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Note

Differences in the amount of *N*-acetyl- and *N*-glycoloyl-neuraminic acid, as well as *O*-acylated sialic acids, of fetal and adult bovine tissues*

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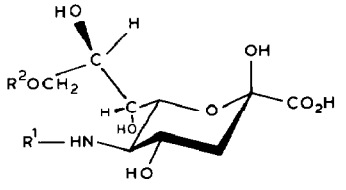
About 25 derivatives of neuraminic acid have been found to occur glycosidically linked to other sugars[1,2], *N*-acetyl- (**1**) and *N*-glycoloyl-neuraminic acid (**2**) being the most widespread species. The expression of *N*-acetyl- (**1**) and *N*-glycoloyl-neuraminic acid (**2**) seems to be developmentally regulated in mammalian tissues, as variable relative amounts of *N*-glycoloylneuraminic acid were found in bovine serum[3,4], gangliosides of rat small intestine[5], and rat colon[6]. In all cases, adult animals have a higher percentage of *N*-glycoloylneuraminic acid than young animals. As fetal bovine tissues have not been investigated so far, we compared the relative contents of *N*-acetyl- (**1**) and *N*-glycoloyl-neuraminic acid (**2**) in corresponding tissues from fetal and adult cows, respectively.



1 $R^1 = COCH_3, R^2 = H$
2 $R^1 = COCH_2OH, R^2 = H$
3 $R^1 = COCH_3, R^2 = COCHOHCH_3$

Scheme 1. Structures of the sialic acids.

The total amount of sialic acids per g of dry weight of the different materials analyzed was higher for samples from fetus than for those from adult cow, ranging from a 1.12-fold excess in cerebellum to 24 times as much in breast muscle (Table I). In all tissues, *N*-acetylneuraminic acid (**1**) was present. The percentage of *N*-glycoloylneuraminic acid (**2**) was significantly higher in the samples from adult animals with a maximal

* Dedicated to Professors Nathan Sharon and Toshiaki Osawa.

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TABLE I

Total amount of sialic acid, *N*-glycoloylneuraminic acid, and percentage of *N*-glycoloylneuraminic acid in various bovine tissues from adult cow and fetus

Source	*Sialic acids (mg)/dry weight (g)*		*Amount of Neu5Gc*			
	Cow	*Fetus*	*Cow*		*Fetus*	
			%	μg/g	%	μg/g
Breast muscle	0.1	2.4	45	45	0	0
Parotis	1.0	12.5	63	630	24	3000
Thymus	0.2	1.1	66	132	49	539
Brain stem	1.8	5.6	22	396	6	336
Heart muscle	0.4	1.2	48	192	24	288
Kidney	2.0	5.3	43	860	12	636
Liver	0.8	2.0	59	472	23	460
Paunch	0.6	1.1	53	318	13	143
Neocortex	3.1	5.5	10	310	5	275
Abomasum	2.0	3.1	46	920	13	403
Colon	1.3	1.9	65	845	15	285
Small intestine	1.2	1.6	59	708	35	560
Cerebellum	2.5	2.8	9	225	5	140

difference in breast muscle where no *N*-glycoloylneuraminic acid (**2**) was found in fetus but 45% in cow. The other values ranged from a 1.35-fold excess in thymus to a 4.33 times higher percentage of *N*-glycoloylneuraminic acid (**2**) in colon. As an example, the thin-layer chromatograms together with the densitometric curves of sialic acids from adult and fetal bovine liver are shown in Fig. 1.

Remarkably, the relative amount of *N*-glycoloylneuramic acid (**2**) is always lower in fetal tissues when compared to adult tissues, and in most cases also the absolute quantity, expressed as μg of *N*-glycoloylneuraminic acid per g of dry weight. However, in parotis, thymus, and heart muscle, its absolute quantity is higher than in the

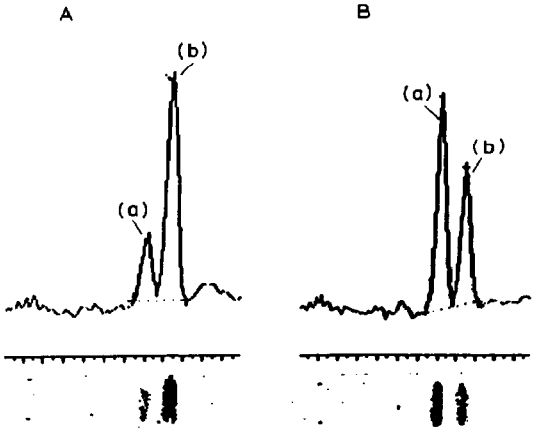

Fig. 1. Thin-layer chromatograms and densitometric curves of sialic acids from liver of calf fetus (A) and adult cow (B): (a) *N*-glycoloylneuraminic acid (**2**) and (b) *N*-acetylneuraminic acid (**1**).

corresponding tissues of adult animals, as can be seen from the data given in Table I. In liver and brain stem, the absolute amounts of *N*-acetyl-(**1**) and *N*-glycoloyl-neuraminic acid (**2**) are similar. These observations are in agreement with data from serum of newborn calf and adult cow, which showed a decrease of total sialic acid content and an increase of the relative amount of *N*-glycoloylneuraminic acid (**2**) with growth of the calf[4].

In the sialic acid fractions from adult bovine tissues, except submandibular gland[7], significant quantities of sialic acids other than *N*-acetyl- (**1**) or *N*-glycoloyl-neuraminic acid (**2**) were not found; only small amounts (<5%) of *N*-acetyl-9-*O*-lactoylneuraminic acid (**3**) were identified by mass spectrometry (see Fig. 2) in bovine tissues, *i.e.*, cerebellum, neocortex, and brain stem. Also, most fetal tissues contain only *N*-acetyl- (**1**) and *N*-glycoloyl-neuraminic acid (**2**); in addition, small amounts (<5%) of *N*-acetyl-9-*O*-lactoylneuraminic acid (**3**) were discovered in fetal liver and parotis. In the samples from fetal brain, however, relatively high proportions of this sialic acid ester were present, with 16% in brain stem, 13% in neocortex, and 14% in cerebellum. Thus, where it is present at all, fetal tissues have a higher percentage of *N*-acetyl-9-*O*-lactoylneuraminic acid (**3**) than the corresponding tissues from adult cow. *O*-Acetylated sialic acids were only found in submandibular glands from cow and fetus. They represent 54% of the total sialic acid fraction of adult cow, in addition to 25% of *N*-acetyl-(**1**) and 21% of *N*-glycoloyl-neuraminic acid (**2**). The corresponding fetal tissue contains significantly less *O*-acetylated sialic acids (15%), together with 64% of *N*-acetyl-(**1**) and 21% of *N*-glycoloyl-neuraminic acid (**2**).

Some preparations also revealed the presence of low amounts (<5%) of the unsaturated sialic acid, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid, by mass-spectrometric analysis. This compound, which cannot occur in glycosilic linkage, was considered as an artifact possibly from the CMP-glycoside of *N*-acetylneuraminic acid[8], or formed during preparation and isolation of sialic acids, especially as its relative amount was low and variable in different preparations from the same source.

The biological function of the observed higher sialic acid content and the lower percentage of *N*-glycoloylneuraminic acid (**2**) in calf fetus reported here or in calf serum reported earlier[4] is not known. Similar to the increased amount of sialic acids found in the embryonic bovine tissues, tumor tissues often have a higher sialic content then normal materials[9–11], possibly due to a higher activity of sialyltransferase[12]. One of the reasons for this may be that D-galactosyl nonreducing terminal sugar groups on cell surface glycoconjugates seem to be responsible for inhibition of cell spreading in culture[13] and, thus, masking of this group by a sialic acid unit might trigger cell growth that is essential for both, embryonic and tumor tissues. Another reason may be the immunosuppresive effect of increased sialylation, which also may be of advantage for embryonic and tumor growth[4,14].

Conversion of *N*-acetylneuraminic acid (**1**) to the corresponding *N*-glycoloyl derivative (**2**), which occurs by the exclusive action of *N*-acetylneuraminate monooxygenase (EC 1.14.99.18)[15] on CMP-*N*-acetylneuraminic acid[16], (a finding later confirmed by other authors[17]), renders the sialyl group less susceptible to the action of the catabolic enzymes, sialidase (EC 3.2.1.18) and *N*-acetylneuraminate lyase (EC 4.1.3.3)[14]. Further-

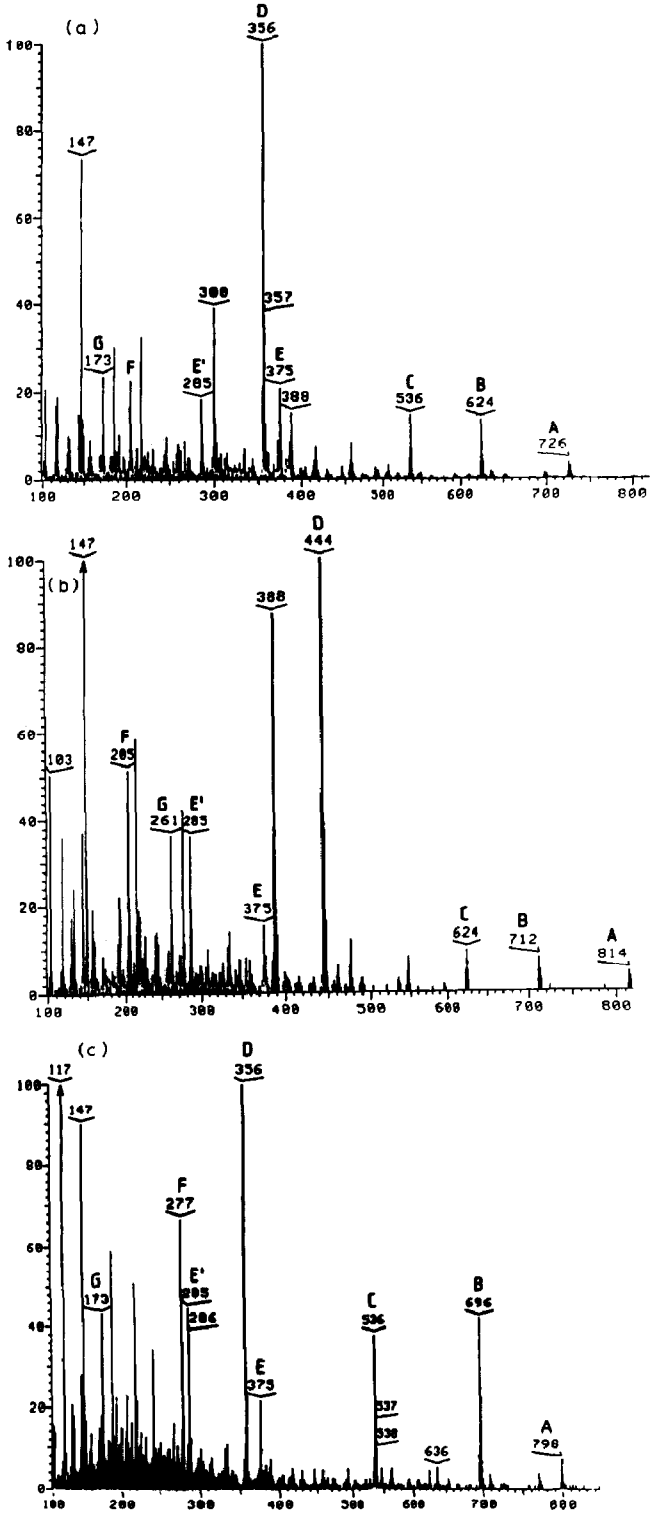

Fig. 2. Mass spectra of the sialic acids found in various tissues from adult cow and calf fetus analyzed as per-*O*-trimethylsilylated derivatives: *N*-Acetyl- (1) (a), *N*-glycoloyl- (2) (b), and *N*-acetyl-9-*O*-lactoyl-neuraminic acid (3) (c). The characteristic fragment ions[28,29] are indicated. The structures are given in Scheme I.

more, interactions with specific receptors, *e.g.*, those recognizing D-galactose, that might also be involved in growth regulation of embryonic tissues, are weaker when *N*-glycoloyl- (**2**) instead of *N*-acetyl-neuraminic acid (**1**) is present[18].

The biological function of *O*-lactoylation is also not yet understood; even the metabolism of this functional group is not known. As this ester substituent is not released by the action of sialate 9-*O*-acetylesterase[19], the presence of a lactoyl residue might offer further advantages for an organism in increasing the life time of the corresponding sialoglycoconjugates. For example, it would prevent the rapid catabolism of this sialic acid derivative and in consequence the recognition of the originally penultimate galactosyl residue by specific receptors[20] and enzymic degradation by glycosidases. As *O*-lactoylation has been found in significant proportions only in fetal brain tissues, it is considered as a further differentiation marker. *N*-Acetyl-9-*O*-lactoyl-neuraminic acid (**3**) has so far been observed in bovine submandibular gland[21], human saliva[22], human nasal mucin[23], and human stomach[24]. The finding of *O*-acetylated sialic acids only in submandibular gland and the low amount in the corresponding fetal tissue is remarkable. Increase with age of the relative amount of *O*-acetylated sialic acids has been observed in chicken erythrocytes[25] and rat colon[6].

In conclusion, it may be rewarding to investigate the way in which the outfit of bovine embryonic tissues having high amounts of sialic acids, especially *N*-acetyl-neuraminic acid (**1**), may be of advantage for rapid growth and cell differentiation. Such studies could provide more insight into the regulation of the biosynthesis of sialic acids in general, and especially of *N*-glycoloylneuraminic acid (**2**), in which the activity of *N*-acetylneuraminate monooxygenase is involved, as well as of *N*-acetyl-9-*O*-lactoyl-neuraminic acid (**3**) and *O*-acetylated sialic acids. The present experiments also show that the expression of these types of sialic acid is tissue specific, as is known from other studies[6], and give support to the assumption that the monooxygenase activity is regulated in a tissue-specific manner.

EXPERIMENTAL

Isolation of sialic acids. — Calf foetus (75-cm long) and materials from adult cow were obtained from the local slaughterhouse. Tissues (3–17 g wet weight) were homogenized in water (10–20 mL) with and Ultraturrax (Janke & Kunkel, Oberstauffen, F.R.G.) and hydrolyzed by the two-step acid hydrolysis procedure described for the liberation of sialic acids, including *O*-acylated derivatives[26]. In brief, conc. formic acid was added to the samples to adjust the pH to ~2, and the material was kept for 1 h at 70°. Following dialysis (4 × 100 mL of water) at 4°, the pH of the retentates was adjusted to 1 by addition of 20% HCl, and the contents were hydrolyzed for 1 h at 80°, and dialyzed again as described above. The dialyzates from both hydrolysis procedures were kept separately, concentrated by rotary evaporation, and lyophilized. The residues were purified by passage through cation- and anion-exchange resins as described by Schauer[26]. Adsorbed sialic acids were eluted from the latter resin with M formic acid. After concentration and lyophilization of the sialic acid-containing eluate, the samples were analyzed.

Sialic acid analysis. — The amounts of sialic acids were related to the dry weights of the corresponding materials. The weights were determined after lyophilization of appropriate amounts of the tissues. Colorimetric quantification of sialic acids was carried out by the orcinol–Fe^{3+}–HCl test or the periodic–thiobarbituric acid assay, with and without preceding alkaline hydrolysis of possibly occurring *O*-acyl groups[26]. H.p.l.c. of sialic acids was performed on Aminex A-29 with 0.75mM Na_2SO_4 as eluent and detection[27] at A_{200}.T.l.c. was performed on 0.2-mm cellulose-covered plastic sheets (Merck, Darmstadt, F.R.G.) with development in 2:1:1 (v/v) propanol–butanol–0.1M HCl, followed by detection with the orcinol–Fe^{3+}–HCl spray reagent[26]. The bands on these chromatograms were quantified and, thus, the relative amounts of sialic acids were estimated by densitometric analysis of the plates using a CD60 densitometer (Desaga, Heidelberg, F.R.G.) and the corresponding software for integration.

G.l.c.–m.s. of the per-*O*-trimethylsilylated samples was performed in a WCOT capillary column with CP-Sil 5 (Chrompack, Müllheim, F.R.G.) in a Varian 3700 gas chromatograph at a carrier gas-flow rate of 1 mL/min, coupled to a Varian MAT 44S/SpectroSpin SS220 mass spectrometer combination, essentially as described[28]. The mass spectra were interpreted on the basis of the well established fragmentation scheme published earlier[28,29].

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