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Low incidence of *N*-glycolylneuraminic acid in birds and reptiles and its absence in the platypus

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Dedicated to Professor Dr. Hans Kamerling on the occasion of this 65th birthday

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ABSTRACT

The sialic acids of the platypus, birds, and reptiles were investigated with regard to the occurrence of *N*-glycolylneuraminic (Neu5Gc) acid. They were released from tissues, eggs, or salivary mucin samples by acid hydrolysis, and purified and analyzed by thin-layer chromatography, high-performance liquid chromatography, and mass spectrometry. In muscle and liver of the platypus only *N*-acetylneuraminic (Neu5Ac) acid was found. The nine bird species studied also did not express *N*-glycolylneuraminic acid with the exception of an egg, but not tissues, from the budgerigar and traces in poultry. Among nine reptiles, including one turtle, *N*-glycolylneuraminic acid was only found in the egg and an adult basilisk, but not in a freshly hatched animal. BLAST analysis of the genomes of the platypus, the chicken, and zebra finch against the CMP-*N*-acetylneuraminic acid hydroxylase did not reveal the existence of a similar protein structure. Apparently monotremes (platypus) and sauropsids (birds and reptiles) cannot synthesize Neu5Gc. The few animals where Neu5Gc was found, especially in eggs, may have acquired this from the diet or by an alternative pathway. Since Neu5Gc is antigenic to man, the observation that this monosaccharide does not or at least only rarely occur in birds and reptiles, may be of nutritional and clinical significance.

1. Introduction

Among the more than 50 species of sialic acids, *N*-glycolylneuraminic acid (Neu5Gc) has been frequently identified in members of the Deuterostome lineage, from echinoderms to mammals.[1–3] However, it was detected neither in microorganisms nor in the few animal species lower than echinoderms, where sialic acids have been found, including insects. There is one exception: *Trypanosoma cruzi*, but this parasite acquires Neu5Gc in addition to *N*-acetylneuraminic acid (Neu5Ac) from the culture medium, since it cannot synthesize this sialic acid itself.[4] Humans and chicken are known exceptions for the regular occurrence of Neu5Gc in higher animals. Man has lost the ability to synthesize this sialic acid during his divergence from the Great Apes about 2–3 million years ago.[5] The reason for this was the loss of a 92 base pair exon of the CMP-Neu5Ac hydroxylase (CMAH) gene, leading to a truncated, enzymatically inactive protein due to a frame shift mutation. CMAH is responsible for the oxidative conversion of Neu5Ac to Neu5Gc requiring the Rieske center, an iron–sulfur cluster, for its activity.[6–8]

The consequences of this defect for human life and development may have been enormous, as discussed by Varki and associates.[9] Microorganisms specifically binding to Neu5Gc on infection were no more hazardous to human life which may have promoted human spreading and development. For example, the major binding protein of *Plasmodium reichenowi*, which causes malaria in Great Apes binds to Neu5Gc and may have threatened ancient man.[10] In contrast, the corresponding protein of *P. falciparum* has a preference for Neu5Ac. Furthermore, an AB_5-toxin secreted by Shiga toxigenic *Escherichia coli* (STEC) and causing gastrointestinal disease, was also found to bind to Neu5Gc.[11]

Although humans can no longer synthesize Neu5Gc, traces of this sialic acid are found in glycans from healthy donors, and higher amounts are found in some tumors.[1,2,12,13] The values vary between less than 1% of the sialic acid fraction and a few percent in some malignant tumors. Evidence for a pathway alternative to the enzymatic oxidation of Neu5Ac by CMAH leading to Neu5Gc has not been found,[14] although metabolic prerequisites exist.[12,15] Different experimental approaches have shown that Neu5Gc can be taken up from orally applied Neu5Gc by humans[16] or rats and mice[17] or from the medium by human cells in culture.[18] It is therefore conceivable that we can acquire Neu5Gc from Neu5Gc-containing foodstuffs, such as red meat, fish, and milk. This is discussed to have pathophysiological consequences and may promote inflammatory diseases including rheumatism and cancer.[9,13,19] The reason is that Neu5Gc is antigenic in humans, leading to the formation of Hanganutziu–Deicher antibodies detectable in variable amounts in blood serum.[12,19]

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Due to the increasing biological and pathological significance of Neu5Gc, which was also shown by inactivation of the CMAH gene in mice,[14] it is important to know whether more vertebrates are unable to synthesize Neu5Gc and whether there is a developmental reason for this. During the early years of analytical determination of sialic acids from a variety of animals, it was found that one of the most important sources for sialic acids, especially Neu5Ac, the mucin glycoproteins from the nests of the swiftlet (*Collocalia fuciphaga* = *Aerodramus fuciphagus*, 'edible bird nest substance, collocalia mucoid') does not contain Neu5Gc.[20–22] It has been known for a long time that glycoproteins from the chicken or other poultry contain only Neu5Ac or only insignificant quantities of Neu5Gc.[23] When, by chance, the analysis of milk of the Australian spiny anteater echidna (*Tachyglossus aculeatus*), which is an early mammal and belongs to the monotremes, proved to lack Neu5Gc,[1] we were prompted to study the platypus (*Ornithorhynchus anatinus*), another still living monotreme, as well as more bird species. The studies were extended to some reptiles and a turtle, because, together with the birds, they belong to the taxon *Sauropsida*. A hypothesis is forwarded that, based on sialic acid and BLAST analysis of the currently available genomic sequence databases, both the sauropsids and the evolutionarily closely related monotremes, which represent ancient mammals, may have lost the capability to synthesize Neu5Gc. Evolutionary aspects and nutritive consequences of this finding will be discussed.

2. Results and discussion

The results of these studies are summarized in Table 1. All taxonomic names are listed in Section 3 ('Animals') and in the Tables. They demonstrate that Neu5Gc is absent in the biological materials analyzed from platypus, most bird and reptile species, as well as from turtle (Figs. 1–4). In two animal species, however, in the egg of the bird, the budgerigar, and in both the egg and tissues of an adult green basilisk, appreciable amounts of these sialic acids were detected by HPLC and confirmed by mass spectrometry. Traces of Neu5Gc, below 1%, were found in some poultry species. In duck intestine, small amounts (about 2%) of the sialic acid fraction were also reported to consist of Neu5Gc.[24] Screening the literature for sialic acid analyses of bird and reptile materials (Table 2) showed, to

Table 1
Sialic acids in tissues, eggs, or mucins from platypus, birds, reptiles, and turtle

Animal	Material or tissue	Neu5Gc	Total sialic acid quantity
Platypus (*Ornithorhynchus anatinus*)	Liver	n.d.[a]	6.4[b]
	Muscle	n.d.	3.5[b]
Chicken (*Gallus gallus*)	Liver	Trace[c]	n.a.[d]
	Muscle	n.d.	n.a.
	Embryonic brain	Trace	n.a.
	Ovomucin	n.d.	n.a.
Duck (*Anatidae*)	Liver	n.d.	n.a..
	Ovomucin	n.d.	n.a.
Turkey (*Meleagrididae*)	Liver	Trace	n.a.
Goose (*Anatidae*)	Liver	Trace	n.a.
	Ovomucin	n.d.	n.a.
Ostrich (*Struthio camelus*)	Liver	n.d.	n.a.
	Ovomucin	n.d.	n.a.
Emu (*Dromaius novae-hollandiae*)	Egg	n.d.	44.3[b]
	Muscle	n.d.	n.a.
Scarlet macaw (*Ara macao*)	Egg	n.d.[*]	6[b]
Budgerigar (*Melopsittacus undulatus*)	Egg[f]	18.8[e,*]	
	Liver[f]	n.d.	3.3[g]
	Muscle[f]	n.d.	5.8[g]
Oriental swiftlet (*Aerodramus/Collocalia species*)	Nest glycoproteins[h]	n.d.	210[g,i]
Swallow (*Hirundo urbica urbica* L.)	Nest glycoproteins	n.d.	n.a.
Green iguana (*Iguana iguana*)	Egg	n.d.[*]	10.3[b]
Agama (*Pogona vitticeps*)	Egg	n.d.[*]	n.a.
Green basilisk (*Basiliscus plumifrons*)	Egg	73[e]	30[b]
	Total freshly hatched animal[j]	n.d.[*]	13[b]
	Liver	33[e,*]	3.4[g]
	Muscle	33[e]	1.6[g]
Anaconda (*Eunectes murinus*)	Liver	n.d.	6.0[g]
Hundred pace viper[j] (*Deinagkistrodon acutus*)	Liver	n.d.[*]	6.0[g]
	Muscle	n.d.	14.7[g]
Taiwan sting snake[j] (*Elaphe carinata*)	Liver	n.d.[*]	20
	Muscle	n.d.[*]	10
Taiwan beauty snake[j] (*Elaphe taeniura*)	Muscle	n.d.[*]	2
Crocodile (*Crocodilus niloticus*)	Muscle	n.d.	n.a.
Turtle (*Cuora amboinensis*)	Liver	n.d.	6.2
	Muscle	n.d.	3.8

[a] Not detectable.
[b] μg sialic acid/mg protein.
[c] Below 0.7% of total sialic acid fraction.
[d] Not analyzed.
[e] Means % total sialic acid fraction.
[f] Egg and tissues were from different animals.
[g] μmoles/g dry tissue.
[h] Three different samples (see Section 3).
[i] Value from Chinese collocalia mucoid.[20]
[j] Relatively large amounts (26–66%) of 7(9)-mono-*O*-acetyl-Neu5Ac were found.
[*] Samples also analyzed by mass spectrometry.

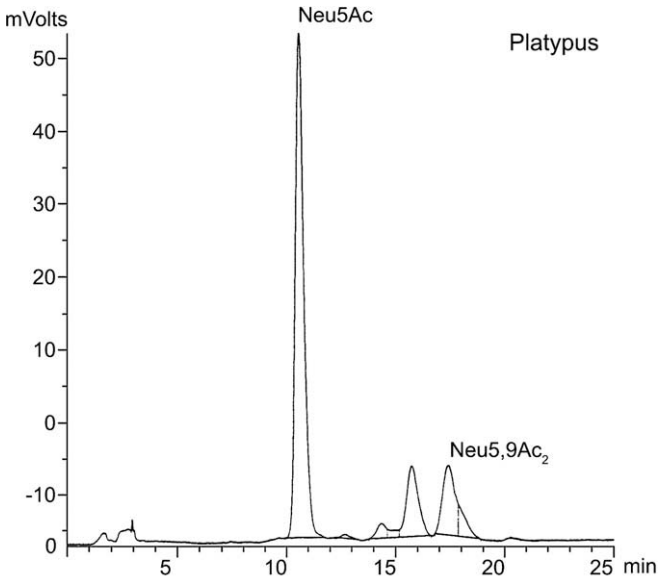

Figure 1. Sialic acid analysis by fluorimetric HPLC of platypus liver. Only Neu5Ac and O-acetylated sialic acids were detected. For methodological details see the text.

our knowledge, that only Neu5Ac has been reported. Neu5Gc has never been reported in larger amounts, with the exceptions mentioned above and in a chicken cell line derived from Marek's lymphoma.[25] Interestingly, the other monotreme species still alive in Australia besides the platypus, the echidna (*T. aculeatus*), also seems to lack Neu5Gc. Sialyllactose of this animal only contains Neu5Ac, as shown by mass spectrometry.[1]

Thus it appears that in monotremes as well as in sauropsids, to which birds, reptiles, and turtles belong, Neu5Gc is rare. Whether the significant relative amounts of Neu5Gc observed in a few cases are de novo biosynthesized in these animals, by the action of the CMP-Neu5Ac hydroxylase or by an alternative pathway, or are taken up from foodstuffs, is not yet known. These possibilities were discussed in Section 1, especially for humans, who usually exhibit only very small amounts of Neu5Gc as a component of their glycoconjugates. It is most likely that both humans and mice and rats can acquire this sialic acid to a low extent from exogenously presented free or bound Neu5Gc. Such a source may also be responsible for the traces of Neu5Gc found in poultry. Whether exogenous Neu5Gc can be accumulated in eggs is speculative, although it appears possible, because eggs cannot synthesize the stored substances by themselves. Both the budgerigar and the basilisks in captivity have access to Neu5Gc-containing food such as cow's milk (glyco-)proteins, fish, or meat, all of which contain Neu5Gc. For example, the Neu5Gc-containing egg investigated originated from a basilisk which had been fed baby mice. Remarkably, a freshly hatched animal from the same clutch of eggs, in one of which Neu5Gc was found, possessed only Neu5Ac (Fig. 4). The adult basilisk (Table 1), which was obtained from a different source, showed both Neu5Ac and Neu5Gc in liver and muscle, and had had access to Neu5Gc-containing food.

A BLAST search against the genomes of the platypus,[34] chicken[35], and zebra finch[36] did not reveal any sequence homologous to the CMP-Neu5Ac hydroxylase. Furthermore, no expression of this hydroxylase gene was found by Southern blot analysis of chicken liver.[37] This explains the absence of Neu5Gc in most of the animals investigated, that is, in birds, reptiles, including the turtle, as well as in the platypus and echidna. However, in order to give more support for the hypothesis that Neu5Gc cannot be synthesized by these animal groups, more chemical, enzymatic, and molecular genetic studies are necessary in more individuals

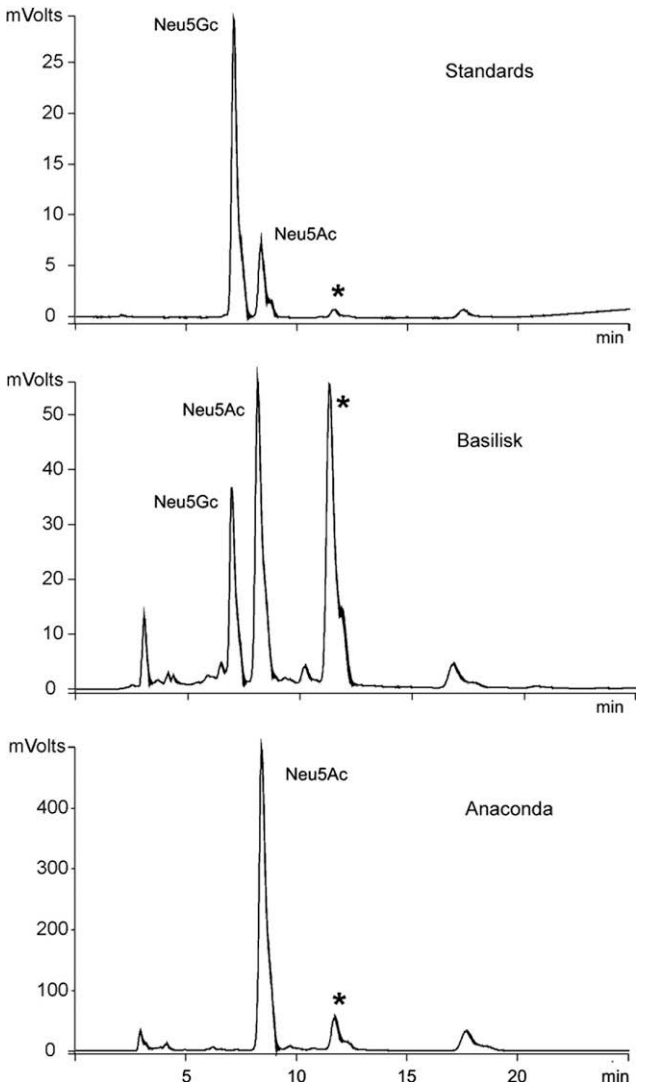

Figure 2. Sialic acid analyses by fluorimetric HPLC of liver from adult green basilisk and anaconda. While the snake only expresses Neu5Ac, the basilisk sample also contains Neu5Gc, which was verified by mass spectrometry. The asterisk indicates a reagent peak. For experimental details see Section 3.2.

and species. One must await genome sequencing of more of these animals, including reptiles, to be able to search for the CMAH gene with the BLAST tool. In the light of the absence of the CMAH sequence in two bird species, that is, the chicken and zebra finch genomes, the finding of Neu5Gc in a budgerigar egg supports the assumption of its nutritional origin. The complete absence of this sialic acid in liver and muscle from an adult budgerigar of the same species supports this assumption. The same may be the case for the basilisk. Surprisingly, in the freshly hatched animal no Neu5Gc was found (Fig. 4).

Birds and reptiles belong to the *Sauropsida*, a systematic group (taxon) of animals derived from the (dino-)saurs and today comprising a very large and diverse group of species. For example, a chicken can be considered as a modern descendant of the dinosaurs.[35] The platypus and echidna are the only remaining members of the taxon *Monotremata*. They represent the first animals, prototherian mammals, which lay eggs and produce milk for their offspring. They diverged from the Therians (*Marsupialia* and *Placentalia*) about 166 million years ago.[34,35] Morphological and molecular genetic research has shown that monotremes have sauropsid-like properties and thus represent a link between sauropsids and modern mammals. The ancestors of the mammals are the synapsids, which

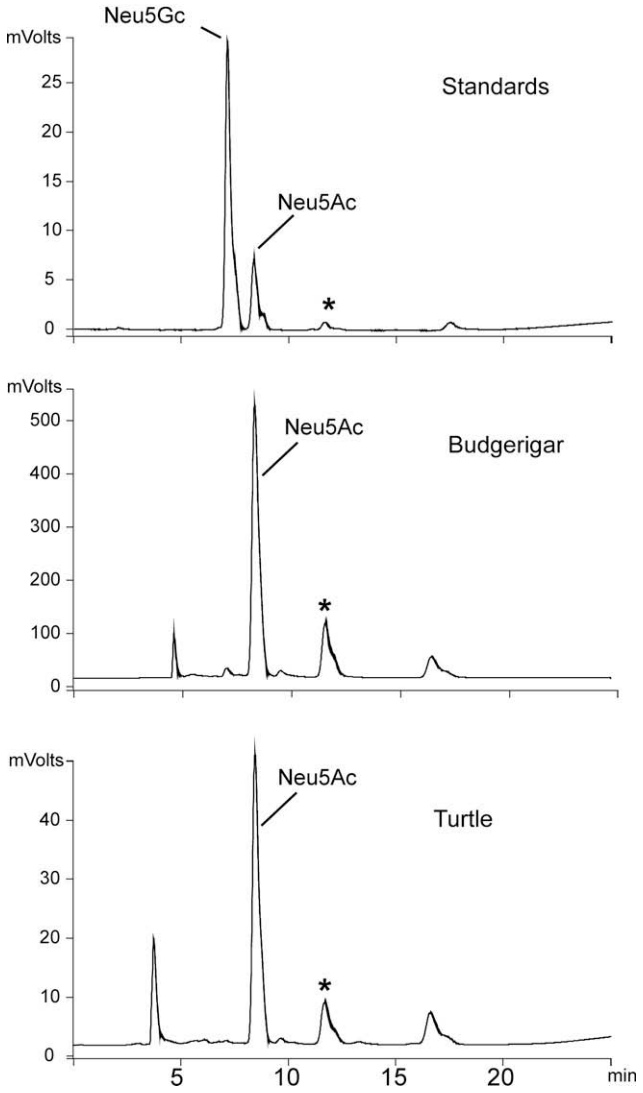

Figure 3. Sialic acid analyses by fluorimetric HPLC of muscle from budgerigar and turtle showing the exclusive presence of Neu5Ac. The asterisk indicates a reagent peak. For experimental details see Section 3.2.

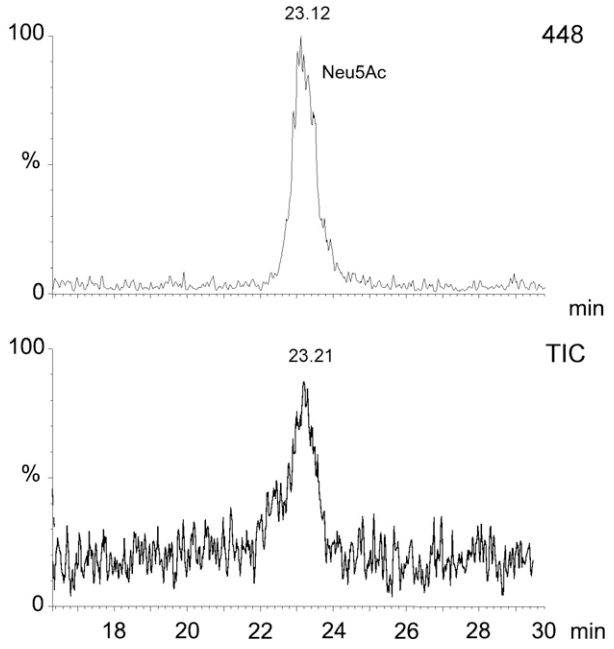

Figure 4. LC–MS detection of the sialic acid isolated from a whole, freshly hatched green basilisk. Comparison of the total ion count (TIC) and reconstruction (with Neu5Ac-specific ion $[M+Na]^+$ at *m/z* 448) chromatograms with those of standard molecules only permitted the identification of Neu5Ac.

separated from animals of the sauropsid lineage around 315 million years ago.[34,35] The platypus and echidna lay eggs like birds and reptiles, and they have a cloaca and many other anatomical and physiological features of reptiles and birds.[34,38] Their reproductive systems show both reptilian origin and mammalian characteristics. It is unique among mammals that the male platypus possesses a venom gland which is reminiscent of that of snakes. These similarities of ancestral bird/reptilia and derived mammalian characteristics are combined in the platypus genome.[34] To this list of similarities the nature of sialic acids can now be added, that is, the absence of Neu5Gc in most of the members of the animal groups investigated and the absence of the CMAH gene in the platypus and two bird species, the only animals of the groups studied in which a BLAST search for the CMAH gene is possible so far. To our knowledge, a reptile genome is not yet available for BLAST analysis. Based on these observations a hypothesis is presented that sauropsids and monotremes do not express Neu5Gc due to the lack of CMP-Neu5Ac hydroxylase.

This low incidence of Neu5Gc in such large animal groups like those of birds and reptiles is striking, since this sialic acid was considered to be the most frequent species after Neu5Ac in vertebrates. Also man so far was considered to be the only mammal in whom Neu5Gc is missing due to a CMAH gene mutation.[8] We now know that there exists at least one more mammal in which Neu5Gc is not expressed, the platypus. The absence of Neu5Gc in echidna milk points in the same direction. We do not know which types of sialic acids were biosynthesized by the (dino-)saurs; however, on the basis of the foregoing discussion it appears likely that they did not express Neu5Gc.

There is another interesting evolutionary point: The hydroxylase CMAH gene is highly conserved from the echinoderms to the mammals with the possible exceptions of the monotremes and

Table 2
Evidence for the exclusive presence of N-acetylated sialic acid in birds and reptiles (collected from the literature or personal communications)

Animal	Material or tissue	References
Oriental swiftlets (Genus *Aerodramus/Collocalia*)	Mucin	20–22
Pigeon (*Columba livia*)	Egg white	26
Cobra (*Najanaja kaouthia*)	Venom	27,28
Viper (*Agkistrodon halys brevicanus stejneger*)	Venom serine protease	29
Chinese silky fowl (*Gallus gallus domesticus brissoon*)	Egg yolk	30
Chicken (*Gallus gallus*)	Eye lens gangliosides	31
Chicken	Embryonated egg Chorioallantoic and amniotic cells	32
Chicken	Colon epithelial cells	33
Quail (*Coturnix japonica*)	Colon epithelial cells	33
Crocodile	Egg	G. Strecker, personal communication

sauropsides. It is shown in Figure 5 that amphibians express Neu5Gc, for example, in oviductal mucins of the fire-bellied toads (*Bombina* species)[39] and the alpine newt (*Triturus alpestris*),[40] as well as the *Therian* mammals, that is, the *Marsupialia* and the *Placentalia*, which follow the monotremes in evolution.[34,38] For example, the sialic acid fraction of the kangaroo was found to contain Neu5Gc (R. Schauer, unpublished), and in the opossum genome the CMAH gene can be detected.[41] All of these studies show that the CMAH activity seems to have been lost in sauropsids, monotremes, and humans.

The sialic acid analyses reported here are of interest not only from an evolutionary point of view, but also from a nutritional aspect. It was mentioned in the introduction that Neu5Gc represents an antigen in humans, which can be acquired from food stuffs such as fish, milk products, and 'red' meat (bovine, ovine, porcine, and caprine) and can be incorporated into cellular glycans. The relative Neu5Gc amounts in fish are variable, although more analytical studies are necessary to unequivocally evaluate this from nutritional aspects. A. Varki and co-workers provided evidence that this may lead to immunoreactions causing inflammation and possibly cancer.[19] The consequence of this would be to prefer 'white' meat or eggs, from poultry, for example, in the diet.

3. Experimental

3.1. Animals

Muscle and liver samples from the platypus (*O. anatinus*) were obtained from a sanctuary with the permission of the Ministry of Natural Resources and Environment of Victoria (Australia) (Permit No. RP-97-193 under the *Wildlife Act* 1975). The tissues were taken from an animal which had to be killed and were sent in dry ice.

Liver samples were taken from chicken (*Gallus gallus*), duck (*Anatidae*), goose (*Anatidae*), turkey (*Meleagrididae*), and the African ostrich (*Struthio camelus*) acquired from local farmers. Chicken embryonic brain was also analyzed on day 15. Eggs from the Australian emu (*Dromaius novae-hollandiae*), the parrot scarlet macaw (*Ara macao*), and the budgerigar (*Melopsittacus undulates*) were obtained from the Gettorf Zoo, Germany. From the budgerigar liver and muscle samples were also prepared, and emu meat (muscle) was bought on a market in Australia. In addition, mucin was extracted from the nest of the European swallow (*Hirundo urbica urbica* L.) after breeding and was purified from debris by centrifugation before sialic acid analysis. Samples of edible birds nest glycoproteins (*Collocalia* mucoid) from Chinese, Indonesian, and Vietnamese swiftlets (*Collocalia/Aerodramus* species), respectively, were also studied.

A muscle sample of the African crocodile (*Crocodilus niloticus*) was obtained from a restaurant in the Netherlands. An adult specimen of the green basilisk (*Basiliscus plumifrons*), which had died from drowning, was obtained in deep-frozen state from Hagenbeck Zoo, Hamburg (Germany). An egg from the same reptile species, as well as a freshly hatched animal, was made available by Johannes Udart, Kiel. He also provided an egg of the bearded agama (*Pogona vitticeps*). Eggs from the green iguana (*Iguana iguana*) were obtained from Winfried Rathje, Kiel.

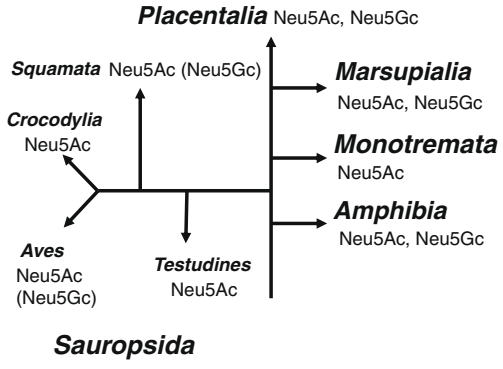

Figure 5. Phylogenetic tree of higher vertebrates and mammals and distribution of Neu5Ac and Neu5Gc. For detailed discussion see the text.

Muscle and liver from four snake species were investigated. Samples from the Taiwan stink snake (*Elapha carinata*), Taiwan beauty snake (*Elaphe taeniura*), and the hundred-pace viper (*Deinagkistrodon acutus*) were sent in freeze-dried form by Professor Albert Wu, Kweisan (Taiwan). Tissue of the anaconda (*Eunectes murinus*) was provided deep-frozen by the Gettorf Zoo.

An adult turtle specimen (*Cuora amboiuensis*) was obtained deep-frozen from Hagenbeck Zoo, Hamburg.

When whole animals were provided, the tissues were prepared by Dr. Heinrich Luttmann, Institute of Zoology, University of Kiel, and immediately frozen before analysis.

It has to be stated that no animal was killed for the purpose of this study; all tissue samples were obtained from animals that died for other reasons.

3.2. Methods

Eggs, after careful removal of the shells, and edible birds nest glycoprotein probes were homogenized in an ultraturrax homogenizer for 1 min with cooling on ice. Fresh or frozen liver and muscle tissue samples were homogenized in an appropriate volume of water using the same instrument at higher setting for 3 min with cooling. The homogenates were either lyophilized or directly processed. Protein concentration was estimated colorimetrically using the micro-BCA assay kit from Pierce (Rockford, IL, USA) in microwell plates according to the manufacturer's instructions. The values were related to a calibration curve with BSA standards for 0–20 μg protein.

Sialic acid analyses were carried out in the total homogenates in order not to miss any sialic acid type present in the biological sources. The homogenates were mixed with equal volumes of 4 M propionic acid giving 2 M acid end concentration, and vortexed for a short time, and the sialic acid glycosidic bonds hydrolyzed for 4 h at 80 °C.[42] Alternatively, hydrolysis was performed in 0.1 N HCl at 80 °C for 1 h.[43] After cooling the hydrolysates were dialyzed in dialysis bags at a cut-off value between 12 and 14 kDa against three times 10-fold volumes of water with gentle magnetic stirring in the course of 12 h. The dialysates were concentrated by rotary evaporation, followed by lyophilization. In order to obtain exact analytical data and to avoid false-positive results, for example, by HPLC, the lyophilized sialic acids, dissolved in 1 mL water, were purified by ion-exchange chromatography. The sequential cation- and anion-exchange chromatographies on Dowex 50 and Dowex 2X8, respectively, have been described in detail.[43] The sialic acid samples were dried before analysis.

The recent analyses of tissue samples from budgerigar, basilisk, snakes, and turtle were carried out as follows: 10 mg of dried tissues were homogenized in water and incubated in 1 mL of 0.1 M TFA at 80 °C for 2 h. The samples were centrifuged at 5000 RPM for 15 min to remove insoluble material, and four volumes of cold ethanol were added to the supernatants. After centrifugation, the ethanolic supernatants were dried, solubilized in water, and successively passed through 50 × 2 (200 × 400 mesh) and 50 × 8 (25 × 50 mesh) Dowex columns (1 mL) (BioRad, Marnes-la-Coquette, France). The columns were eluted with three column volumes of water, concentrated to 200 μL and analyzed by fluorimetric HPLC as follows.

Sialic acids were analyzed both quantitatively and qualitatively in different ways using Neu5Ac and Neu5Gc standards. For quantitative analysis the colorimetric orcinol/ferric ion/HCl assay,[43]

known as the 'Bial'-assay, was microwell adapted and carried out in 50-μL volumes each. The microwells were read at 572 nm. Neu5Ac at a concentration range between 2 and 10 μg served as reference. HPLC also allowed for the quantitative estimation of the sialic acid after proper calibration. The sialic acid types present in the biological sources, especially with regard to the occurrence of Neu5Gc, were analyzed by TLC, HPLC, and HPLC coupled with mass spectrometry (LC–MS). Sialic acids were chromatographed on cellulose thin-layers and developed in 1:2:1 butanol–propanol–0.1 N HCl, which achieved good separation of Neu5Ac and Neu5Gc. Staining was done with diluted Bial reagent.[43]

For HPLC analysis sialic acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB, Dojindo Laboratories, Tokyo, Japan) according to Hara et al.[44] and were separated isocratically on a C_{18} reversed phase (RP) HPLC column (250 × 4.6 mm, 5 μm) by a solvent mixture of 7:9:84 acetonitrile–methanol–water and were identified by referring to the elution positions of standard Neu5Ac and Neu5Gc derivatives or by co-chromatography with these standards. DMB-derivatized sialic acids were also analyzed by LC–MS using a Hewlett–Packard Model 1100 liquid chromatography (Palo Alto, USA) coupled to a Quattro II tandem quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray-ionization (ESI) source and equipped with a Micromass MassLynk data system. Separations were achieved on a C_{18} reversed-phase (RP) HPLC column (250 × 4.6 mm, 5 μm) with identical conditions as described above. The column eluent was first directed to a UV detector set at 206 nm and then to a pneumatically assisted ESI interface operating with a capillary voltage at 3.2 kV and temperature at 110 °C. The cone voltage was maintained at 60 V. The mass spectrum was acquired from *m/z* 250 to 800 with a scan duration of 3 s and a scan delay of 0.1 s.

Some sialic acid samples were analyzed by the GC–MS method of Zanetta et al.[45] after volatilization of the monosaccharides by methyl esterification using diazomethane and by derivatization using heptafluorobutyric anhydride.

The specificity of the sialic acid analyses, especially for the more indirect methods such as TLC and HPLC, was increased by enzymatic degradation of sialic acids by Neu5Ac-pyruvate lyase[43] and running this assay in parallel. Decline of the corresponding peaks confirmed their sialic acid nature.

A BLAST search according to Altschul et al.[46] was carried out for the identification of significant homology to the CMP-Neu5Ac hydroxylase of the genomic databases from the platypus,[34] chicken[35], and zebra finch (*Taeniopygia guttata*),[36] using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast/). The *Asterias rubens*[47] CMP-Neu5Ac hydroxylase protein sequence, which is highly conserved throughout the deuterostome lineage, was used as reference.

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