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Molecular adaptation of the blood of Antarctic teleosts to environmental conditions

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Abstract: Following the break-up of Gondwana, the drift of Antarctica to its present position and the establishment of the Antarctic Convergence, fish evolution was characterized by adaptation to progressive cooling of the environment. The decrease of erythrocyte number and haemoglobin concentration in the blood of Antarctic teleosts raises several questions concerning the physiology of respiration and the enzymatic role of erythrocytes. Our study of the molecular basis of cold adaptation includes the relationship between molecular structure and biological function of haemoglobins. Species of the suborder Notothenioidei, largely confined within the Convergence, have only one major haemoglobin, which displays the Root effect in oxygen binding; on the other hand, Zoarcidae (a family found at all latitudes) have four or five haemoglobins, only one of which displays the Root effect. In addition, our data indicate that the physiological relevance of erythrocyte-like cells, present in very small number in the blood of haemoglobinless Channichthyidae, may be linked to higher content of enzymes, such as glucose-6-phosphate dehydrogenase, in comparison with erythrocytes of red-blooded fishes.

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Introduction

Over a period of 300 Ma, during the Palaeozoic and Mesozoic, Antarctica was part of the supercontinent of Gondwana, and experienced much warmer climates than currently (King 1958, Adie 1970, Wilson 1963, Craddock 1970, Dietz & Sproll 1970, Frakes & Crowell 1970, Hayes & Pitman 1970, Schopf 1970, Kennett 1977). The break-up of Gondwana began 135 Ma (Jurassic–Cretaceous boundary) and the final separation occurred in the early Tertiary. Antarctica was probably close to its present position approximately 65 Ma, with glaciation and ice sheet formation possibly beginning as early as 40 Ma (Tanner 1968, Denton *et al.* 1970, Le Masurier 1970). Cooling followed the opening of the Drake Passage, which permitted the development of the Circum-Antarctic Current and of the resulting Antarctic Convergence near the Oligocene–Miocene boundary, 25–22 Ma (Barker & Burrell 1977, Kennett 1977).

Today Antarctica is characterized by extreme climatic conditions. Throughout the year, the temperature of the Antarctic waters, in which no fish from temperate waters could survive, is –1.87°C, the equilibrium temperature of the ice–salt water mixture. Nevertheless, the oxygen-rich Antarctic waters support a wealth of marine life. During the period of increasing geographic and climatic isolation, Antarctic teleosts, similar to arctic fishes (Scholander *et al.* 1953), developed cold adaptation (Wohlschlag 1964), the evolutionary counterpart of cold acclimation. This enabled the fish fauna to tolerate the cooling of the environment. An essential feature of cold adaptation is freezing resistance to the ambient temperature (which is well below the normal freezing point of body fluids) by means of the synthesis of a group of 'antifreeze' glycoproteins. Their presence in the blood and in other fluids lowers the freezing point in a non-colligative fashion (DeVries & Lin 1977, DeVries 1980).

Antarctic fishes generally show a decrease in the number of erythrocytes and in the amount of haemoglobin in their blood (Everson & Ralph 1968, Hureau *et al.* 1977, Wells *et al.* 1980) in comparison with fishes of temperate and tropical waters (Coburn & Fischer 1973, Larsson *et al.* 1976). This decrease, producing much lower blood viscosity and therefore easing the demand on the heart, may be considered a component of cold adaptation. The total lack of haemoglobin (Ruud 1954) and the very small number of erythrocyte-like cells (Hureau *et al.* 1977) in the blood of the 17 species of the family Channichthyidae represent the extreme stage of such evolution.

In view of these considerations, a thorough investigation was initiated on the relationship between the molecular structure and the oxygen-binding properties of haemoglobins isolated from the blood of several species of teleosts from Antarctic waters. The aim of this study was to gain an insight into the molecular basis of this adaptation, as well as into the evolutionary history of the development of these features in fishes, during the isolation that followed the separation and drift of Antarctica from Gondwana.

Experimental evidence on metabolic rates in polar fishes generated the concept of metabolic cold adaptation (Scholander *et al.* 1953, Wohlschlag 1964). Physiologically, an organism is considered cold adapted if its resting or standard metabolic rate is higher than that predicted by

extrapolation from temperate or tropical organisms, assuming the Arrhenius relationship between temperature and metabolic rate. According to this criterion, fishes of several arctic and Antarctic families are cold adapted, since they show a metabolic rate three times higher than the rate that would be expected by extrapolation at 0°C.

The concept of metabolic cold adaptation has been questioned by Holeton (1973, 1974) and evidence both for and against an elevation of basal metabolic rate in polar marine ectotherms has been reviewed recently (Clarke 1983, Macdonald *et al.* 1987). No conclusive and generally acceptable argument either for or against this proposition has so far been published. If cold adaptation is indeed linked to profound variations in the metabolic rate, then evidence for the molecular basis of adaptation may come from studies of the structure–function relationship in enzymes controlling key metabolic pathways for organisms living under totally different conditions.

Following this reasoning, several enzymes of special metabolic importance were considered. One of these was glucose-6-phosphate dehydrogenase, which catalyses the oxydation of D-glucose-6-phosphate to D-glucono-δ-lactone-6-phosphate by $NADP^+$ or NAD^+, the first reaction of the hexose monophosphate shunt. The main function of this shunt in the erythrocytes is to generate NADPH as well as some 5-phosphoribosyl pyrophosphate. In turn, NADPH is the coenzyme of glutathione reductase and is required for the optimal functioning of catalase; both enzymes thus play an essential role in the physiological viability of the erythrocytes.

The haemoglobinless blood of fishes of the family Channichthyidae contains erythrocyte-like cells, which account for only 5% of the erythrocytes normally present in red-blooded Antarctic teleosts (Hureau *et al.* 1977). Therefore, the assessment of the presence and concentration of glucose-6-phosphate in the blood cells of this family of unique vertebrates was an essential, preliminary condition for the subsequent structure–function studies.

Materials and methods

Materials

Specimens of marine teleosts were collected by bottom trawling in Dallmann Bay and at Low Island (63°25'S, 62°15'W), Antarctic Peninsula, and by the use of gill nets, set 100 m deep on the bottom, in the vicinity of Terra Nova Bay station (74°42'S, 164°07'E). At the latter and at Palmer station (64°46'S, 64°03'W), fishes were kept in aquaria supplied with running sea-water. During a visit to McMurdo station, the blood of three additional species was kindly supplied by A.L. DeVries. Fish nomenclature follows Fischer & Hureau (1985).

The purification of haemoglobins and the functional studies were carried out either at Palmer station or at Terra Nova Bay; the dried globins were brought back to Naples and used for subsequent separation and characterization.

Cellulose acetate was from Gelman Int. Ltd; DEAE cellulose (DE 52) from Whatman; glucose-6-phosphate and $NADP^+$ from Boehringer. All other reagents were of the highest purity commercially available.

Preparation and purification

Blood was drawn from the caudal vein of unanaesthetized fishes by means of heparinized syringes. The erythrocytes were prepared and lysed as previously described (D'Avino & di Prisco 1988). When necessary in functional studies, endogenous organic phosphates were eliminated by running the haemolysates through a small column of Dowex AG 501 X8 (D), a mixed bed ion-exchange resin ('stripping').

Haemoglobins were purified by ion-exchange chromatography of the haemolysates, according to previously described procedures (D'Avino & di Prisco 1985, di Prisco 1988).

Globin mixtures were prepared by removing haem from each purified haemoglobin by the acid-acetone precipitation procedure (Rossi Fanelli *et al.* 1958). Globin chains were then purified by high-performance liquid chromatography (HPLC) on reverse-phase columns (D'Avino & di Prisco 1988).

The oxygen binding in haemoglobin was measured as previously described (di Prisco *et al.* 1988).

Results

Haemoglobin

The present investigation consists of data collected on 24

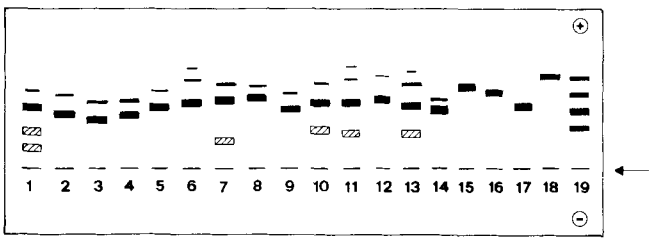

Fig. 1. Sketch of cellulose acetate electrophoresis of haemolysates of Antarctic fishes. Nototheniidae: 1. = *Notothenia coriiceps neglecta;* 2. = *N. rossii;* 3. = *N. gibberifrons;* 4. = *Notothenios nudifrons;* 5. = *N. larseni;* 6. = *Pagothenia hansoni;* 7. = *Trematomus newnesi;* 8. = *P. bernacchii;* 9. = *P. borchgrevinki;* 10. = *T. nicolai;* 11. = *T. centronotus;* 12. = *T. loennbergi;* 13. = *T. eulepidotus;* 14. = *Paranotothenia angustata;* Harpagiferidae: 15. *Harpagifer bispinis;* 16. = *Artedidraco skottsbergi;* Bathydraconidae: 17. = *Parachaenichthys charcoti;* Rajidae: 18. *Raja georgiana;* Zoarcidae: 19. = *Lycenchelys nigripalatum.* Additional minor components, observed in some species, are also sketched. The arrow indicates origin.

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species from five Antarctic families (Nototheniidae, Bathydraconidae, Harpagiferidae, Zoarcidae and Rajidae) during the course of seven field seasons (four at Palmer station, the remaining three at Terra Nova Bay). All haemolysates have been examined by electrophoresis on cellulose acetate at pH 9.0 (D'Avino & di Prisco 1988). Fig. 1 displays a sketch of the electrophoretic patterns of 13 nototheniids, a bathydraconid, two harpagiferids, a zoarcid, a rajid and a non-cold-adapted notothenid (*Paranotothenia angustata*), commonly found in the southern waters of New Zealand. A qualitatively similar haemoglobin pattern was observed in the haemolysate of all nototheniids, indicating the presence of a major component (Hb 1, 85–90% of total) and often of a second, more anodal one, Hb 2 (5–10%). Minor, less anodal components were observed in some cases, accounting however for less than 1% of total. A single haemoglobin was observed in all other cases, with the exception of the zoarcid (four components).

The haemoglobins from 15 species were purified by ion-exchange chromatography and the purified components were obtained in crystalline form in nine cases. Table I summarizes part of the experimental evidence outlined in this communication; the blood of two additional species of Zoarcidae also contained multiple haemoglobin components. The oxygen-binding results (Bohr and Root effects) will be discussed later.

Table I. Summary of haemoglobin characteristics in Antarctic fishes.

Family	Species	Location[1]	No. of Hb components[2]	Bohr effect	Root effect
Nototheniidae	*N. coriiceps negl.*	AP	2**	+	+
	N. rossii	AP	2**	+	+
	N. gibberifrons	AP	2**	+	+
	N. nudifrons	AP	2		
	N. larseni	AP	2		
	P. hansoni	AP, RS	2*		+
	P. bernacchii	RS	1**		+
	P. borchgrevinki	RS	2		
	T. newnesi	RS	2**		
	T. nicolai	RS	2*		
	T. centronotus	RS	2		+
	T. loennbergi	RS	2**		
	T. eulepidotus	RS	2*		
	D. mawsoni	RS	1*		+
Bathydraconidae	*P. charcoti*	AP	1*	+	+
	G. acuticeps	RS	1**		
	C. mawsoni	RS	2*		+
Harpagiferidae	*H. bispinis*	AP	1		
	A. skottsbergi	AP	1		
	H. velifer	RS	1		+
Rajidae	*R. georgiana*	AP	1		
Zoarcidae	*L. nigripalatum*	AP	4		
	L. dearborni	RS	4**		–, +
	A. brachycephalus	RS	5**		–, +

[1] AP = Antarctic Peninsula; RS = Ross Sea.
[2] Detected by electrophoresis on cellulose acetate.
* Obtained in purified form by ion-exchange chromatography.
** Purified Hb components, obtained in crystalline form.

The suborder Notothenioidei includes three families of red-blooded fishes (Nototheniidae, Bathydraconidae and Harpagiferidae) and the haemoglobinless family Channichthyidae. Fig. 2 illustrates the haemoglobin elution pattern from the haemolysate of one species, the notothenid *Notothenia coriiceps neglecta*, showing the separation of the two components Hb 1 and Hb 2. The elution of the five components detected in the haemolysate of the zoarcid *Austrolycichthys brachycephalus* (suborder Zoarcoidei) is reported in Fig. 3.

As indicated in Table I, the functional properties of the haemoglobins of all Notothenioidei studied appeared to be finely regulated both by pH and by the physiologically active organic phosphates (di Prisco *et al.* 1988), showing a negative, large and steep alkaline Bohr effect, indicative of

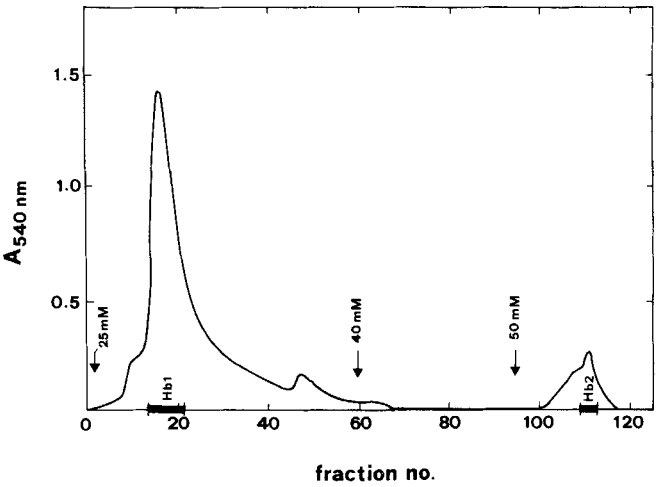

Fig. 2. Ion-exchange chromatography of the haemolysate from 11 ml blood of the notothenid *N. coriiceps neglecta*, on a column of DE 52 (2.2 × 20 cm), equilibrated with 10 mM Tris-HCl pH 7.6. Stepwise elution with increasing concentrations of the same buffer, as indicated. Fraction vol, 12 ml.

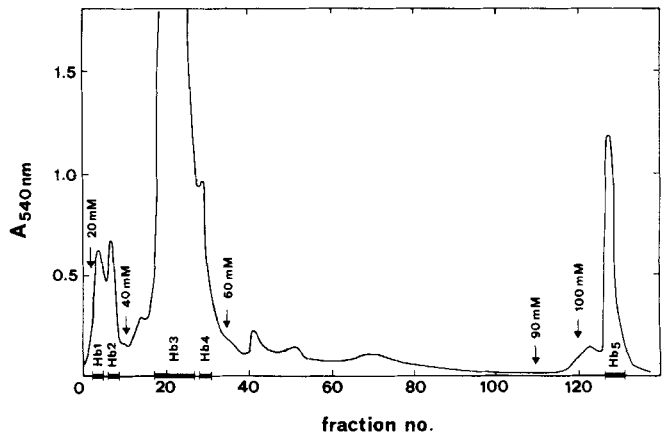

Fig. 3. Ion-exchange chromatography of the haemolysate of the zoarcid *Austrolycichthys brachycephalus*. Experimental conditions as in Fig. 2.

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a sharp pH dependence of the oxygen affinities. In fact, the reduction of the oxygen affinity at low pH (Root effect; Root 1931), corresponding to an exaggerated Bohr effect, was observed in the intact erythrocytes, 'stripped' haemolysates and purified Hb 1 and (when present) Hb 2 of all Notothenioidei; an example is shown in Fig. 4. In Zoarcidae, on the other hand (Fig. 5), only the more acidic components displayed the Root effect, whereas the oxygen affinity of the more basic ones appeared to be much less dependent on pH and organic phosphates. Zoarcidae, therefore, not only diverge from the general trend of having only one major haemoglobin, but appear also to be the only family having functionally distinct components.

Glucose-6-phosphate dehydrogenase

The enzyme was isolated from the erythrocytes of the nototheniids *N. coriiceps neglecta* and *N. gibberifrons* and from the blood of three species of Channichthyidae, *Chaenocephalus aceratus, Pseudochaenichthys georgianus* and *Champsocephalus gunnari* (di Prisco 1986). In 23 experiments with individual *C. aceratus* (Table II) the total activity found in the blood was an average of 1400 arbitrary units per ml, whereas a value of 7000 was found in the red-blooded nototheniids. Thus, the channichthyid blood contains only 20% of the activity present in an equal volume of notothenid blood. However, the volume of channichthyid blood is up to four times that of teleosts with haemoglobin (Hemmingsen & Douglas 1977). On a weight basis, the two families then have a relatively similar level of glucose-6-phosphate dehydrogenase. The blood of Channichthyidae contains a concentration of erythrocyte-like cells equal to 5% of the erythrocytes in red-blooded Antarctic fish families (Hureau *et al.* 1977); it follows that a channichthyid cell contains almost five times the activity associated with a notothenid erythrocyte.

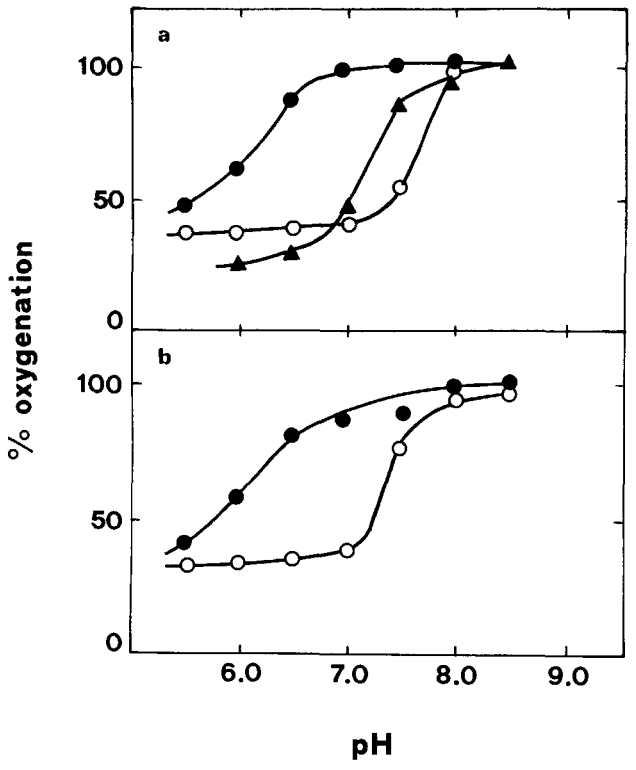

Fig. 4. Oxygen saturation of *N. coriiceps neglecta* haemoglobin as a function of pH (Root effect). **a.** 'stripped' haemolysate and **b.** purified Hb 1, in the absence (●) and presence (O) of 3 mM inositol hexaphosphate. ▲ = Erythrocytes in isotonic buffer.

Discussion

The first teleosts appeared about 200 Ma (Triassic). These fishes continued to evolve through the Cretaceous; the first fossil records of most percoid families appear in the Eocene.

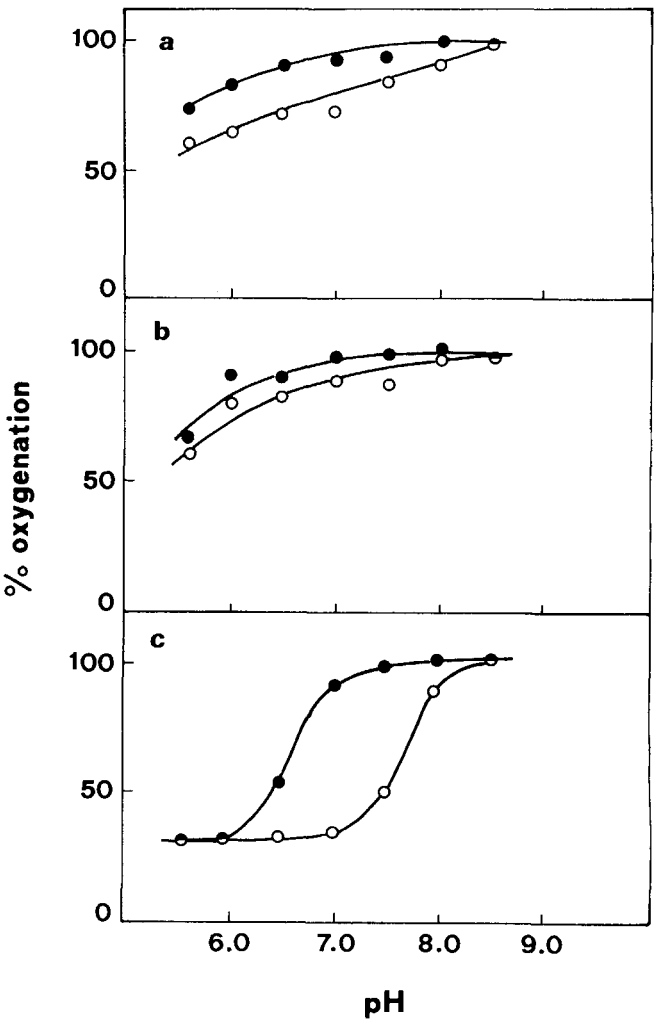

Fig. 5. Root effect of *A. brachycephalus* **a.** 'stripped' haemolysate, **b.** Hb 3 and **c.** Hb 5, in the absence (●) and presence (O) of 3 mM inositol hexaphosphate.

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Table II. Glucose-6-phosphate dehydrogenase activity in the blood of Nototheniidae and Channichthyidae.

Species		Arbitrary units/ml*
C. aceratus	(n = 23)	1400
N. coriiceps neglecta	(n = 25)	7000
N. gibberifrons	(n = 18)	7000

* Activity assays were made at 22°C, as described (Camardella *et al.* 1981). Values are corrected for 6-phosphogluconate dehydrogenase.

Notothenioidei probably originated in the lower Tertiary in Antarctic waters. Fishes of this suborder account for over 65% of the total Antarctic fish fauna and compose 86% of the endemic genera (DeWitt 1971). The extremely high endemism found in the Antarctic fish fauna may be attributable to the long isolation of the region during the cooling process south of the Convergence.

Our experimental evidence, gathered in two widely separated areas (the Antarctic Peninsula and the Ross Sea), suggests that during the period of isolation within the Convergence, the blood and haemoglobins of Antarctic Notothenioidei acquired some common features clearly differentiating them from fishes of temperate and tropical climates. Blood contains fewer erythrocytes and less haemoglobin, with a resultant decrease in its viscosity. On the other hand, at the temperature of the Southern Ocean, oxygen is more soluble in sea-water and, moreover, the oxygen affinity for haemoglobin becomes higher. Haemoglobin diversity is very limited; usually there is only one (major) component, which invariably displays the Root effect and a finely pH-regulated oxygen affinity. Characteristically, most fish haemolysates contain multiple components (Riggs 1970) that in fast swimmers often show functional differences in oxygen binding. Such a functional variety is probably not required in an environment with relatively constant physico-chemical features; therefore, Antarctic fishes do not need more than one haemoglobin type.

The family Zoarcidae, which belongs to a different suborder (Zoarcoidei), is not endemic and is found at all latitudes, including the north polar region. In three Antarctic zoarcid species, four or five haemoglobins were detected and purified although, in each species, only one of these components displayed the Root effect. When compared with Notothenioidei, these findings suggest separate evolutionary pathways following suborder divergence. In support of this hypothesis, it seems pertinent to stress that, in addition, Notothenioidei have glycoproteins (AFGP) for freezing resistance, whereas in Zoarcidae this is provided by a peptide with a totally different molecular structure (DeVries 1980). Thus, it is likely that family diversification in Notothenioidei took place after the cooling event which led to the appearance of AFGP. 'Antifreeze' gene evolution in arctic and Antarctic fishes has been the object of recent studies (Scott *et al.* 1986).

The nototheniid *P. angustata* found in both the New Zealand and Patagonian regions, probably established itself in the former region when the Campbell Plateau was still relatively close to West Antarctica in the lower Tertiary (Andersen 1984). Although not cold adapted, *P. angustata* is similar to Antarctic Notothenioidei in having Hb 1 (90%) and Hb 2 (5%). Moreover, the amino acid composition of the α- and β-chains of Hb 1 (D'Avino & di Prisco 1988) suggests a very high homology of the amino acid sequence with those of haemoglobins from Antarctic Notothenioidei. It is worth mentioning that at the end of the Miocene (5 Ma) and during the Pliocene, the Convergence may have moved northward up to 39°S, the present latitude of northern New Zealand (Kennett 1968), greatly facilitating migration of fish fauna.

The pale-whitish blood of teleosts of the family Channichthyidae contains a small number of cells, morphologically similar to erythrocytes (Hureau *et al.* 1977), of unknown physiological significance. Erythrocytes contain high concentrations of haemoglobin and have therefore an essential role in oxygen transport; the lack of this protein in Channichthyidae rules out, of course, the possibility of this role. On the other hand, erythrocytes also store several enzymes of great metabolic significance. Glucose-6-phosphate dehydrogenase is one of these.

Our findings show that an erythrocyte-like cell from a channichthyid may contain up to five times the amount of glucose-6-phosphate dehydrogenase activity associated with a 'red' notothenid cell. This conclusion offers an explanation for the functional significance of the residual cells in Channichthyidae. On one hand, the drastic reduction in their number, made possible by the elimination of haemoglobin, results in low blood viscosity, hence low flow resistance, greatly facilitating the pumping of the blood. A greater amount of glucose-6-phosphate dehydrogenase in the remaining cells, together with an increased blood volume, contributes on the other hand to maintaining the required physiological level of the erythrocyte enzyme.

The evolutionary trends of Antarctic teleosts include specialization achieved by freezing resistance, by tendency to neutral buoyancy (Eastman & DeVries 1981) and by improved swimming ability, derived by fusion of caudal skeleton elements (Andersen 1984). The modification of the haematological characteristics may be considered an additional form of specialization, for optimal adjustment of the physiology of oxygen transport and circulatory functions to the climatic conditions of the environment.

Fossil records linking the early Devonian fish population of Gondwana with the Eocene teleosts are unknown. Therefore, palaeontology is unable to provide insight into the evolution of cold adaptation. Despite the difficulties, valuable indications on the evolutionary trends may be gathered by integrating the biochemistry of macromolecules and metabolites, in progress in our laboratory, with gene expression and with the physiology of living organisms.

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