performed using the Universal Peroxidase Kit (Immunotech GmbH, Hamburg, Germany), according to the manufacturer's protocol with 3-amino-9-ethylcarbazole (AEC) as the chromogenic substrate. Briefly, the lectins and the antibody were diluted in PBS to give final concentrations of 20 µg/ml MAA, 20 µg/ml SNA, 10 µg/ml LFA, and 40 µg/ml MAB 735. The solutions were incubated overnight at 4°C with sections, which were preliminarily rehydrated and blocked with the manufacturer's protein-blocking reagent. After washing, the sections were incubated with undiluted biotinylated secondary antibodies (for lectins this step was omitted), followed by incubation with the streptavidin-peroxidase reagent and AEC-staining.

Non specific, sialic acid-independent staining was assessed using either inhibitory sugars or enzyme treatment of the sections. In the first controls, lectins and antibodies were preincubated overnight at 4°C in PBS containing an excess of the corresponding inhibitory sugar. The composition of these preincubation mixtures was as follows: 1) 0.5 mg/ml SNA, 5 mg/ml α2,6-sialyllactose purified from colostrum [17]; 2) 0.25 mg/ml LFA, 5 mg/ml Neu5Ac (Serva, Heidelberg, Germany); 3) 3.2 mg/ml MAb 735, 16 mg/ml colominic acid (Sigma Chemicals Ltd, Deisenhofen, Germany). Before use,

the solutions were diluted to give the working concentration of the lectins or antibody, described above. In the second set of controls, the sections were treated with enzymes. The sections were dewaxed and rehydrated to 50% ethanol and washed in PBS prior to all enzyme incubations. For the following *Vibrio cholerae* sialidase (VCS) treatment, the sections were briefly washed in VCS-buffer (50 mM Na-acetate, pH 5.5, 9 mM $CaCl_2$, 154 mM NaCl) and incubated for 20 hours at 37°C with 50 μU of VCS in 50 μl of VCS-buffer (Behringwerke AG, Marburg, Germany). Enzyme control sections were incubated under the same conditions with 50 μl VCS-buffer. The treatment of sections with bacteriophage endo-*N*-acylneuraminidase [18] was performed at 4°C for 20 hours using 0.14 μg of enzyme in 50 μl PBS. The control sections for enzyme treatment were incubated under the same conditions with 50 μl PBS.

Further histochemical staining with lectins or the antibody were performed as described above.

### Electron microscopy

Larvae were fixed in freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate-buffer, pH 7.4, washed in 0.1 M phosphate buffer, pH 7.4, for several hours, immersed in 2% osmium tetroxide, and dehydrated in ascending concentrations of ethanol. The resulting samples were embedded in LR White (London Resin Company Ltd., Reading, UK). In semithin sections, Malpighian tubules were identified, and ultrathin sections were cut with a diamond knife on a Reichert ultramicrotome. The sections were double contrasted with saturated uranyl acetate and lead citrate and viewed in a Philips 300 electron microscope.

### Purification of sialic acids

Lyophilised whole cicada larvae (1 g dry weight) were homogenised in 15 ml water with a Potter-Elvehjem homogeniser at 4°C and sialic acids were hydrolytically removed using a two-step procedure. In the first hydrolysis, the homogenate was mixed with the same volume of 4 M propionic acid and sialic acids were released by incubation at 80°C for 4 hours [19]. Following dialysis (3 × 250 ml of water, 4°C, 20 h), the retentate was adjusted to pH 1 by the addition of 1 M HCl, hydrolysed for 1 h at 80°C and dialysed again as described above. The dialysates from both hydrolyses were separately concentrated by rotary evaporation, and lyophilised. The residues from both hydrolyses were pooled and the sialic acids were enriched using Dowex 50W × 8 and Dowex 2 × 8 ion-exchange resins (BioRad, Munich, Germany), as previously described [20]. Adsorbed sialic acids were eluted from the anion-exchange resin with 1 M formic acid.

For mass spectrometric analysis of the sialic acids, a portion of the above eluate (about 15 μg of sialic acid) was again applied to calibrated Dowex 2 × 8 column (0.7 × 5 cm), and the column was developed with 60 ml gradient from 0 to 2 M formic acid, at a flow rate of 0.25 ml/min. The sialic acid-containing fractions were analysed by HPLC as described below, pooled and lyophilised.

### TLC of sialic acids

About 10 μg of sialic acids, purified from the cicada hydrolysate (see above), Neu5Ac and Neu5Gc standards were applied to a 10 cm × 10 cm cellulose HPTLC plate. The samples were chromatographed in butan-1-ol : propan-1-ol : 0.1 M HCl 1 : 2 : 1 (v/v/v). Sialic acid-containing bands were visualised with the orcinol/$Fe^{3+}$/HCl reagent [20].

### HPLC analysis and quantification of sialic acids

The 1,2-diamino-4,5-methylene dioxybenzene (DMB) derivatives of sialic acids were analysed by HPLC with fluorescence detection on a reversed-phase column calibrated with authentic standards, as previously described [21]. Sialic acid quantification was carried out by the periodic acid/thiobarbituric acid assay with Neu5Ac as standard [20].

### Lyase treatment

About 0.3 μg of sialic acids, partially purified from the cicada hydrolysates, were incubated at 37°C for 2 h with 25 mU of acylneuraminate-pyruvate lyase from *Clostridium perfringens* (Sigma Chemicals Ltd, Deisenhofen, Germany), as previously described [22].

### Mass spectrometry

For gas–liquid chromatography-mass spectrometry analyses 10–700 μg of purified sialic acids (see above) were dried over $P_2O_5$ and pertrimethylsilylated with a mixture of pyridine–hexamethyldisilazane–chlorotrimethylsilane, 5 : 1 : 1 (v/v/v), for 2 h at room temperature. GLC-MS analysis was performed on a Fisons Instruments GC8060/MD800 system (InterScience) equipped with a CPsil5CB column (25 m × 0.25 mm, Chrompack Nederland B.V.). The oven temperature was programmed from 180 to 280°C at 4°C/min. Electron impact mass spectra were recorded at a mass range of m/z 100–750 as described previously [23].

## Results

### Histochemistry

The results of the histochemical experiments are presented in the Table and Figures 1–3. Sections of the cicada larvae, covering all regions of the body, were investigated with lectins from *Maackia amurensis*, specific for Neu5Acα2,3Galβ1,4Glc/ GlcNAc [24], *Sambucus nigra*, specific for Neu5Acα2,6Gal/GalNAc [25], and *Limax flavus* which recognises both Neu5Ac and Neu5Gc [26]. The mouse monoclonal antibody MAb 735, which is specific for homopolymers of α2,8-linked sialic acid (polysialic acid),

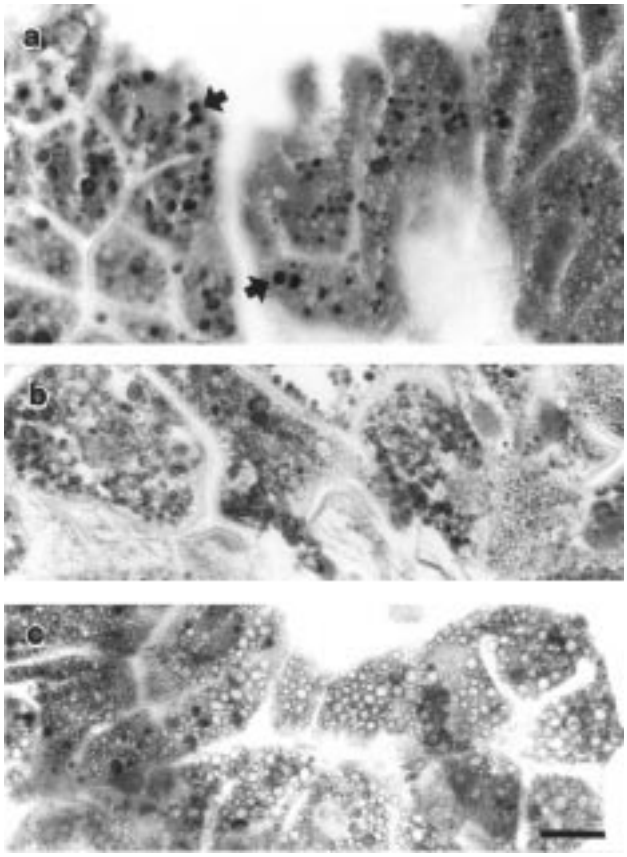


**Figure 1.** Cytochemical detection of sialic acid in *Philaenus spumarius* larvae using *Limax flavus* lectin: (a) Positive staining of granules (arrows) of the Malpighian tubules; (b) Negative control after pretreatment of the sections with *Vibrio cholerae* sialidase; (c) Negative control after preincubation of *Limax flavus* lectin with Neu5Ac. Magnification: × 350; bar: 250 μm.

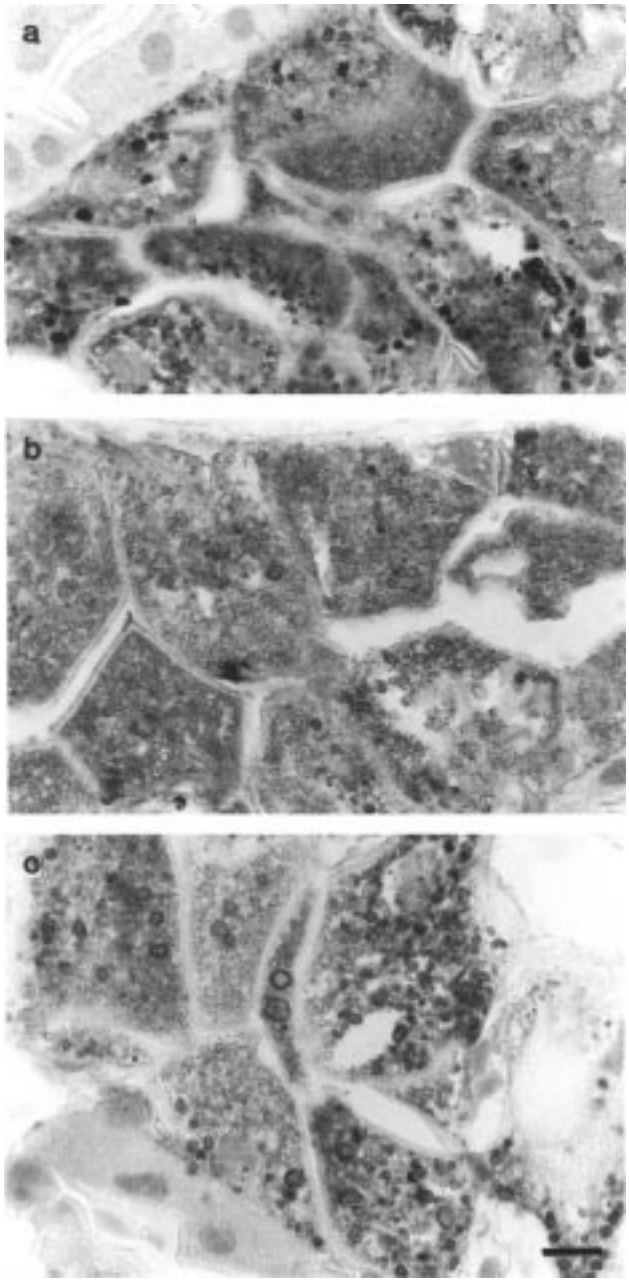


**Figure 2.** Cytochemical detection of sialic acid in *Philaenus spumarius* larvae using *Sambucus nigra* lectin: (a) Positive staining of granules of the Malpighian tubules: (b) Negative control after pretreatment of the sections with *Vibrio cholerae* sialidase; (c) Negative control after preincubation of *Sambucus nigra* lectin with an excess of α2,6-sialyllactose. Magnification: × 350; bar: 250 μm.

was also used. While staining was observed with LFA, SNA and the monoclonal antibody (Figs. 1–3, Table), MAA revealed no detectable binding, even when used at various concentrations (data not shown). The binding of SNA and LFA was either abolished or significantly reduced by the specific enzymatic removal of bound sialic acids from the sections using VCS. Preincubation of the lectins with an excess of α2,6-sialyllactose (SNA) or Neu5Ac (LFA) completely abolished lectin binding (Figs. 1–2, Table).

Accordingly, the immunohistochemical staining with the monoclonal antibody MAb 735 was also abolished, either by pretreatment of the sections with endo-*N*-acylneuraminidase or by preincubation of MAb 735 with colominic acid (the *Escherichia coli* K1 capsular polysaccharide, composed of homopolymers of α2,8-linked Neu5Ac) (Fig. 3, Table).

Closer inspection of the stained sections revealed that the lectins and the antibody used all labelled the concrement vacuoles in the Malpighian tubules. These stained structures appeared as secretory granules composed of concentric lamellae. These granules were heterogeneous in form and varied in diameter from 1.8 to 3.4 μm (Figs. 1–4). In order to exclude the possibility of a microbial origin of this sialic acid, the ultra-thin sections of the Malpighian tubules were studied by electron microscopy (Fig. 4). The micrographs revealed no evidence for a localised viral or bacterial infection.

The lectin and antibody staining was restricted to distinct regions of the Malpighian tubules, consisting of actively-secreting epithelial cells. Beyond this area, the same secretory

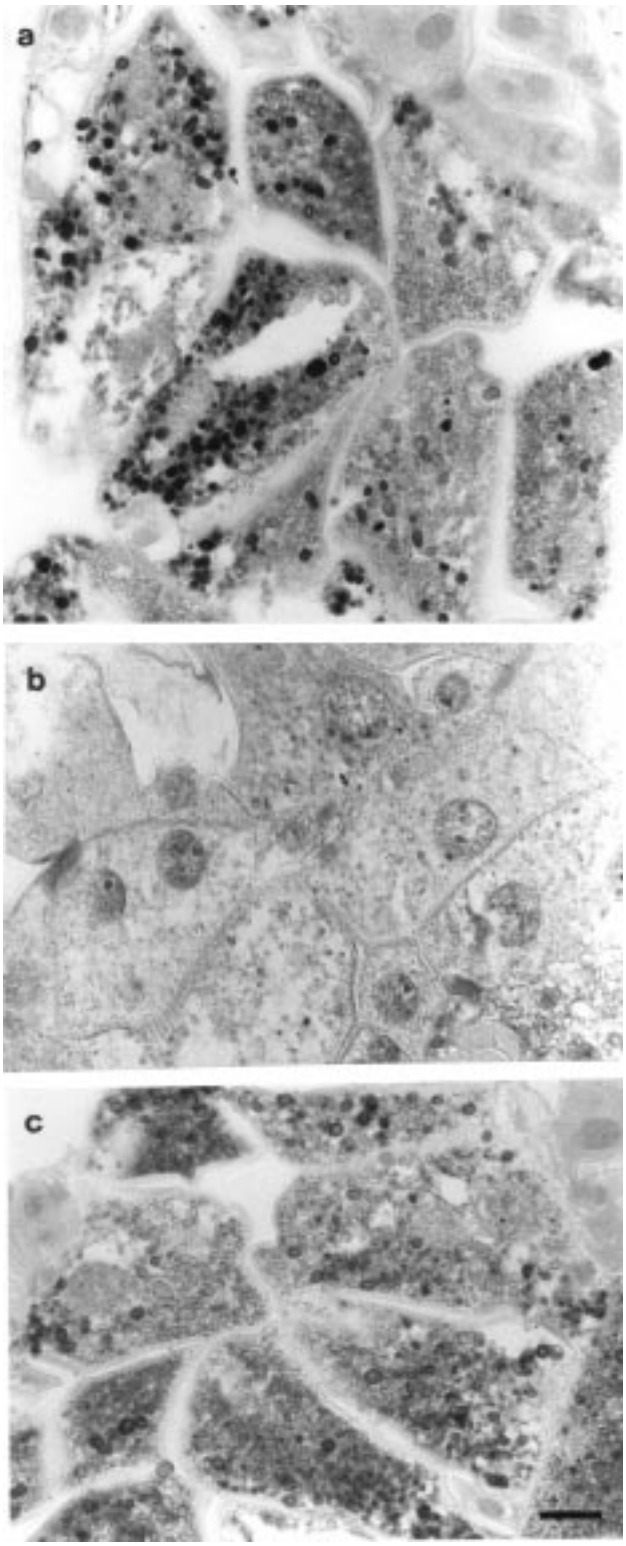


**Figure 3.** Immunohistochemical detection of sialic acid in *Philaenus spumarius* larvae using the monoclonal antibodies MAb 735 specific for polysialic acid: (a) Positive staining of granules of the Malpighian tubules; (b) Negative control after pretreatment of the sections with endo-*N*-acylneuraminidase; (c) Negative control after preincubation of MAb 735 with an excess of colominic acid. Magnification: × 350; bar: 250 μm.

**Table 1.** Lectins and antibodies used for the histochemical investigations

| *Lectins or antibodies* | *Specificity* | *Control* [a] | *Staining* |
|---|---|---|---|
| MAA | Neu5Ac$\alpha$2, 3Gal$\beta$1,4Glc/ GlcNAc | | − |
| SNA | Neu5Ac$\alpha$2, 6Gal/GalNAc | | + |
| | | +$\alpha$2,6 sialyllactose | − |
| | | VCS treatment | − |
| LFA | Neu5Ac, Neu5Gc | | + |
| | | +Neu5Ac | − |
| | | VCS treatment | decreased |
| MAb 735 | homopolymers of $\alpha$2,8-linked sialic acids | | + |
| | | + colominic acid | − |
| | | Endo-N treatment | − |

[a] Preincubation with sugars and treatment with enzymes were performed as described in Materials and methods: neighbouring sections were used for the main staining and corresponding control.

cells were not stained. Co-existing with the stained concrement vacuoles were vacuoles which did not react positively with the sialic acid-specific reagents (Figs. 1–3).

## Sialic acid analysis

Material giving a positive orcinol reaction was enriched from homogenates of whole cicada larvae after release using the two-step acid hydrolysis described in Materials and Methods. Analysis of the potential sialic acids by thin-layer chromatography followed by orcinol staining revealed only one band with an $R_f$ value and colour corresponding to that of the authentic Neu5Ac standard (data not shown).

The HPLC analyses of the DMB-derivatives of the enriched sialic acids are presented in Figure 5a. The chromatogram exhibits well resolved peaks, and the peak with the retention time of 8.17 min coincided with that of the DMB-derivative of Neu5Ac, both in a separate run and in co-chromatography experiments. To confirm that this peak corresponds to Neu5Ac and not to an unidentified $\alpha$-keto acid, the putative sialic acid enriched from the larvae was incubated with acylneuraminate-pyruvate lyase from *Clostridium perfringens* prior to derivatisation. After this enzymatic treatment, the peak corresponding to Neu5Ac was markedly diminished (Fig. 5b).

The amount of sialic acid in the larvae was 30 μg per gram wet weight, according to the colorimetric quantification with the periodic acid/thiobarbituric acid test.

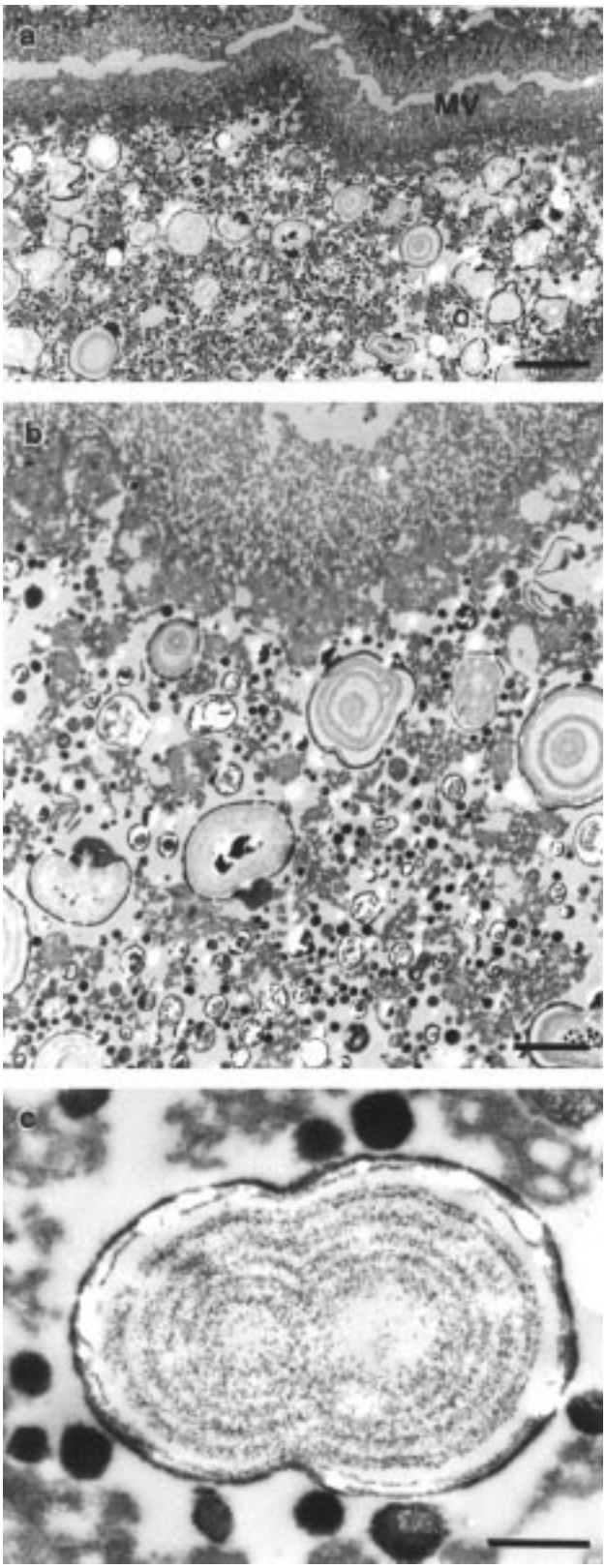


**Figure 4.** Electron microscopy of the Malpighian tubules of the cicada *Philaenus spumarius*, showing concentrically layered inclusions. MV: microvilli. Magnification a: ×1680, bar: 5 μm, b: ×5245, bar: 2 μm; c: ×33500, bar: 400 nm.

Structural analysis

Unequivocal evidence for the presence of Neu5Ac in the partially purified acid extract of the larvae was provided by gas–liquid chromatography combined with electron-impact mass spectrometry. The resulting mass spectrum given in Figure 6 exhibits the fragment ions characteristic for the pertrimethylsilylated derivatives of Neu5Ac. The fragment ions B to H indicated in this figure correspond to the fragmentation scheme of Neu5Ac presented in [5].

## Discussion

We report the occurrence of the sialic acid Neu5Ac in larvae of the cicada *Philaenus spumarius*. The presence of Neu5Ac in fractionated acid hydrolysates of whole larvae was unequivocally confirmed chemically by (i) TLC followed by orcinol staining, (ii) HPLC analysis of the DMB-derivatised hydrolysate before and after treatment with acylneuraminate-pyruvate lyase (Fig. 5), and (iii) gas liquid chromatography–mass spectrometric analysis of the pertrimethylated sialic acid (Fig. 6). The histochemical analysis of larvae sections with sialic acid-specific lectins (Figs. 1, 2) and the polysialic acid specific antibody (Fig. 3) provided further evidence for the presence of Neu5Ac in the cicada larvae.

Members of the order *Homoptera*, including cicada larvae, all feed on plants. Plants are known not to contain sialic acids [5]. *Philaenus spumarius* larvae produce copious amounts of foam, derived from plant sap. The foam collected from the plant *Anthriscus vulgaris*—the most frequent host plant in the Kiel area for this developing insect—did not contain sialic acids as was investigated by the TLC and the periodic acid/thiobarbituric acid assay. Thus, the uptake of sialic acid by the cicada larvae with its food can be excluded. The electron microscopic studies did not reveal any evidence for a microbial infection in the Malpighian tubules (Fig. 4), thus excluding the possibility of a viral or bacterial origin of this monosaccharide. Hence, Neu5Ac in cicada larvae is very likely of endogenous origin. However, metabolic and enzymatic studies are necessary to confirm this assumption.

The localisation of sialic acid was restricted to organelles referred to as concrement vacuoles within cells of the Malpighian tubules. These are primarily excretory and osmoregulatory organs in arthropods, but they also have secretory functions. Only a certain segment of this organ was stained, in agreement with the fact that different regions of the Malpighian tubules may have various functions [27].

The concrement vacuoles are produced in the endoplasmic reticulum as morphologically heterogeneous globules with concentric lamination. The lamellae of the concrement vacuoles consist of acid mucopolysaccharides and can also bind various substances such as waste products of metabolism and ions. These materials may be temporarily stored in the apex of the cells, for example for the following stage in the development of the insect, or secreted into the lumen of

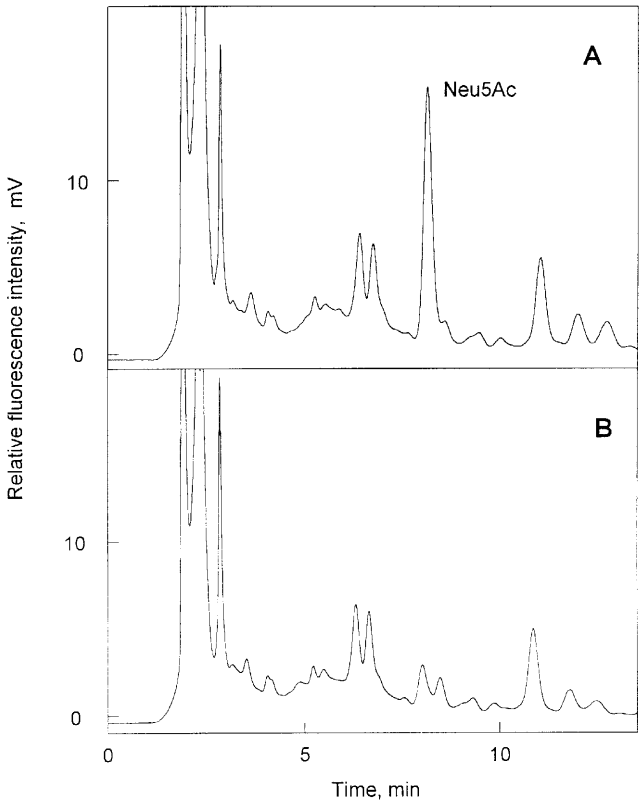


**Figure 5.** HPLC analysis of fluorescent derivatives of the sialic acids purified from the *Philaenus spumarius* larvae (a); the same analysis after preincubation of sialic acids with 25 mU of *Clostridium perfringens* aldolase (b).

the Malpighian tubules [27]. Although nothing is known about the composition of the secretion from the Malpighian tubules in cicada larvae, the same organ of larvae of the bumblebee, *Bombus atratus*, produces a mucous secretion composed of carboxylated and sulphated acid glycosaminoglycans, neutral polysaccharides and protein(s) [28]. Interestingly, in earlier studies by Lison on the *Bombus atratus* secretion in the Malpighian tubules, the presence of sialyl residues was proposed, based on the histochemical staining with toluidine blue solution at pH 2.5 [28].

At present, nothing is known about the glycoconjugates containing the Neu5Ac residues detected in *Philaenus spumarius*, and we can only speculate about the role of the sialic acid in these vacuoles. According to the histochemical data, Neu5Ac in the cicada larvae was present glycosidically bound in Neu5Acα2,8-Neu5Ac-linkages of PSA and in α2,6-linkages of glycoconjugates, and no evidence for α2,3-linkages was obtained.

The sialylated structures observed in the cicada larvae are very common in glycoconjugates of deuterostome animals. Neu5Ac in an α2,6-linkage to Gal or GalNAc is found in most glycoproteins and some gangliosides [5]. Similarly, polysialic acid is a glycan component of neural cell adhesion molecules and sodium channel proteins [29]. Protein-bound PSA also was found in cortical alveolar glycoproteins in eggs from several species of fish [30]. After fertilisation, the polysialoglycoproteins are transported into the perivitelline space where they are involved in osmoregulation. One could speculate that PSA in the cicada Malpighian tubules may be involved in the osmoregulatory functions of this organ.

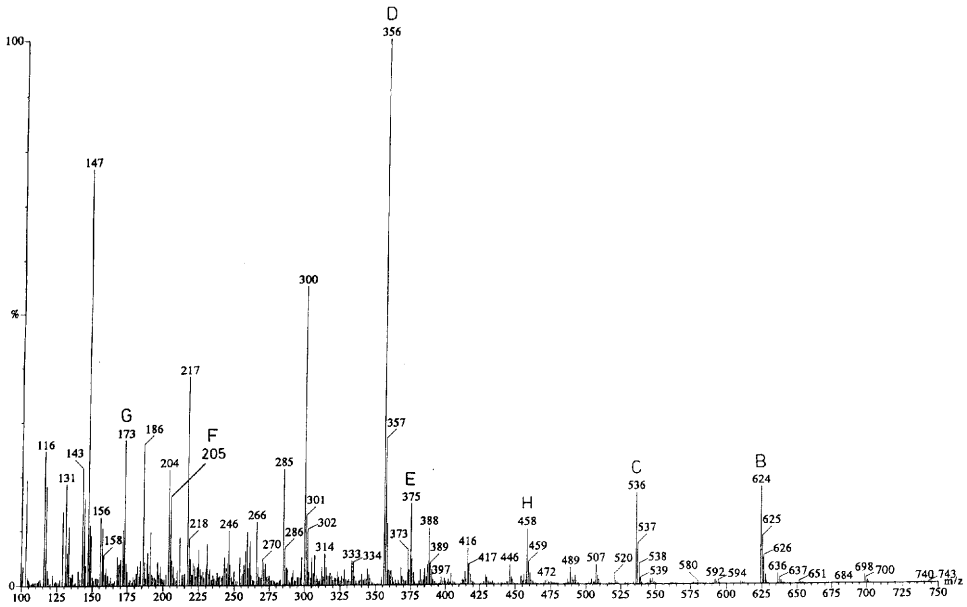


**Figure 6.** Electron-impact mass spectrum (70 eV) of *N*-acetylneuraminic acid purified from *Philaenus spumarius* larvae as per-*O*-trimethylsilyl derivative after gas–liquid chromatography-mass spectrometry. The characteristic fragment ions B through H are indicated.

The presence of sialic acid in insects can now be regarded as an established fact. The available data also suggest that sialylated glycoconjugates may only be expressed very locally in specific phases of an insect's life-cycle. Moreover, the general observation that recombinant mammalian and viral glycoproteins produced by baculovirus-infected insect cell lines do not possess sialic acid may result from the down-regulation of their biosynthesis in cultured cells derived from adult animals. Indeed, there is some evidence to suggest that infection of whole insect larvae with baculovirus vectors coding for mouse interleukin-3 and human placental, secreted alkaline phosphatase resulted in the formation of the respective sialylated recombinant glycoproteins [31,32]. The absence of sialic acid residues in glycoproteins produced by baculovirus-infected cell lines may also be due to their removal by cellular or viral exoglycosidases [8]. Thus, endogenous sialidase activity was reported in several insect cell lines [33], and a sialidase released by the salivary glands of the insect *Triatoma infestans* was purified and characterised [34].

In contrast to the limited expression of sialic acids in insects, sialylated glycoconjugates have been found in most deuterostomes, in all tissues of both adult and developing organisms. To what extent other insect species and other animals of the protostomate lineage possess sialylated glycoconjugates is unknown and remains to be established by systematic studies at all stages of animal development using modern techniques of sialic acid analysis. Furthermore, biochemical and molecular biological investigations on the biosynthetic pathway of sialylated glycoconjugates in insects might yield information about the origin(s) of sialic acid biosynthesis in animals.

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