

Variability in Brain Ganglioside Composition: A Further Molecular Mechanism Beside Serum Antifreeze-Glykoproteins for Adaptation to Cold in Antarctic and Arctic-Boreal Fishes

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Summary. Gangliosides and sialoglycoproteins from brain, liver and muscle have been isolated from 6 Antarctic fish species from the suborder Notothenoids and from 4 Arctic-boreal fish species. In addition freezing and melting points from serum of both groups were examined in order to determine the presence of protein antifreezes. In comparison with eurythermic fishes of temperate climates in both groups the phylogenetical adaptation to cold is correlated with a significantly higher concentration of gangliosides in the brain. The ganglioside concentration of liver in Antarctic fish, but not in Arctic species, is 3 to 5fold higher than in mammals (rat); in muscle the ganglioside content is increased only in red-blooded Antarctic fish as compared with mammals. The concentration of neuronal sialo-glycoproteins generally is lower in Antarctic fish than in other marine teleosts; in muscles the content is 2 to 3fold higher than in mammals. The molecular composition of brain gangliosides is characterized by an extreme high polarity which is due to an equipment with highly sialylated fractions (40 to 50% higher sialylated than tetrasialogangliosides). There are distinct differences between the freezing and melting point of blood serum, especially in the Antarctic species in favour of the existence of protein antifreezes. The results are discussed with regard to the fact that the extremely high polarity of brain gangliosides reflects a very efficient mechanism on molecular level to keep the neuronal membrane functional under low temperature conditions.

Introduction

The fish fauna inhabiting Antarctic waters became isolated by the establishment of a circum-antarctic ocean

circulation in the mid-oligocene, about 25 million years ago (Kennett 1977).

During this period the suborder of Notothenoidei became the dominant group of Antarctic fishes: Of 120 species 100 species (83%) are endemic, and 75 of these are Notothenioids (De Witt 1971). The evolution of Antarctic fishes and their final distribution has been strongly influenced by low temperature and extensive areas of perennial sea ice (Andriashev 1970). The present sea water temperatures in Antarctica range from about 4°C in the vicinity of the Antarctic Convergence (Holm-Hansen et al. 1977), to -1.9°C in deep coastal basins (Andriashev 1977). These temperature limits correspond closely to the thermal tolerance limits for the Antarctic species (-2.5 to +6°C; Somero and De Vries 1967; Wells 1979).

This is in contrast to the corresponding waters of the northern hemisphere, where such an isolation is not very well expressed and where water temperature can change markedly in the course of the season.

Life in both environments requires adequate adaptations, which may comprise biochemical, physiological and behavioral strategies. One expects a more homogeneous and rigid set of biochemical tools well adapted to low temperature as a basis for the life in the temperature-constant Antarctic Ocean and a more heterogeneous and flexible set in regions with marked temperature changes. This hypothesis could be verified by investigations on several peculiarities in Antarctic and Arctic-boreal fish species, e.g. concerning hematology and freezing resistance.

It is well known that the hematocrit and the concentration of hemoglobin in fish depend on the environmental temperature and oxygen levels (Raschack 1969): Low hematocrit and low hemoglobin (even loss of hemoglobin and erythrocytes) correlate with low temperature and corresponding high oxygen levels. These parameters which are seasonally variable in northern latitudes were found to be relatively constant in the Antarctic. Thus a

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constantly low hematocrit is characteristic for Antarctic fish species and is expressed extremely in the white blooded fish family Channichthyidae, lacking completely red blood cells hemoglobin.

Another peculiarity in Antarctic and some Arctic-boreal fish is the development of a freezing resistance due to freezing point depressing peptides and proteins (De Vries 1983; De Vries and Lin 1977; Schneppenheim and Theede 1982). The synthesis of such macromolecules in fish was shown to be correlated with winter conditions in Arctic-boreal waters, whereas antifreeze synthesis in Antarctic fish is not seasonally dependent (Duman and De Vries 1974).

Neurophysiological changes associated with temperature adaptation of temperate animals have been described many times (for review: Lagerspetz 1974; Prosser and Nelson 1981). Far less is known about neurophysiological mechanisms of cold adaptation in Antarctic poikilotherms. Only Macdonald (1981) and Macdonald and Montgomery (1982) reported that in Antarctic animals, propagation of action potentials persists down to the point at which cell membranes are disrupted by freezing of body fluids, that conduction velocity changes less with temperature than in warm-adapted animals, and that the upper thermal limit of spike activity is lowered. These phenomena can be observed also in short-term cold adaptation of temperate poikilotherms. Therefore it seems likely that the underlying mechanisms of adaptation are quite similar in both groups.

Gangliosides (sialic or neuraminic acid-containing glycosphingolipids) are essential constituents of the cell membranes, especially of synaptic terminals. In vertebrates they are possibly involved in specific neuronal functions such as synaptic transmission (Rahmann 1983; Rahmann et al. 1976, 1982; Römer and Rahmann 1979) and adaptive neuronal processes in response to environ-

Table 1. Systematics of the Antarctic and list of the Arctic-boreal fish species investigated in this study

| | |
|-------------------------|--|
| Antarctic | |
| Suborder Notothenoidei | |
| Family Nototheniidae: | |
| | <i>Trematomus eulepidotus</i> Regan, 1914 |
| | <i>Trematomus hansonii</i> Boulenger, 1902 |
| Family Bathyracidae: | |
| | <i>Racovitzia glacialis</i> Dollo, 1900 |
| | <i>Prionodraco evansii</i> Regan, 1914 |
| Family Channichthyidae: | |
| | <i>Chionodraco rastrospinosus</i> |
| | De Witt and Hureau, 1979 |
| | <i>Chaenodraco wilsoni</i> Regan, 1914 |
| Arctic-boreal | |
| | <i>Myoxocephalus scorpius</i> (= sculpin) |
| | <i>Aspitrigla obscura</i> (= gunard) |
| | <i>Gadus morhua</i> (= cod fish) |
| | <i>Pleuronectes platessa</i> (= plaice) |

mental temperature changes (Breer and Rahmann 1976; Hilbig et al. 1979; Hilbig and Rahmann 1979; Rahmann 1978, 1980, 1981, 1982, 1983; Rahmann and Hilbig 1980, 1981, 1983; Geiser et al. 1981; Rösner et al. 1979; Rahmann et al. 1984): Distinct correlations between the molecular ganglioside composition and the state of thermal adaptation of temperate vertebrates had been discovered with the general tendency that "the lower the environmental (\triangleq body) temperature – the higher is the polarity of brain gangliosides".

On this basis the present study should be an approach to explain an adequate mechanism on molecular level of neuronal membranes in Antarctic and Arctic-boreal fish species, seen in context with the two different environments. In addition to this the resistance to freezing, expressed by the presence of protein antifreeze, should

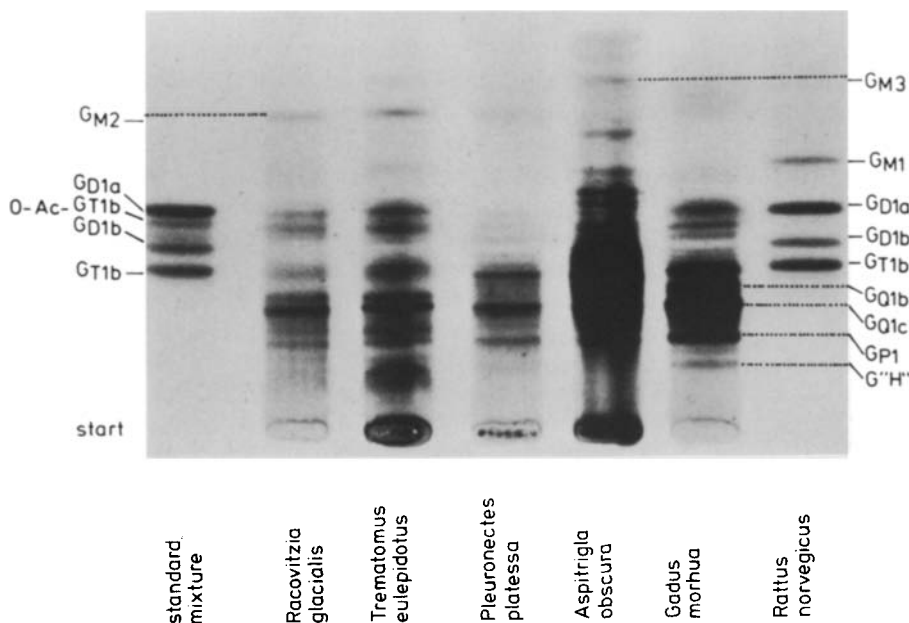


Fig. 1. Brain ganglioside pattern (HP-TL chromatogram) of two Antarctic fishes, three Arctic-boreal fishes (rat and standard mixture for identification of fractions)

Table 2. Freezing point (fp) and melting point (mp) determination in the blood of Antarctic and Arctic-boreal fish. D = difference between mp and fp; n = number of individuals; temperatures are given in °C. SD are about ±0.04°C for mp and ±0.1–0.2°C for fp

| Species | mp | fp | D | n |
|---------------------------------|-------|-------|------|---|
| <i>T. eulepidotus</i> | -0.82 | -1.84 | 1.02 | 4 |
| <i>T. hansonii</i> | -0.90 | -1.85 | 0.95 | 9 |
| <i>R. glacialis</i> | -0.86 | -1.78 | 0.92 | 3 |
| <i>P. evansi</i> | -0.88 | -1.62 | 0.74 | 3 |
| <i>Chio. rastrorpinosus</i> | -0.88 | -1.68 | 0.80 | 7 |
| <i>Chaeno. wilsoni</i> | -0.92 | -1.72 | 0.80 | 2 |
| <i>M. scorpius</i> ^a | -0.74 | -1.44 | 0.70 | 9 |
| <i>P. platessa</i> ^a | -1.10 | -1.88 | 0.78 | 3 |

^a Repo et al., unpublished

be determined in order to outline the ability of fish to live at temperatures even below 0°C.

Material and Methods

Collection of Fishes

The material for this study derived from two German Antarctic expeditions: *Trematomus hansonii* and *Chionodraco rastrorpinosus* from a fishery research cruise with *FRV Walther Herwig* in 1977/78, the other species from a "Pre Site Survey" for the first German Antarctic base with *MS Polarsirkel* in 1979/80.

The fishing areas were quite different; the *Walther Herwig* material had been caught in subantarctic and periantarctic waters, the material from the *Polarsirkel* in the very extreme south of the Weddell Sea at the

Filcher/Ronne ice shelf. All of these species belong to three families of the suborder Notothenoidei (Table 1).

The species from the Weddell Sea lived at constant temperatures near the freezing point of seawater (-1.85°C), whereas the species caught on the *Walther Herwig* cruise lived in slightly "warmer" waters (0–2°C). The four Arctic-boreal species were caught in the North Sea.

The homeotherm *Rattus norvegicus* completed the list of species, now representing three different strategies of thermal adaptational concepts: cold stenothermy in Antarctic fish, relative eurythermy in Arctic-boreal fish and homeothermy in the rat.

Freezing and Melting Point Determination

Immediately after the catch blood was taken from the heart of the fish using heparinized capillaries. The capillaries were centrifuged and the supernatant was taken for freezing and melting point determination, by means of a microcryoscope as described by Schneppenheim and Theede (1982). The fish was then frozen and stored at -20°C for transport until ganglioside investigation.

Ganglioside Analysis

The brains of all species investigated were dissected at 0°C and homogenized in 5 ml of distilled water at 4°C. The homogenates were lyophilized and extracted according to Svennerholm and Fredman (1980). Total lipid-bound sialic acid was determined according to Svennerholm (1957). The delipidized residues were hydrolyzed at 80°C for 2 h in 0.5 M H₂SO₄ to liberate proteinbound sialic acid, and centrifuged for 20 min at 5,000 g. Aliquots of the supernatants were taken for the determination of sialic acid according to Jourdian et al. (1971). Aliquots of purified gangliosides containing about 5 µg sialic acid (e.g. N-acetylneuraminic acid, NeuAc) were separated by thin-layer chromatography on pre-coated silica gel plates (HP-TLC; 0.2 mm; Merck, Darmstadt, FRG). Two different solvent systems were used: Chloroform-methanol-12 mM MgCl₂-33% NH₄OH (60:35.5:7.5:0.5; v/v/v/v) and chloroform-methanol-12 mM MgCl₂ (60:30:8; v/v/v; Rösner 1981). Each sample was chromatographed sequentially in either system.

Table 3. Concentration of ganglioside- and proteinbound sialic acid in brain, muscle and liver of Antarctic and Arctic-boreal fishes. n.d. = not determined

| | Species | Concentration of NeuAc (µg/g fresh wt) bound to | | | | | |
|--------------------------|-----------------------------------|---|----------|----------|-----------|----------|----------|
| | | Gangliosides | | | Proteins | | |
| | | Brain | Muscle | Liver | Brain | Muscle | Liver |
| Antarctic fish | White blooded | | | | | | |
| | <i>Chionodraco rastrorpinosus</i> | 466 ± 58 | 5 ± 2 | 177 ± 82 | 516 ± 63 | 148 ± 17 | 260 ± 28 |
| | <i>Chaenodraco wilsoni</i> | 283 ± 35 | 17 ± 8 | n.d. | 393 ± 47 | 156 ± 39 | n.d. |
| | <i>Racovitzia glacialis</i> | 733 ± 86 | n.d. | n.d. | 443 ± 51 | n.d. | n.d. |
| | <i>Prionodraco evansii</i> | 620 ± 74 | 8 ± 3 | n.d. | 338 ± 47 | 134 ± 28 | n.d. |
| Antarctic fish | Red blooded | | | | | | |
| | <i>Trematomus hansonii</i> | 506 ± 61 | 61 ± 22 | 279 ± 88 | 367 ± 45 | 98 ± 21 | 179 ± 26 |
| | <i>Trematomus eulepidotus</i> | 477 ± 58 | 67 ± 28 | 302 ± 79 | 395 ± 48 | 95 ± 18 | 198 ± 21 |
| Mammal | Arctic boreal fish | | | | | | |
| | <i>Pleuronectes platessa</i> | 380 ± 85 | n.d. | 19 ± 8 | 808 ± 110 | n.d. | 85 ± 21 |
| | <i>Myoxocephalus scorpius</i> | 590 ± 68 | n.d. | n.d. | 800 ± 104 | n.d. | n.d. |
| | <i>Aspitriglia obscura</i> | 286 ± 35 | 11 ± 4 | n.d. | 616 ± 72 | 138 ± 19 | n.d. |
| | <i>Gadus morhua</i> | 370 ± 42 | 25 ± 11 | 19 ± 4 | 650 ± 71 | 146 ± 17 | 118 ± 23 |
| <i>Rattus norvegicus</i> | 836 ± 109 | 13 ± 5 | 635 ± 32 | 170 ± 25 | 43 ± 12 | 119 ± 24 | |

Separated bands of gangliosides were treated with resorcinol reagent (Svennerholm 1957; Fig. 1). Their respective concentrations were determined by densitometric scanning at 580 μm (chromatogram spectrophotometer KM₃, Zeiss). The peak areas were integrated by means of a digital integrator (MOP-AM 02, Kontron). The fractions were identified, as far as possible, by comparison with standards (Seromed, München) and named according to Svennerholm (1963) following Yu and Ando's (1980) pathway of ganglioside biosynthesis. In addition to the wellknown gangliosides, in some species highly sialylated fractions were detected which have not yet been identified completely until now ($\text{Gx}_1 - \text{x}_4$). Since the ratio of sialic acid residues to the ceramide moiety in these fractions had been determined by Rösner (1981), Gx_1 seems to be a penta-sialoganglioside ($\text{G}_{\text{Plc}}?$); Gx_2 may represent hexa- and Gx_3 and Gx_4 even higher sialylated (hepta-) sialogangliosides. Furthermore some less polar sialylated (hepta-) sialogangliosides. Furthermore some less polar mono- and disialogangliosides ($\text{Gy}_1 - \text{Gy}_3$) were found, which have not yet been identified until now and which have variations in their ceramide moiety.

Results

a) Determination of Freezing and Melting Points from Serum of Antarctic and Arctic-Boreal Fishes

In order to compare physiological parameters for the ability of both groups of fishes to live at temperatures around or below zero degree in a first approach their resistance to freezing, expressed by the presence of protein antifreezes in their serum was investigated. For this freezing point (fp) and melting point (mp) values for all species except the gunard (*Aspitrigla obscura*) and codfish (*Gadus morhua*) had been determined. (It must be noted that the antifreeze-values from the northern species derived from different material than those of the ganglioside results).

The analysis indicates the presence of special antifreeze substances – expressed as differences between fp and mp in the blood plasma (thermal hysteresis) – in all of the eight species (Table 2). Even the blood of *M. scorpius*, caught in winter exhibited a significant hysteresis, although previous authors (Schneppenheim and Theede 1982) did not find such an effect in the blood of cold acclimated sculpins.

All these data concerning significant differences between the freezing and melting point of the blood speak in favour of the existence of freezing-point depressing peptides (FPDP) or antifreeze-glycoproteins, which in the meantime for most of the species investigated had already been described (for ref., see Schneppenheim and Theede 1982).

b) Concentration of Ganglioside- and Protein-Bound Sialic Acid in Antarctic and Arctic-Boreal Fishes

In the brain of Antarctic and Arctic-boreal fishes the concentration of total macromolecularly bound sialic acids varies from about 680 $\mu\text{g/g}$ fresh wt in the white blooded ice fish *Chaenodraco wilsoni* to about 1,390 μg in the Arctic sculpin (*Myoxocephalus scorpius*). As can be followed from Table 3 brain ganglioside content in the Antarctic fishspecies ranges from 283 (*Chaenodraco*) to

733 μg (*Racovitzia*) and in the Arctic-boreal species from only 280 (gunard) to 590 μg (sculpin). The concentration of protein bound sialic acid, on the other hand, varies in the brain of Antarctic fish between about 340 (*Prionodraco*) to about 520 $\mu\text{g/g}$ fresh wt. in *Chionodraco*, in the Arctic-boreal species, however, from about 620 μg (gunard fish) to 800 μg in the sculpin. With these values in both categories the concentration of neuronal sialoglycoproteins is much higher than that in mammals (rat: 170 μg). This phenomenon is in full agreement with former data indicating a reduction of the NeuAc-proteins during phylogeny (Rahmann and Hilbig 1983). No seasonal variations neither in the content of brain gangliosides nor in that of sialoglycoproteins of the boreal plaice (*Pleuronectes platessa*) were found.

In extraneuronal organs of the vertebrates generally the concentration of sialoglyco-macromolecules is very low: gangliosides in muscle tissue range between 5 and 30 μg , in liver about 60 μg ; sialoglyco-proteins amount in muscles about 150 μg and in liver between 100 and 250 μg . In Antarctic fish of the genus *Trematomus*, however, the ganglioside content of the muscles is significantly higher ($P < 0.001$) and makes up to about 65 μg sialic acid. In the liver of the two *Trematomus* species and also in that of the white blooded *Chionodraco* the highest ganglioside content of all vertebrates investigated until now was found with values between about 180 and 300 $\mu\text{g/g}$ fresh wt.

The concentration of sialoglyco-proteins of muscle tissue from both *Trematomus* species is significantly lower than that from all other bony fish. The concentrations of protein bound sialic acid in liver exhibits a great variability but they do not show any distinct correlations with temperature or with the level of systematical organization.

From these data it can be concluded, that the adaptation of the Antarctic fish to extreme environmental temperature of about 0°C is correlated with significantly higher concentrations of ganglioside-bound sialic acid not only in the brain but (in the case of the red blooded Antarctic fish) also in muscle and liver. The concentration of neuronal sialoglycoproteins generally is lower in Antarctic fish as compared with other marine teleosts.

c) Composition of Brain Gangliosides of Antarctic and Arctic-Boreal Fishes

The molecular composition of the individual brain ganglioside fractions, differing in their number of sialic acid residues showed remarkable differences between the cold-stenothermic Antarctic fishes and the more eurythermic Arctic and boreal fishes on the one side, and the homeothermic mammals (e.g. rat chosen as standard), on the other (Figs. 1 and 2). The ganglioside pattern of the Antarctic fish species is composed of a large number of extremely high polar fractions with more than four sialic acid residues. In the other fishes the polarity is reduced, but not to such a high degree as in mammals.

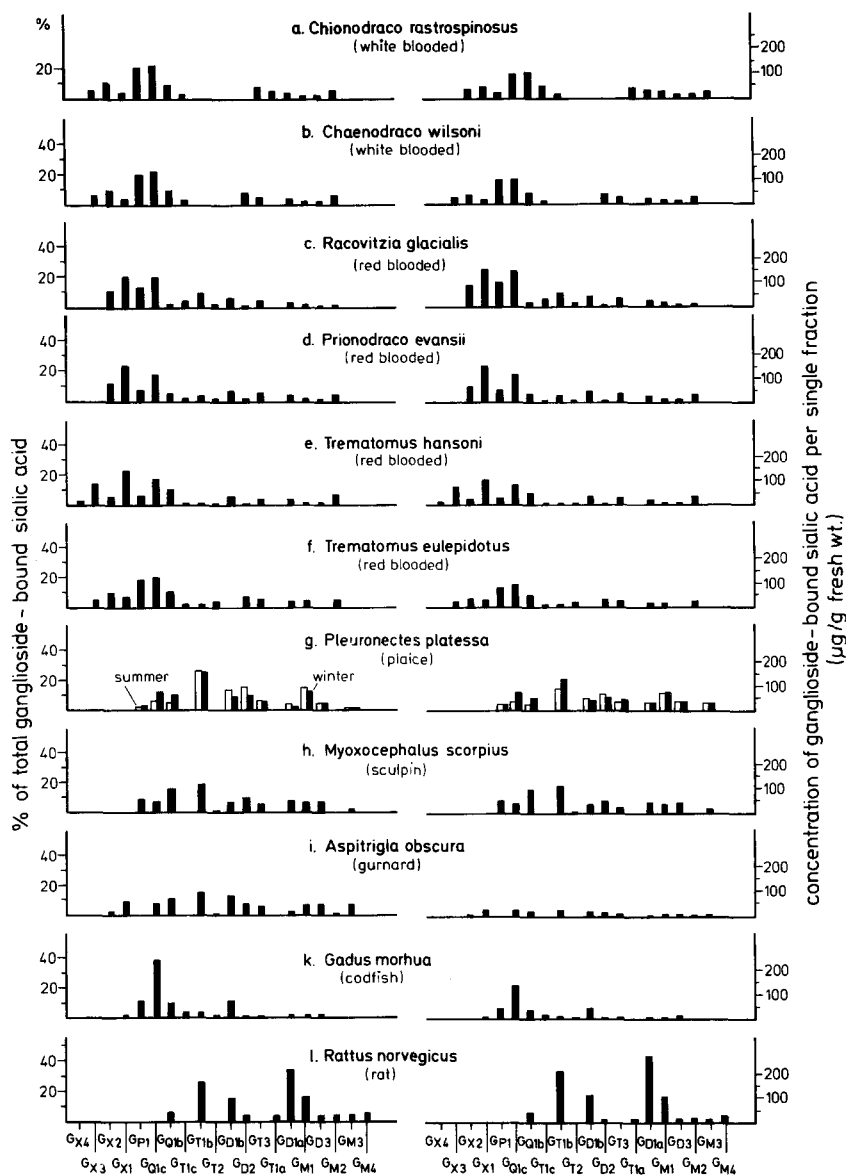


Fig. 2. Relative composition (%) and concentration ($\mu\text{g/g}$ fresh wt) of neuronal ganglioside-bound sialic acid per single fraction (G_{M4} to G_{X4}) of six Antarctic fishes, four Arctic-boreal fishes and the rat for comparison

The relative composition of the brain gangliosides of the different species of white and of red blooded Antarctic fish is almost very similar. Their pattern generally consists of highly sialylated fractions migrating on TLC slower than tetrasialogangliosides (G_Q) and amounting about 40 to 50% of all gangliosides. For instance the brain of *Trematomus hansonii* contains the following five fractions being higher sialylated than G_Q : G_{P1} – 6.1%, G_{X1} – 21.0%, $G_{X2} \cong G_{H?}$ – 5.1%; $G_{X3} \cong G_{S?}$ – 14.8%, G_{X4} – 2.7% (Fig. 2). The brain ganglioside pattern of the more eurythermic Arctic and boreal species according to the high amount of tetra-sialogangliosides in comparison with that of the rat (only one tetra-fraction: G_{Q1b} !) is much more polar, but by far not to such an amount as that of the Antarctic fish. Additionally the ganglioside pattern of Arctic fish is characterized by very low amounts of alkali labile O-acetylated fractions, whereas in other boreal fish e.g. the cod fish the O-

acetylated fractions can comprise up to 25% of total NeuAc concentration (Ghidoni et al. 1984).

In the eurythermic plaice seasonal changes in the pattern composition of brain gangliosides occurred (Fig. 2g): In adaptation to the cold the relative concentration of the tetra-sialogangliosides G_{Q1c} and G_{Q1b} increased, concomitantly the content of the less polar di-sialogangliosides G_{D1b} , G_{D2} and G_{D1a} decreased. These sialylation changes cause a higher polarity of the neuronal membranes in winter in quite a similar way as it had been described already for fresh water fishes (carp, goldfish, trout, perch: Hilbig et al. 1979).

Discussion

It is wellknown that the hematocrit and the concentration of hemoglobin in fish depend on the ambient temperature and oxygen levels (Raschack 1969): Low hemato-

crit and low hemoglobin (even loss of hemoglobin and erythrocytes) correlate with low temperature and corresponding high oxygen levels. These parameters which are seasonally variable in northern latitudes were found to be relatively constant in the Antarctic. Thus a low hematocrit is characteristic for Antarctic fishes and is extremely expressed in the white blooded fish family *Channichthyidae*, lacking completely red blood cells hemoglobin.

Another peculiarity in Antarctic and Arctic-boreal fish is the development of a freezing resistance due to freezing point depressing peptides and glycopeptides (De Vries and Lin 1977; Schneppenheim and Theede 1982; De Vries 1983). The synthesis of such macromolecules in fish was shown to be correlated with winter conditions in Arctic-boreal waters, whereas antifreeze synthesis in Antarctic fish is not seasonally dependent (Duman and De Vries 1974). The results of this investigation are in full agreement with the former data. Significant differences between the freezing and melting points of blood serum were found especially in all Antarctic fish. From this the existence of freezing-point-depressing peptides or antifreeze-glycoproteins, which enable those cold-water fishes to survive at temperatures around the freezing point of seawater, again had been confirmed.

Neurophysiological changes associated with temperature adaptation of temperate animals have been described many times (for review: Lagerspetz 1974; Prosser and Nelson 1981). Far less is known about neurophysiological mechanisms of adaptation to cold in Antarctic species. In those animals, however, the propagation of action potentials persists down to the freezing point, the conduction velocities change less with temperature than in warm-adapted animals, and also the upper thermal limit of spike activity is lowered (Macdonald 1981).

Since the ability of vertebrates to adapt to fluctuations in their environment is mainly based upon adaptive alterations within the CNS, where the synapses had been shown to be the most sensitive structures it is assumed that physico-chemical changes in synaptic membranes might be responsible for the adaptation of transmission to changes in the ambient temperature. With regard to this gangliosides were found to possess modulatory functional properties for synaptic transmission during temperature adaptation (for review: Rahmann 1978 – 1983; Rahmann et al. 1982, 1984): Distinct correlations between the brain ganglioside composition and the state of thermal adaptation were found. The general trend is: "The lower the environmental (\pm body) temperature – the higher is the polarity of the brain gangliosides." The polarity changes in the various vertebrates can be obtained either by a changed degree of sialylation (more or less NeuAc residues) or by N-acetylation or O-acetylation of the sialic acid residues of gangliosides (Rahmann et al. 1984). This principle was proved for ectothermic vertebrates after seasonal acclimatizations and during experimentally induced acclimation to temperatures near the limit of thermal tolerance. An open question,

however, remains the well known species variation of the gangliosides and the differentiation between environmental and genetical expression of the metabolic pathways under these extreme stenothermic conditions in the Arctic and especially Antarctic oceans. The "c-" pathway of ganglioside biosynthesis, which is extremely expressed in polar fish is generally typical for lower aquatic vertebrates (Rahmann and Hilbig 1983).

According to recent physico-chemical in vitro-studies gangliosides reveal a high and specific ability to complex with Ca^{2+} -ions. These Ca^{2+} -ganglioside-complexes were highly sensitive to variations in the ambient temperature (Probst and Rahmann 1980; Mühleisen et al. 1983; Probst et al. 1983; Rahmann et al. 1984). Generally these results were taken as evidence that synaptic membranes either from cold- or from warm-adapted animals may have similar Ca^{2+} -binding abilities and by this viscosities, generated by changes in the polarity of gangliosides (Rahmann 1983; Rahmann et al. 1983).

The variability in the equipment of synaptic membranes with gangliosides of different polarity obviously had been of great evolutionary advantage in so far as it enables the synaptic transmission process to proceed always effectively even under extreme temperature conditions. On this basis it is quite obvious to realize that the molecular composition of brain gangliosides especially in the Antarctic fish is characterized by an extreme high polarity which is due to gangliosides containing more than 40 to 50% of fractions with five and even more negatively charged sialic acid residues.

Summarizing, the present results concerning the extreme high polarity of neuronal gangliosides in cold-water fishes, which complement one another with the occurrence of protein antifreezes in the serum of the same species investigated speak in favour of the existence of an additional mechanism on molecular level that enables cold-water fishes to keep their neuronal membranes functional even under the extreme low temperatures of the poles.

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