REGULATION OF ANTIFREEZE POLYPEPTIDE BIOSYNTHESIS IN THE WINTER FLOUNDER (PSEUDOPLEURONECTES AMERICANUS)

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REGULATION OF ANTIFREEZE POLYPEPTIDE BIOSYNTHESIS IN THE WINTER FLOUNDER (Pseudopleuronectes americanus)

BY

©Ronald Mitchell Fourney, B.Sc., M.Sc.

A thesis submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Department of Biochemistry
Memorial University of Newfoundland
January 1984

St. John's Newfoundland

ABSTRACT

The heterogeneity of antifreeze polypeptides (AFP) in the Newfoundland winter flounder Pseudopleuronectes americanus was analyzed by reverse phase high performance liquid chromatography (HPLC) and by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Seven AFP components could be readily resolved. Five of the components were similar in molecular weight (3,300) and amino acid composition. Two of the AFP components were larger (4,500) and contained valine. The two major components (components 6 and 8) were identical to those reported earlier from our laboratories (Davies et al., Proc. Natl. Acad. Sci., USA 79, 335, 1982).

Serum AFP were isolated from winter flounder collected from Nova Scotia, New Brunswick and Long Island (New York) and compared to the AFP found in Newfoundland winter flounder. These peptides were analysed by reverse phase HPLC, and showed nearly identical elution profiles to that of Newfoundland AFP. Individual components from New Brunswick and Long Island had antifreeze activity and agreed with the activity measurements previously established for the Newfoundland AFP. Amino acid analysis of the major AFP components from the different flounder populations indicated that they had similar compositions. A minor variation which included valine in one AFP component was noted in the New Brunswick sample suggesting that a limited amount of genetic polymorphism may be present in the winter flounder population from New Brunswick. In general, there appears to be little or no variation in the structure of the AFP from winter flounder inhabiting different geographical habitats. In view of the similarity of the serum AFP components and in light of recent information on the structure and genomic organization of AFP genes, it is likely that the winter flounder AFP components are products of an AFP gene family.

The seasonal synthesis of AFP in winter flounder is influenced by photoperiod and comes under the control of the pituitary gland. The effects of hypophysectomy (hypex) and long day length on the accumulation of antifreeze messenger RNA (mRNA) in the liver were examined. Hypophysectomy resulted in a significant decrease in serum freezing temperature, and increases in liver weights, total liver Poly $(A)^+$ RNA and AFP mRNA accumulation. The identity of the AFP mRNA in hypex animals was confirmed by gel electrophoresis, cell-free translation and Northern blot hybridization techniques. Cytoplasmic-dot hybridization analysis indicated that the AFP mRNA level in hypex fish approximated that observed in winter animals actively synthesizing AFP. These experiments utilized a nick-translated CT5, an antifreeze protein cDNA clone. An increase in AFP mRNA was detectable as early as the first day after hypophysectomy and by day 7 reached 25% of the level found in fish actively synthesizing AFP mRNA during the winter months. Since AFP mRNA is found at very low levels in the control flounder, this suggests that its accumulation after hypophysectomy depends on accelerated transcription. The pituitary gland appears to regulate the liver AFP mRNA level by a negative transcriptional control mechanism.

The effect of photoperiod on the seasonal accumulation of winter flounder AFP mRNA in the liver was examined. Flounder maintained under conditions of 15 h long day length have both a delayed appearance and decreased accumulation of AFP mRNA. AFP mRNAs were identified and their concentrations measured by a cytoplasmic dot-hybridization method and Northern blot hybridization utilizing a nick-translated E_3, an antifreeze genomic clone. December flounder maintained under conditions of long day length demonstrated the most significant decrease in AFP mRNA levels. It was estimated that these fish contained less than 0.6% the AFP mRNA normally found in control December fish. The seasonal fluctuation of AFP mRNA in both experimental and control fish match closely, but preceed, the rise and fall of plasma AFP levels. These results suggest that long day length suppresses the rate of transcription of antifreeze genes, and support the hypothesis that photoperiod may act as the initial cue for entraining the precise activation of AFP synthesis possibly through a pituitary mediator. A model for the regulation of winter flounder AFP gene expression is presented.

ACKNOWLEDGEMENTS

Many people have contributed to the successful completion of this investigation and to them I am greatly indepted. Foremost, I wish to thank my wife Anne, whose constant support, patience and confidence have provided me with a source of encouragement and motivation. I wish to thank my supervisor Dr. C.L. Hew for his continual support. My thanks are also due to Drs. G.L. Fletcher and W.S. Davidson and members of their laboratories for their assistance and suggestions in many aspects of this study. I wish to also express my deepest gratitude to Dr. D.R. Idler and Marine Science Laboratory staff and Dr. B. Sells, Dr. C. Bird and Mr. F. Jacobs for their assistance and use of laboratory facilities. I am indepted to Mr. D. Hall and the Department of Biochemistry for my amino acid analysis. A special thank you is extended to Maureen James for her careful typing of this thesis and to Dr. M. Wiegand for his many helpful suggestions.

Financial support from Dr. C.L. Hew (research funds) and Memorial University (M.S.R.L. Graduate Scholarship and the University Fellowship) is gratefully acknowledged and sincerely appreciated.

This thesis is dedicated to my father and was defended in his memory.

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LIST OF ABBREVIATIONS

AFP	antifreeze polypeptide(s)
AFGP	antifreeze glycopolypeptide(s)
HPLC	high performance liquid chromatography
hypex	hypophysectomy; removal of pituitary gland
poly $(A)^+$	polyadenylated
A	absorbance
A_{280}	absorbance at 280 nanometers
A_{260}	absorbance at 260 nanometers
A_{230}	absorbance at 230 nanometers
E_{230}	extinction coefficient at 230 nanometers
nm	nanometer
c	centi, 10^{-2}
m	milli, 10^{-3}
µ	micro, 10^{-6}
L	litre
m	meter
OD	optical density
g	gram(s)
M	molar
N	normal
mol	mole(s)
g	acceleration due to gravity
°C	degrees Celsius
min	minute(s)
h	hour(s)

Ci	Currie(s)
cpm	counts per minute
$[^{3}H]$	tritium
$[^{35}S]$	sulfur-35
$[^{32}P]$	phosphate-32
NMR	nuclear magnetic resonance
CD	circular dichroism
S	Svedberg unit
rpm	revolutions per minute
(w/v)	weight/volume
(v/v)	volume/volume
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
cDNA	complementary deoxribonucleic acid
CT5	a specific antifreeze protein cDNA clone
F8	a specific antifreeze genomic clone
bp	base pairs
kb	killo base pairs
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
Rf	mobility relative to bromophenol blue
TCA	trichloracetic acid
SSC	buffer; 0.15M NaCl/0.015M Trisodium citrate pH 7.0
o/oo	parts per thousand
≤	less than or equal to
≥	greater than or equal to

~	approximately
SE	standard error
♀	female
♂	male

CHAPTER I

GENERAL INTRODUCTION

Temperature is of primary importance in limiting the distribution of animals and their activity. All life processes require an aqueous medium. The phase changes of water below 0°C, from liquid to ice, cause extensive changes in the physical state of the organism or cell and irreversible changes in internal processes such as osmoregulation and excretion. The survival of all organisms inhabiting subzero environments depends on their ability to deal with the freezing of water and the prevention or tolerance of cryoinjury associated with this event. To avoid freezing many fish such as the winter flounder Pseudopleuronectes americanus have evolved a unique set of polypeptides which act as "biological antifreezes".

Mechanisms of cryoinjury

In contrast to cold injury which many organisms can tolerate (Hochachka and Somero, 1973), freezing injury is, in most cases, lethal. There are numerous theories for the mechanism of freezing injury (Mazur, 1970); however, two hypothesis predominate. The first involves intracellular ice formation and the mechanical damage caused by ice crystals large enough to cause injury through the disruption of cellular components (Mazur, 1970; Bank, 1973). The second mechanism involves extracellular ice formation which results in cellular dehydration and is associated with the loss of a critical amount of cell water (Prosser, 1973).

In man's search for cryoprotectants considerable effort has been expended in trying to understand the growth and size of intracellular ice crystals. It is thought that the freezing of intracellular fluids occurs following nucleation within the cell or by the penetration of ice crystals through the cell membrane from the extracellular environment (Franks and Skaer, 1976). The most probable reason for intracellular ice injury is likely to be due to the ice crystals physically alterating the various cellular and subcellular components (DeVries, 1974; Franks, 1975). Structural alterations and functional changes in plasma membranes are the major targets implicated in this injury (Trump et al., 1965; Heber, 1968).

More recently Farrant (1977) has suggested that injury by intracellular ice formation may also be caused by alteration of water transport such that osmotic movements of water into and out of cells virtually cease. This process is considered to be more rapid than any movement of water across cellular membranes particularly at subzero temperatures. The lack of water movement would lead to a gross imbalance of electrolytes and cause changes in the hydration properties of proteins. The breakdown of membrane structure would be the main result of such events.

Although the exact mechanism of intracellular ice injury remains in question, the rate of cooling and repeated freeze-thaw cycles appear to be main factors promoting this injury. Cooling rates exceeding a critical value, which is characteristic of a particular cell type, produce intracellular ice because water cannot leave the cell rapidly enough to build extracellular ice crystals (Mazur, 1963; Mazur, 1977). For example, some intertidal invertebrates exposed to high chill factors at low tide experience high cooling rates. Intracellular ice formation takes place and the organism dies. However, the same invertebrates exposed to a slower cooling rate due to changes in wind velocity or warmer seasonal temperatures, are not as susceptible to intracellular ice formation and its corresponding injury (Murphy, 1983). Repeated freeze-thaw cycles promote intracellular ice injury. Although the reason for this is unknown, Fishbein and Winkert (1977) suggest that both physical disruption of membranes and detrimental extraction of bound water from membrane proteins are major factors contributing to this injury.

Extracellular ice formation is the second major mechanism of cryoinjury. Extracellular ice formation results in cellular dehydration. When extracellular water freezes the mole fraction of extracellular water declines. Intracellular water diffuses down its concentration gradient into the extracellular space. This results in the loss of a critical amount of cellular water (Mazur, 1963; Mazur, 1977). In effect, extracellular ice formation represents a form of dessication since cells become hypoosmotic with respect to the external environment. The outcome of such dehydration is thought to result in severe structural changes in cellular proteins (Levitt, 1962), changes in ionic strength, pH, and electrolyte imbalance (Lovelock, 1953) and the eventual disruption of normal membrane permeability (Lovelock, 1957; Meryman, 1968; 1971). In general, extracellular ice formation causes irreversible damage to cellular constituents as well as extracellular components.

Biological adaptations to freezing

Freezing is lethal to most cellular organisms, and species inhabiting the subzero environment have evolved different adaptive mechanisms in order to survive. Homeothermic ("warm-blooded") vertebrates are not in danger of freezing because they have developed a sophisticated means of generating heat and so are able to maintain their body temperatures well above the freezing point of their body fluids. Poikilothermic ("coldblooded") organisms have evolved other means of adaptation. These include behavioural avoidance, ability to tolerate limited freezing, increased synthesis of small molecular weight compounds, ability to supercool, synthesis of ice-nucleating factors, and the synthesis of specific macromolecules which act as organic antifreezes.

Behavioural avoidance or the seasonal occupation of ice-free habitats represents one of the strategies adapted by organisms inhabiting potentially subzero habitats. Many sub-Arctic marine teleosts such as the threespine stickleback, Gasterosteus aculeatus and the starry flounder, Platichthys stellatus change their osmoregulatory patterns and avoid subzero marine environments by migrating into warmer fresh water (Smith and Paulson, 1977). The longhorn sculpin, Myoxocephalus octodecemspinosus and the crescent gunnel, Pholis laeta migrate away from shallow, ice-laden water into deeper, ice-free water taking advantage of the fact that this water is generally warmer and ice crystal nucleation is inhibited by increasing hydrostatic pressure and convection currents (Leim and Scott, 1966; Dayton et al., 1969; Smith and Paulson, 1977). Freezing avoidance does not necessarily require long distance migration but may involve highly specific habitat selection. The Antarctic fish, Notothenia kempi lives in an ice covered ocean near the Antarctic Circle by remaining in a +2°C layer of water that exists year round at a depth of 100-200 meters (DeVries, 1982; DeVries and Eastman, 1982).

Various organisms such as the Arctic beetle Pytho americanus (Ring and Tesar, 1980) and intertidal mussels and snails (Murphy and Pierce, 1975; Murphy, 1979) can actually tolerate some frost and a certain percentage of freezing. Although the exact mechanisms are unknown the larval and adult stages of P. americanus appear to use glycerol and perhaps hemolymph sugars as natural cryoprotectants to allow limited freezing to occur without death (Ring and Tesar, 1980). The intertidal mussel, Mytilus edulis and the subtidal clam, Venus mercanaria are exposed to the freezing atmosphere in the winter twice daily as a result of the rise and fall of the tide. These organisms can tolerate up to 64% of the water in their tissues being frozen (Williams, 1970; Murphy, 1983). Resistance to freeze injury in these invertebrates appears to be associated with a mechanism involving structural or tissue solute changes which are dependent on a shift from an aerobic to an anaerobic metabolism. The exact nature of these changes and the nature of an oxygen-dependent freeze injury is not understood (Murphy, 1983).

Many organisms, including those with less advanced circulatory systems, achieve a certain degree of freezing resistance by increasing tissue levels of sugars, polyhydric alcohols (eg. glycerol), lipids, sodium chloride and other small molecules (Potts and Parry, 1964; Danks, 1978; Feeney and Yeh, 1978; DeVries, 1980). These small compounds will lower the freezing point of tissue liquids by normal colligative properties. Colligative freezing point depression is nearly proportional to the molar concentrations of these additives. This route can only be used to a certain extent in most animals because it raises the osmotic pressure. The ability to supercool, or the cooling of body fluids below the freezing point in a liquid state, is thought to be stabilized by this solute effect (Umminger, 1969, 1978; DeVries, 1980). Biological systems in the absence of ice nucleation promoters can be supercooled to a limited extent. Species as diverse as the deep water fish Icelus spatula from Hebron

Fjord, Labrador (Scholander et al., 1957) and the larvae of the Alaskan beetle, Pytho deplantitus which can depress its supercooling point to -54°C (Ring and Tesar, 1981), have utilized the freezing point depression of small molecules and the ability to supercool as an effective mechanism against freezing. However, the supercooled state is thermodynamically unstable and in the presence of seed ice crystals, supercooled organisms such as the Arctic fish Icelus spatula will quickly freeze (Scholander et al., 1957). Feeney and Yeh (1978) have suggested that living organisms can allow supercooling to occur more easily by compartmentalization with membrane systems. This would restrict nucleation centers to particular regions.

Some organisms have exploited the ability to exist in the supercooled state a step further by evolving a specific set of ice-nucleating factors. These compounds assist freezing such that the extensive supercooled state which promotes lethal intracellular freezing is avoided in favour of a controlled localized extracellular ice formation which can be tolerated to a certain extent (Farrant, 1980). Examples of these molecules are found on a seasonal basis in Afro-alpine plants (Krog et al., 1979) and many insects (Zacharissen and Hamel, 1976; Duman and Horwath, 1983).

One of the most intriguing adaptive mechanisms evolved in organisms living in subzero habitats is the occurrence of a unique class of specific macromolecules capable of acting as organic antifreezes. These proteins and polypeptides occur in numerous organisms, and are essential in preventing the animal from freezing. Biological antifreeze molecules act in a non-colligative manner to lower the freezing point depression of body and cellular fluids below that of their surrounding environment (for reviews see: Feeney and Yeh, 1978; DeVries, 1980, 1982, 1983; Hew, 1981; Duman and Horwath, 1983). These antifreezes have been reported in species as diverse as Antarctic fishes (Feeney and Yeh, 1978; DeVries, 1983), many Arctic and sub-Arctic fish (Hew, 1981; DeVries, 1983), the intertidal mussel Mytilus edulis (Theede et al., 1976), the spiders Philodromus sp. (Philodromidae) and Clubiona sp. (Clubionidae) (Duman, 1979a) and numerous insects (Duman, 1977a, 1977b, 1979b, 1982; Danks, 1978; Duman and Horwath, 1983; Hew et al., 1983).

Antifreeze proteins are an intriguing adaptive mechanism with many interesting features. The remainder of this chapter will emphasize the structure, genetics, and biochemical diversity of the antifreezes found in teleosts. In addition, the mechanism most likely to be involved in regulating the concentration of these proteins will be discussed.

Biological antifreezes in fish

Fishes are the only poikilothermic vertebrates which inhabit subzero environments. With the exception of myxinoids and elasmobranchs (i.e. hagfish, sharks and rays), fish are hypoosmotic to their marine environment and risk freezing in the polar oceans. Many polar and north-temperate marine fishes live at temperatures between -1.4°C and -1.8°C near the freezing point of saltwater (-1.9°C). The body fluids of most temperate marine teleosts freeze at temperatures between -0.5 and -0.9°C (Holmes and Donaldson, 1969). In temperate fish, sodium chloride represents the most abundant electrolyte in the blood and accounts for 85% of the freezing point depression (Gordon et al., 1962; Feeney and Yeh, 1978). The remainder of the freezing point depression has been attributed to other small molecules and salts such as glucose, free amino acids, urea, potassium and calcium (Potts and Parry, 1964; Dobbs and DeVries, 1975). Fish inhabiting freezing environments have elevated concentrations of sodium chloride in comparison to temperate teleosts (DeVries and Lin, 1977a; Fletcher, 1977; Fletcher, 1981). However, the concentration of this electrolyte accounts for only 30 to 50% of the observed freezing point depression and the concentrations of other ions do not vary with respect to habitat (Fletcher, 1977, 1981; Feeney and Yeh, 1978; DeVries, 1980). The colloidal blood fraction of a variety of fish from polar and subpolar oceans contains antifreeze macromolecules which depress the freezing temperature of their body fluids and are believed to be essential for their survival in ice-laden seawater (Feeney and Yeh, 1978; DeVries, 1980, 1982, 1983; Hew, 1981).

Historical perspectives

Scholander and co-workers (1953, 1957; Gordon et al., 1962) reported the initial observations of freezing resistance in marine fishes. They observed that the blood sera of Arctic fish had a lower freezing temperature than did the blood sera of fish from more temperate zones. More importantly, they noted that the decreased serum freezing temperature was not due to inorganic salts, but rather to macromolecules found in a fraction of serum that is soluble in trichloracetic acid. It was concluded, because of the inability to precipitate this fraction by trichloracetic acid, that these macromolecules were not proteins and that they probably lowered the freezing point by a colligative process. Other investigators soon made similar observations. In the Norwegian boreal and Arctic fishes of the Barents Sea, Eliassen and co-workers (1960) noted that these fish responded to subzero temperatures in much the same manner as described by

Scholander et al. (1957) and concluded that these fish survived by supercooling. Raschack (1969) attributed a lowering of plasma freezing point in the sculpin, Myoxocephalus scorpius (caught in the Baltic sea), to an increase in concentration of non-dissociated organic compounds. It was assumed that these compounds acted through solute activity and a rise in body fluid osmolarity. Similarly, Pearcy (1957) observed that the elevated freezing point depression in the blood sera of the winter flounder, Pseudopleuronectes americanus inhabiting subzero waters, could not be explained by elevated levels of sodium chloride. However, Pearcy also concluded that the flounder probably survived due to undetected elements acting in a predictable colligative manner.

The undetected elements accounting for the significant freezing point depression in fish serum were resolved by DeVries and Wohlschlag (1969). These investigators reported the isolation of a macromolecular antifreeze from the blood of Antarctic fishes inhabiting the Ross Sea. The antifreeze was a glycoprotein containing only two amino acids, alanine and threonine. These macromolecules could account for over 30% of the freezing point depression attributed to the sera. DeVries, Feeney and co-workers (1970) characterized these glycoproteins in the Antarctic fish, Trematomus borchgrevinki and Dissostichus mawsoni and found them to have unusual chemical and physical properties. In contrast to most proteins, these glycoproteins were soluble in 10% Trichloroacetic acid. In addition the glycoproteins contributed negligibly to the osmolarity of the blood but exhibited a major depression of the freezing point of water beyond what would be expected to occur by a normal colligative effect. DeVries (1971) demonstrated that these antifreezes lowered the freezing point of a solution but not its melting point, which further illustrated the non-colligative freezing point depression effect of these so called "antifreezes". Confirmation of antifreeze substances in the blood of northern fishes was soon made by other investigators (Scholander and Maggert, 1971; Hargens, 1972).

Antifreeze proteins which lacked a carbohydrate moiety were first reported in the blood serum of the winter flounder, Pseudopleuronectes americanus by Duman and DeVries (1974a). It was estimated that 40% of the freezing point depression of the blood serum from winter flounder can be attributed to the presence of these antifreeze proteins.

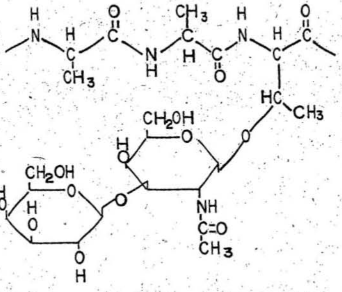
Extensive investigations carried out over the past decade have resulted in the discovery of numerous non-glycoprotein antifreezes in Arctic, sub-Arctic and at least one Antarctic fish. In addition, several slightly different glycopeptide antifreezes from Arctic and Antarctic fishes have been reported. To date, no antifreezes have been found in either freshwater fishes or in temperate fishes which never inhabit ice-laden environments. It is believed that these antifreeze proteins are necessary for the survival of many polar and subpolar fish. Unlike the antifreeze glycoproteins from the Antarctic fish, the flounder antifreeze consists of a group of peptides which are synthesized seasonally. Winter flounder require antifreeze peptides and synthesize them only during the winter months (Duman and DeVries, 1974a, 1974b; Hew and Yip, 1976; Fletcher, 1977). It was the seasonal biosynthesis of the flounder antifreeze peptides that first prompted Hew and co-workers (Hew and Yip, 1976; Hew et al., 1978) to study the regulation of this unique class of proteins.

Two basic types of biological antifreezes have been isolated from polar and north-temperate (sub-Arctic) fishes: the antifreeze glycopeptides (AFGP), and the antifreeze peptides (AFP). These two types differ in their amino acid composition, secondary structure, and absence or presence of carbohydrate. All known antifreezes appear to operate via a similar non-colligative mechanism which lowers the freezing temperature of the serum without lowering the melting temperature. The following section provides a brief survey of known teleost antifreezes. The major biochemical characteristics are described with an emphasis on the structural and chemical studies which have been carried out to elucidate the unique structure function relationship of these molecules.

Glycoprotein antifreezes

AFGP have been identified and charaterized from two members of the family Nototheniidae, *Trematomus borchgrevinki* and *Dissostichus mawsoni* from the Antarctic and five members of the family *Gadidae*, saffron cod (*Eleginus gracilis*), Atlantic cod (*Gadus morhua*), polar cod (*Boreogadus saida*), Labrador rock~~-cod~~ (*Gadus ogac*) and frost fish (*Microgadus tomcod*) (DeVries *et al.*, 1970; Shier *et al.*, 1972; 1975; Raymond *et al.*, 1975; Van Voorhies *et al.*, 1978; Osuga and Feeney, 1978; Hew *et al.*, 1981; Fletcher *et al.*, 1982b). All of the glycoprotein antifreezes have similar, if not identical, structures in which the basic repeating unit is a glycotripeptide of alanine-alaninethreonine with the disaccharide β-D-galactopyranosyl-(1→3)-2-acetamide-2-deoxy-α-D-galactopyranose linked to the threonine residue (Fig. 1). Electrophoretic analysis separates the Notothenidae glycopeptides into 8 separate components ranging from 2600 to 33,000 daltons in which the tripeptide unit is repeated up to 50 times

Figure 1. Basic repeating structural unit of the antifreeze glycopeptide. The polypeptide is composed of a simple Ala-Ala-Thr, with Thr glycosidically linked to the disaccharide galactosyl-N-acetylgalactosamine. Two additional Ala are at the COOH-terminal end.



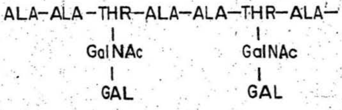

ALA–ALA–THR–ALA–ALA–THR–ALA–
GalNAc
GAL
GalNAc
GAL

Table 1. Properties of antifreeze glycoproteins and glycopeptides (AFGP)

Component	Relative Amount in Serum (%)	Approx # (f) Glycotripeptides Ala-Ala-Thr	Molecular wt			Relative Activity
			(g)	a	b	
1	2	?	32200		33700	
2	4	35	29200	25000	28800	Strong
3	12	30	26000	21000	21500	Strong
4	12	24	18000	17000	17000	Strong
5	12	16	11400	11000	10500	Strong
6	8	9	-	6000[d]	7900	Weak[e]
7	20	5[c]	-	3288[d]	3500	Weak[e]
8	30	4[c]	2500	2646[d]	2600	Weak[e]

(a) and (b) for Antarctic glycoprotein antifreezes.

(a) From Feeney, 1982
(b) From DeVries, 1982
(c) Based on amino acid sequence
(d) AFGP 6-8 have Pro substituting for Ala following Thr
(e) Small components have 25% the antifreeze activity of large AFP on a weight basis
(f) Complete sequence has two additional Ala
(g) Molecular weights for frostfish, *Microgadus tomcod* from Fletcher *et al.* (1982b)
Note: These values are included for comparison of molecular weights and do *not* bear any relationship on other values included in the table.

(Modified from Feeney, 1982).

(Table 1) (DeVries et al., 1970; 1971; Feeney, 1982). The carboxyl terminal of each polymer ends with one or two alanyl residues. The smaller glycopeptides (2600-8000 molecular weight) are composed of the same repeating glycotripeptide with the exception that proline occassionally replaces alanine (Lin et al., 1972; Morris et al., 1978; Osuga and Feeney, 1978). Amino acid sequence determination show that the positions of the proline vary from species to species (Morris et al., 1978; Osuga and Feeney, 1978; Hew et al., 1981).

Although the smaller glycopeptides (6 to 8) are present in the serum at concentrations several times the combined concentrations of the other glycopeptides, they contribute less to the overall antifreeze activity of the serum (DeVries et al., 1970; Feeney and Yeh, 1978; Schrag et al., 1982). The larger glycopeptides (1 to 5) lower the freezing point more than the small ones on a weight basis (Schrag et al., 1982). The glycopeptides are present in the blood at concentrations of 3.5% (w/v) contributing 30% of the freezing point activity of the serum. Glycopeptide 8 in Nototheniid fish appears to be a mixture of three identical-sized molecules (Lin et al., 1972; Morris et al., 1978).

The saffron cod, Eleginus gracilus from the Bering Sea (Raymond et al., 1975) and the tomcod, Microgadus tomcod (Fletcher et al., 1982b), also have similar glycopeptide antifreezes. They differ from those of the Antarctic fishes and other polar cods in several respects. They have a different number of electrophoretic variants (3 to 6) with different molecular weights and in the smaller glycopeptides arginine replaces one or two of the threonine residues (Raymond et al., 1975; Fletcher et al., 1982b; O'Grady et al., 1982c). It has also been reported by DeVries

(1982) that the AFGP from the saffron cod and tomcod have less non-colligative antifreeze activity than equivalent concentrations of antifreezes isolated from Antarctic fish and other Arctic Gadoids.

Extensive chemical and physical studies have been conducted on AFGP. Physical studies which include Raman spectroscopy, quasi-elastic light scattering, nuclear magnetic resonance spectra (NMR) and high vacuum circular dichroism spectra suggests that some of the AFGP have a partially extended conformation (Tomimatsu et al., 1976; Ahmed et al., 1975, 1976, 1981; Bush et al., 1981). However, other investigations using techniques which include x-ray diffraction and natural abundance C-13 NMR yield less definite information on AFGP secondary structure (Raymond et al., 1977; Franks and Morris, 1978; Berman et al., 1980). The exact nature of the secondary structure of AFGP remains in question. Degradation of the glycopeptide chains causes extensive reduction in antifreeze activity (Komatsu et al., 1970; Chuba et al., 1973; Feeney and Yeh, 1978). This implies that antifreeze activity resides in the macromolecule itself and not in the glycotripeptide unit. Chemical and enzymatic modifications of the carbohydrate side chain, such as periodate oxidation and acetylation, show that the hydroxyl groups of the carbohydrate moiety are important for the glycoprotein antifreeze function. These studies have been reviewed by Feeney and Yeh (1978).

From an evolutionary standpoint, the glycopeptides pose an interesting story. All antifreeze glycopeptides are composed of the basic tripeptide unit of alanine-alanine-threonine with a disaccharide linked to the threonine unit. All members of the Antarctic Nototheniidae have identical glycopeptides and some members of an unrelated family in the opposite hemisphere (Gadidae) have nearly the same glycopeptides (Osuga and Feeney, 1978; Van Voorhies et al., 1978; Hew et al., 1981). In contrast, two members of the same family (Gadidae), the sympatric species tomcod and saffron cod, have evolved antifreeze glycopeptides with minor differences. This suggests that less variability is tolerated in some fish occupying certain habitats and that the basic tripeptide unit is highly conserved in all AFGP. The minor variability that is noted is attributed to a few residues found in the smaller components. The significant similarity in AFGP poses an intriguing question with respect to the function and the evolution of their antifreeze genes.

Peptide antifreezes

Antifreeze polypeptides (AFP) have been identified in, and isolate[illegible] from a number of north-temperate and Arctic fish, including two members of the family Pleuronectidae (winter flounder, Pseudopleuronectes americanus, and the Alaskan plaice, Pleuronectes quadritaberculatus), three cottids (Bering Sea sculpin, Myoxocephalus verrucosus, shorthorn sculpin, Myoxocephalus scorpius, and the sea raven, Hemitripterus americanus) and three zoarcids (ocean pout, Macrozoarces americanus, Antarctic eelpout, Rhigophila dearborni and the polar eelpout Lycodes polaris). In contrast to AFGP, considerable diversity in size, composition and structure is found in the peptide antifreezes (Table 2). The data have recently been reviewed by several authors (Feeney and Yeh, 1978; DeVries, 1980, 1982, 1983; Hew, 1981).

Antifreeze polypeptides from the winter flounder Pseudopleuronectes americanus) have been extensively studied (Duman and DeVries, 1974a, 1976; Hew and Yip, 1976; Fletcher, 1977, 1981; Lin and Gross, 1981; Davies et al., 1982). In spite of these studies, considerable controversy exists

Table 2. Properties of fish antifreeze proteins (AFP)

	Polypeptide Antifreezes							
Family	Pleuronectidae		Cottidae			Zoarcidae		
	Winter Flounder(a)	Alaskan Plaice(b)	Bering Sea Sculpin(c)	Shorthorn Sculpin(d)	Sea Raven(e)	Ocean Pout(f)	Antarctic Eelpout(g)	Polar Eelpout(h)
Amino Acids (mol%)								
Asx	11.5	6.4	6.1	6.4	10.7	12.0	NR	5.5
Thr	10.7	12.8	6.1	6.4	7.9	11.4		5.6
Ser	3.0	2.1	2.0	1.6	8.2	9.7		1.9
Pro	0	0	0	1.6	6.7	8.9		0
Glx	2.8	2.1	6.1	3.2	9.1	12.4		9.3
Gly	0.0*	0	2.0	1.6	8.1	6.4		3.7
Ala	60.6	72.4	63.3	60.8	14.4	11.2	Major amino acid	51.6
1/2 Cys	0	0	0	0	7.6	0.8		0
Val	0	0	0	0	1.2	11.0		1.9
Met	0	0	2.0	2.4	5.4	1.2		1.9
Ile	0	0	2.0	1.6	1.7	6.8		3.7
Leu	5.6	0	6.1	4.0	6.2	4.7		9.3
Tyr	0	0	0	0	1.2	1.3		0
Phe	0	0	0	0	2.0	1.5		0
Lys	2.7	2.1	2.0	8.0	2.1	6.2		3.7

Table 2. (continued)

Family	Polypeptide Antifreezes							
	Pleuronectidae		Cottidae			Zoarcidae		
	Winter Flounder(a)	Alaskan Plaice(b)	Bering Sea Sculpin(c)	Shorthorn Sculpin(d)	Sea Raven(e)	Ocean Pout(f)	Antarctic Eelpout(g)	Polar Eelpout(h)
His	0	0	0	0	2.5	0	NR	0
Trp	0	0	0	0	2.8	ND		0
Arg	2.7	2.1	2.0	2.4	2.3	0.5		1.9
Number of components	2(a) 3(i)	3(b)	6(c)	3(j)	2(e)	8(k)	4	NR
Size of components	3,300-8,000(a)(i)	3,000 to 8,000	~5,000	~10,000	~14,000	5,000-6,000(k)	~6,000(l)	~5000
Secondary structure	α-Helix	ND	ND	α-helix	β-structure	non-repeat(*)	ND	ND
Sensitivity to Dithiothreitol	No	ND	ND	No	Yes	No	ND	ND

(a) *Pseudopleuronectes americanus* (Davies et al., 1982) average amino acid composition
(b) *Pleuronectes quadritaberculatus* (DeVries, 1980)
(c) *Myoxocephalus verrucosus* (DeVries, 1980)
(d) *Myoxocephalus scorpius* (Hew et al., 1980)
(e) *Hemitripterus americanus* (Slaughter *et al.*, 1981)
(f) *Macrozoarces americanus* (Hew, 1981)
(g) *Rhigophila dearborni* (DeVries, 1980)
(h) *Lycodes polaris* (DeVries, 1980)
(i) (DeVries, 1982)
(j) (Fletcher *et* al., 1982a)
(k) (Hew *et* al., 1984)
(l) (Eastman *et al.*, 1979)

* The c-terminal glycine was removed by post-translational modification
* (Slaughter, personal communication)
NR Not reported
ND Not determined

over the number, size and amino acid sequence of the flounder AFP. A major fraction containing molecules of approximately 10,000 daltons, as determined by gel filtration analysis, was described by Hew and Yip (1976). This fraction was further separated into two smaller components of similar size (Davies et al., 1982). DeVries (1982) reported the presence of three AFP (3200-8000 daltons) in winter flounder inhabiting waters off the coast of Nova Scotia. In addition, primary sequence studies on the AFP are in disagreement (Lin and Gross, 1981; Davies et al., 1982). The small size, and unusual gel filtration properties of these polypeptides (Davies et al., 1982) have contributed to this controversy. The poor staining qualities of flounder AFP following electrophoresis (DeVries and Lin, 1977a; Sclater, 1979; Pickett et al., 1983) and the abnormal solubility in most protein precipitating agents (DeVries and Wohlschlag, 1969; Feeney and Yeh, 1978) have also delayed the complete characterization of these proteins.

The amino acid composition of winter flounder AFP is unusual. Flounder AFP contains only 9 different amino acids of which alanine accounts for 60% of the residues (Duman and DeVries, 1974a, 1976; Hew and Yip, 1976; Davies et al., 1982). Most of the remainder are polar residues such as lysine, serine, aspartate, glutamate and threonine. Unlike the AFGP, the secondary structure of flounder AFP is well described. Both viscosity and circular dichroism (CD) studies indicate that these polypeptides exist primarily as an α-helical (greater than 80%) configuration at -1°C (Ananthanarayanan and Hew, 1977a; Raymond et al., 1977).

DeVries (1980) has reported that the Alaskan plaice, Pleuronectes quadritaberculatus, has evolved peptide antifreezes which share close identity with those of the flounder. These similarities include: size and number of peptides, protein sequence and amino acid composition. The only major difference appears to be the lack of leucine in the Alaskan plaice. It is interesting to note that a close relative, the American plaice (Hippoglossoides platessoides), found off the coast of Labrador and Newfoundland, lacks antifreeze proteins (Fletcher, personal communication).

Antifreeze polypeptides have been identified and characterized from two sculpins, Bering Sea sculpin (Myoxocephalus verrucosus) (Raymond et al., 1975; Raymond, 1976) and the shorthorn sculpin (Myoxocephalus scorpius) from the waters of Newfoundland and Ellsmere Island (Hew et al., 1980; Fletcher et al., 1982a). There are at least six electrophoretic variants of approximately the same molecular weight (5000 daltons) isolated from the Bering Sea sculpin and three different components identified by high pressure liquid chromatography (~ 10,000 daltons) from the two populations of shorthorn sculpins. Differences in isolation and identification techniques precludes accurate comparisons of size and number of AFP between these two different species. The sculpins M. scorpius and M. verrucosus share close taxonomic identity and their AFPs exhibit similar amino acid compositions. It is interesting to note that the AFP from the sculpins and the winter flounder have similar amino acid compositions and secondary structure. Sculpin antifreezes contain approximately 60% alanine and are rich in the polar residues aspartate, threonine, glutamate and lysine. They differ from the flounder AFP in that they also contain the nonpolar amino acids proline, methionine and isoleucine (Hew et al., 1980; Fletcher et al., 1982a). Sculpin AFP have a high α-helical secondary structure and have an antifreeze activity similar to flounder AFP (Hew et al., 1980; DeVries, 1982, 1983; Fletcher et al., 1982a). The structural and biochemical homologies noted between the sculpin and flounder antifreezes have prompted Hew and co-workers (Hew et al., 1980; Fletcher et al., 1982a) to suggest that they may belong to the same family of AFP.

The AFP isolated from the sea raven, Hemitripterus americanus, are different from all other fish antifreeze proteins (Slaughter et al., 1981). The sea raven contains one major protein of molecular weight ~14,000 and differs from other AFP in its amino acid composition, secondary structure and immunological specificity. Amino acid analysis of sea raven antifreeze showed that it contains a large proportion of half-cystine, hydrophilic amino acids, and only an average amount of alanine. As noted previously, sculpin and flounder AFP contained 60% alanine and no half-cystine residues. Furthermore, the sea raven AFP protein is sensitive to sulfhydryl reducing agents. Circular dichroism studies indicated the absence of significant amounts of α-helix and the possible presence of β-structure. Antibodies raised against sea raven AFP did not cross-react with AFP from shorthorn sculpin and winter flounder. Consequently, it is suggested that sea raven AFP represents a separate type of fish antifreeze.

The structural diversity of AFP has been further illustrated in the recent description of another type of antifreeze found in the Newfoundland ocean pout (Macrozoarces americanus). In a previous report the serum of the ocean pout was reported to contain an antifreeze (Duman and DeVries, 1975) but only recently has this AFP been identified and investigated

(Hew et al., 1984). The ocean pout contains a complex mixture of at least 8 AFP components of similar size (6000 daltons). These are judged to be separate entities based on polyacrylamide gel electrophoresis, ion-exchange chromatography and reverse phase high pressure liquid chromatography. These components fall into two distinct groups, based on their ion-exchange chromatography behaviour and immunological properties. Amino acid analysis demonstrated that ocean pout AFP contains most of the twenty amino acids but lacks the abundance of alanine found in the flounder and shorthorn sculpin polypeptides as well as the high half-cystine residues reported in sea raven antifreezes. The ocean pout AFP also appears to be unique with respect to its non-repeating structure which is different from the secondary structures reported for the other AFP (based on CD studies, Don Slaughter personal communication).

In addition to the ocean pout, two other fish antifreezes from the family Zoarcidae, have been described. AFP from the polar eelpout (Lycodes polaris) (DeVries, 1980) has an average molecular weight of 5000 and exhibits a similar amino acid composition to that reported for sculpins and flounder. The polar eelpout has a high amount of alanine and polar residues but differs in that it also contains leucine and valine residues. Neither the exact number of components nor the secondary structure for the AFP of Lycodes polaris has been reported. The Antarctic eelpout, Rhigophila dearborni has four electrophoretic variants, containing 12 amino acids of which the principal component is alanine (DeVries, 1980). Like the AFP from L. polaris, the AFP of the Antarctic eelpout also contains valine. Rhigophila dearborni has the singular distinction of being the only Antarctic fish identified to date that does not contain a glycopeptide antifreeze. At this time it is difficult to make a rigorous comparison between the zoarcids mentioned above since the AFP characteristics for R. dearborni and L. polaris have not been fully reported. However, from the limited comparison which can be made, it appears that considerable AFP diversity is present in this family of fishes.

The recent description of yet another type of AFP from the oceanpout Macrozoarces americanus, has added additional complexity in understanding the mode of action of antifreezes. Initial analysis of antifreeze glycopeptides from Arctic and Antarctic fishes suggested that a common, highly conserved structure of a repeating glycotripeptide may be a universal feature of all teleost antifreezes. The discovery of a different AFP from flounder and shorthorn fishes suggested that the larger amount of alanine may be necessary for activity since it was a common characteristic in both AFP and AFGP. In fact, the demonstration of freezing point depression from a synthetic polypeptide containing alanine (65%) and aspartic acid (35%) seemed to support this contention (Ananthanarayanan and Hew, 1977a). The recent isolation of AFP from the sea raven and ocean pout, however, demonstrates that their functional activity is not dependent upon an abundance of alanine. The discovery of sea raven AFP, which has an unusually high half-cystine content and a moderate amount of alanine, represents yet a third class of AFP. The insect antifreeze isolated from Tenebrio molitor (Patterson and Duman, 1979; Schneppenheim and Theede, 1980; Duman, 1982) and from the spruce budworm, Chonistenura fumiferra (Hew et al., 1983) also have a high half-cystine content and may be similar to that of sea raven AFP. The characterization of ocean pout AFP as being completely different from all other known biological antifreezes is good evidence for a fourth class of antifreeze. The significant concentrations of alanine, threonine and aspartic acid in all of the above AFP may suggest a common role for their activity requirements (Hew, 1981). Definitive evidence for this will require further considerations concerning their conformation and orientation in ice during freezing interactions. At the present, although all known fish biological antifreezes share a common non-colligative affect on freezing point depression, the structural and biochemical diversity of AFP and AFGP make it difficult to propose that they operate via a common mechanism.

Functional activities of antifreeze proteins

All known antifreeze proteins (both AFGP and AFP) share the following characteristics:

(1) Their effect on freezing temperature are non-colligative; i.e. they lower the freezing point much more than would be expected on the basis of the osmolality of their solutions.

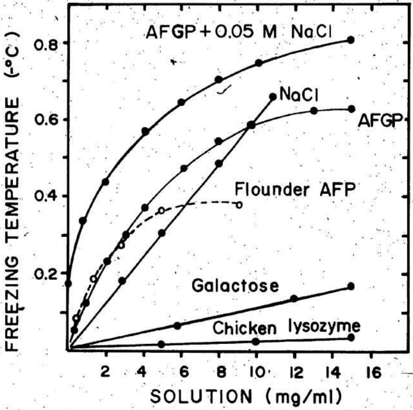
(2) They have a thermal hysteresis, or the ability to depress the freezing temperature without affecting the melting point. Melting temperatures are affected in a colligative manner.

(3) Freezing point depression due to antifreeze proteins is additive with that due to solutes having colligative effects, i.e. antifreeze proteins depress the freezing temperature additively with salt.

(4) Plots of thermal hysteresis or freezing point depression versus antifreeze concentrations are convex rather than linear.

To date, the only function attributed to antifreeze proteins is one of preventing the body fluids from freezing. One of the most significant properties of these molecules is their ability on a molal basis of depressing the freezing point of a solution 200 to 300 times more than what is expected from normal colligative properties (DeVries et al., 1970; DeVries, 1971). Figure 2 demonstrates that the active antifreeze

Figure 2. Effects of antifreezes, salts and proteins on freezing. (Flounder data from Slaughter *et al.* (1981), all other data adapted from Feeney et al. (1981), Figure 2).


AFGP + 0.05 M NaCl
NaCl
AFGP
Flounder AFP
Galactose
Chicken lysozyme
FREEZING TEMPERATURE (-°C)
0.8
0.6
0.4
0.2
2
4
6
8
10
12
14
16
SOLUTION (mg/ml)

peptides are much more effective in lowering the freezing temperature than calculations from their molecular weight would suggest or when compared to other comparably sized proteins or a comparable weight of sodium chloride (DeVries et al., 1970; Feeney et al., 1981). It has been calculated that a concentration of winter flounder antifreeze of 25 mg/ml would on a colligative basis, only be expected to contribute 0.005°C to the freezing point depression of a solution (~~Slaughter~~ and Hew, 1981). Yet winter flounder serum samples taken in mid-winter contain 10 mg/ml of AFP which changes the freezing temperature by -0.65°C (Fletcher, 1977). It is this unusual freezing point depression characteristic which has been termed "antifreeze activity" where it is stressed that these macromolecules lower the freezing temperature in a non-colligative manner. Presumably, if antifreeze proteins acted through a colligative means to attain their equivalent non-colligative antifreeze activity, the osmotic pressure exerted by the concentrations of these substances would be in excess to what the organism could tolerate.

Pauling (1953) has defined the freezing point of a solution as the temperature at which the vapour pressure of the solid phase (ice) is equal to the vapour pressure over the liquid phase. This means that if the system is in thermal equilibrium between solid and liquid phases, the freezing point and the melting point would be the same. In salt solutions and other biological solutions lacking antifreezes the equilibrium freezing point can be estimated by determining the melting point of a small ice crystal provided the size of the crystal is small relative to the volume of the solution and the rate of warming, or cooling is slow. This relationship does not hold for antifreeze solutions. Solutions containing glycoprotein or peptide antifreezes have a significant difference or "hysteresis" between the freezing temperature and the melting point. In addition, antifreezes have little or no effect on the melting temperature of the solution (DeVries, 1971; Feeney and Hoffmann, 1973; Slaughter and Hew, 1981). Determination of the freezing points of solutions containing biological antifreezes reveal that the melting point of the solid phase (seed ice crystal) does not change and occurs at a temperature predicted by a colligative relationship; however, the freezing point (temperature at which the ice crystal propagation occurs) is much lower than the melting point (DeVries, 1971; Scholander and Maggert, 1971; Raymond and DeVries, 1972; Feeney and Hofmann, 1973; Tominatso *et al.*, 1976; Slaughter and Hew, 1981). For example, DeVries (1971) and Hargens (1972) have demonstrated that in the blood serum from the Antarctic fish inhabiting -1.9°C water, the initial ice crystal or "seed crystal" melts at approximately -1.0°C while ice crystal freezing (rapid propagation) occurs at -2.2°C. In comparison, the affect on winter flounder is not as large. Flounder inhabiting water temperatures of -1.2°C to -1.4°C have a serum freezing point of -1.47°C, a serum melting point of -0.71°C and a resulting thermal hysteresis of -0.76°C (DeVries, 1974). The antifreeze's unique characteristic of thermal hysteresis is considered to be closely tied to the non-colligative mechanism of freezing point depression (Feeney and Yeh, 1978; DeVries, 1980, 1982, 1983).

Two other distinct activity characteristics of antifreeze molecules are evident in Figure 2. Plots of thermal hysteresis (or freezing point depression) versus antifreeze concentrations are convex rather than linear. There is a significant saturation effect above certain concentrations of antifreezes. This generally becomes evident at concentrations greater than 6 mg/ml for most antifreezes. This saturation phenomenon is absent in solutions containing other molecules such as sodium chloride, that utilize colligative properties to effect a freezing point depression (Feeney and Yeh, 1978; Feeney et al., 1981; Slaughter and Hew, 1981; DeVries, 1983). This suggests that the mechanisms for non-colligative freezing point depression are unique. The other characteristic shown in Figure 2 is that antifreezes depress the freezing temperature of a solution additively with salt. This is not surprising since two independent mechanism are involved: (1) non-colligative antifreeze activity and (2) colligative or solute concentration effect of salts. This additive effect is critical for the survival of the organism. In many situations, the temperature of the teleost habitat would require that antifreeze proteins be supplemented by other serum components (e.g. salts) in order to give the necessary protection from freezing.

It should be noted that several investigators have reported other activity characteristics attributed to some antifreeze molecules such as formation of ice lattice patterns, unusual crystal growth, and potentiation of antifreeze activity by small AFGP (Feeney and Yeh, 1978; Osuga et al., 1978; Feeney, 1982). Some of these characteristics (i.e. potentiation of antifreeze activity) remain highly controversial as to their nature and presence (Osuga et al., 1978; Schrag et al., 1982; Schrag and DeVries, 1983). In many cases the reports have been limited to a few studies and it is not known if the particular characteristic holds true for all antifreeze proteins.

The unusual activity characteristics attributed to both AFGP and AFP has generated considerable scientific interest on their mode of action. Mechanisms for their action have been proposed that include the absorption of antifreeze molecules to ice and the inhibition of ice crystal growth (Raymond, 1976; Raymond and DeVries, 1977), and the inhibition of nucleation (Feeney and Yeh, 1978). Considering the structural and biochemical diversity exhibited by fish antifreezes, it is difficult to envision a common mechanism of action. In view of this, it has been suggested that antifreeze molecules may have attained common functions through similar stearic properties achieved by different conformational means (Hew, 1981). The theoretical aspects of antifreeze mechanisms have been extensively reviewed by several authors (Feeney, 1974, 1982; Raymond, 1976; Raymond and DeVries, 1977; Feeney and Yeh, 1978; Franks and Morris, 1978; Yeh and Feeney, 1978; DeVries, 1980, 1982, 1983).

Presence of antifreezes and measurement of their activity

It is not surprising that the ability to avoid freezing and the presence of different amounts of serum antifreezes is directly correlated with the environment. Fish inhabiting the coldest marine environments, such as Antarctic and high Arctic waters, have more serum antifreezes than teleosts living in polar and sub polar waters (DeVries, 1980). For example, Antarctic fish live in waters with an average temperature of -1.8°C and contain over 25 mg per ml of serum AFGP (Feeney and Yeh, 1978), whereas winter flounder inhabit waters with an average temperature of -1.4°C in the winter, and possess 6 to 11 mg per ml of serum AFP (Slaughter and Hew, 1982). In addition, fish living in waters that have a marked fluctuation in temperature are capable of seasonally regulating their antifreeze biosynthesis. Therefore, it appears that nature has finely adjusted the biological adaptation of antifreezes to meet specific habitat requirements. As noted by DeVries (1980):

> "In general, there is agreement between the organismal freezing point of a fish and the temperature at which ice will propagate in its blood or extracellular fluid. For almost all cold water fishes the blood freezing points are a few tenths of a degree lower than the freezing temperature of the specimen, indicating that freezing is probably initiated in some fluid other than the blood. There is also a correlation between the blood freezing point, the fishes' freezing temperature and environmental temperature."

To date, most investigations of fish antifreezes have utilized blood serum and its presence in other body fluids has largely been ignored. From a technical point of view, this is not surprising. The ease with which blood can be sampled, the serum analysed for antifreeze activity, and the potential for large scale isolation have been the main factors precluding detailed investigation of antifreezes in other body tissues and fluids. Yet, it is obvious that fish living in subzero waters face the risk of ice-nucleation and cryoinjury in many parts of the body. For example, freezing could occur in the intestinal fluid following ingestion of ice during feeding or at the water-integument surface of the gills.

DeVries (1982) has briefly reviewed the existence of eight antifreeze glycopeptides found in the blood, pericardial fluid, coelomic fluid, intestinal fluid, bile and cerebral spinal fluid of Antarctic fishes. Interestingly, only the smaller glycopeptides (under 7000 daltons) are present in the intestinal fluids of these fish. The cerebral spinal fluid, bile and egg fluids of the shorthorn sculpin, M. scorpius, and winter flounder, P. americanus, also contain antifreezes which are similar, if not identical, to those found in blood (Fletcher, personal communication). In contrast to many Antarctic fish, no antifreezes have been detected in the intestinal fluids of these two fish. It has also been reported that ice propagation is inhibited by peptides with antifreeze properties at the membrane-cytoplasm interface in skin integument of the shorthorn sculpin (Schneppenheim and Theede, 1979). This would undoubtedly be important for areas of the body such as the gill filaments, which have maximum exposure, to the surrounding freezing waters and are not covered by protective scales or mucous.

For many fish we still lack adequate information on which tissues or fluids have antifreezes. The information that is available, has led to some interesting questions concerning the nature and distribution of these antifreezes. For example, the presence of an egg antifreeze is the first indication of an intracellular antifreeze. The mode of action and biosynthesis of this antifreeze is extremely interesting. The recent discovery of antifreezes in the intestinal fluids of polar fishes (O'Grady _et al._, 1982b) deserves special consideration due to the nature of its renal conservation and lack of degradation. At present, it is not clear why the many forms of antifreeze exist and whether they all play a role in protection of the body fluids from freezing.

It is difficult to compare the potential range of antifreeze activity possessed by the different teleost antifreezes since studies have involved different means of measurement. Antifreezes are large macromolecules that remain in the non-dialyzable fraction of serum following dialysis with a membrane of 3000 molecular weight cut off. Thermal hysteresis measurements and differences detected in freezing point depression between dialyzed and non-dialyzed serum are excellent indicators of biological antifreezes (Table 3). In many studies, however, the contribution of

Table 3. Presence of biological antifreezes in fish: detected by freezing point depression and thermal hysteresis measurements*.

7

Species	Percent antifreeze contribution to freeze point depression of blood serum	Location sample taken	Freezing temperature -(C°) of blood plasma(A)		Thermal hysteresis -(C°) of plasma(B)	
			before dialysis	after dialysis	freezing point	melting point
Homo sapiens(1) (humans)	NP	-	0.56	0.01	-	-
Salmo gairderii(1) (rainbow trout)						
Mallotus villosus(2) (capelin)	NP	Arctic	1.18	0.01	-	-
Boreogadus saida(2) (polar cod)	>33	Arctic	2.11	0.63	-	-
Dissostichus mawsoni(2)	-	Antarctica	1.99	0.49	-	-
Chaenocephalus aceratus(3)	-	Antarctica	1.50	0.6	-	-
Gymnodraco acuticeps(4)	>60	Antarctica	-	-	1.33	0.03
Trematomus borchgrevinki	>30	Antarctica	2.07(1)-2.7(3)	0.56(1)-1.3(3)	1.31(4)	0.03
Trematomus bernachi(4)	>40	Antarctica	-	-	1.29	0.04
Trematomus loennbergii(5)	>60	Antarctica	-	-	1.15	0.05
Trematomus centronotus(5)	-	Antarctica	-	-	1.46	0.05
Trematomus hansoni(4)	>60	Antarctica	-	-	1.28	0.05
Trematomus lepidorhinus(5)	-	Antarctica	-	-	1.09	0.04
Rhigophila dearborni(4) (Antarctic eelpout)	>50	Antarctica	-	-	0.67	0.06

Table 3 (continued)

Species	Percent antifreeze contribution to freeze point depression of blood serum	Location sample taken	Freezing temperature -(C°) of blood plasma(A)		Thermal hysteresis -(C°) of plasma(B)	
			before dialysis	after dialysis	freezing point	melting point
Myoxocephalus aeneus(5) (grubby sculpin)	-	Nova Scotia	-	-	0.70	0.04
Myoxocephalus verrucosus(6) (Bering Sea sculpin)	-	Arctic	-	-	2.3(c)	0.9
Myoxocephalus scorpius (shorthorn sculpin)	>25(7)	Newfoundland and Arctic	1.3(7)	0.4	0.79(5)	0.22
Eleginus gracilis(6) (saffron cod)	-	Arctic	-	-	2.1(c)	1.1
Gadus ogac(3) (Labrador rock cod)	-	Arctic	-	-	2.2(c)	1.1
Microgadus tomcod(8) (frost fish or tomcod)	-	Newfoundland	-	-	0.92(c)(d)	0.72
Gadus morhua(9) (Atlantic cod)	-	Newfoundland	-	-	1.01(c)(d)	0.75
Osmerus mordax(5)	>32	Nova Scotia	-	-	0.4	0.01
Macrozoarces americanus(10) (oceanpout)	>40	Newfoundland(10) Nova Scotia(5)	1.7	0.68	0.41(5)	0.02
Anarchichas lupus(5) (wolffish)	-	Nova Scotia	-	-	0.42	0.01
Limanda ferruginea(5) (yellowtail flounder)	-	Nova Scotia	-	-	0.27	0.01

Table 3 (continued)

Species	Percent antifreeze contribution to freeze point depression of blood serum	Location sample taken	Freezing temperature -(C°) of blood plasma(A)		Thermal hysteresis -(C°) of plasma(B)	
			before dialysis	after dialysis	freezing point	melting point
Pseudopleuronctes americanus (winter flounder)	>40(12)	Newfoundland Nova Scotia	1.4-1.5(11)	0.69	0.65(5)	0.01
Hemitripterus americanus(13) (sea raven)	>35	Newfoundland(12) Nova Scotia(5)	1.2(13)	0.5	0.4(5)	0.02

* Antifreezes present unless otherwise noted. Comparison of antifreeze-activity cannot easily be made between species since many variations involving sample preparation and technique differences have been noted. This table only indicates the presence of antifreezes.

(1) Feeney and Yeh (1978)
(2) Osuga and Feeney (1978).
(3) DeVries (1980)
(4) DeVries and Lin (1977b)
(5) Duman and DeVries (1975)
(6) Raymond *et al.* (1975)
(7) Fletcher *et al.* (1982a), Hew *et al.* (1980)
(8) Fletcher *et al.* (1982b)
(9) Hew *et al.* (1981)
(10) Hew *et al.* (1984)
(11) Fletcher (1977); Hew and Fletcher (1979)
(12) Duman and DeVries (1976)
(13) Slaughter *et al.* (1981)
NP No antifreezes present
(A) Measurements made on freezing point osmometer unless otherwise noted
(B) Measurements made by ice-crystal observation method on dialyzed serum unless otherwise noted
(C) Serum most likely not dialyzed
(D) Slaughter and Hew (1981) modified freezing point osmometer technique

serum electrolytes and other small molecules is noted but largely ignored since the major changes in thermal hysteresis or freezing point depression is due to antifreezes. Consequently, the investigator can often get an early indication of the presence of antifreezes in crude sera. Accurate estimations of antifreeze concentrations requires the subtraction of the contribution of dialyzable solutes or the use of a purified, dialyzed sample.

All known biological antifreezes appear to operate via a non-colligative mechanism. They exhibit a freezing point depression without affecting the melting temperature of a solution. Two methods have been used for detecting antifreezes and measuring their relative activity. One method utilizes the freezing point osmometer (i.e. from Advanced Instruments Inc., Needham Heights, Mass.) which determines the freezing temperature of a solution by sensing the heat of fusion during ice formation (Hew and Yip, 1976; Feeney and Yeh, 1978). A second means of measuring biological antifreezes employs a microscopic observation method in which ice crystal growth and shrinkage is determined as a function of temperature (DeVries, 1971; Scholander and Maggert, 1971; Feeney and Hofmann, 1973). This method allows an accurate measurement of thermal hysteresis on small quantities of sample (e.g. Clifton Nanoliter Osmometer, Clifton Technical Physics, Hartford, N.Y., USA). Both methods offer a different set of advantages and disadvantages (Table 4) depending on the nature of the investigation. There is a good correlation between thermal hysteresis activity (using the ice crystal observation method) and measurement of freezing point depression (using freezing point osmometer), provided that the antifreeze concentration of the solution is within the

Table 4. Techniques of antifreeze protein measurement: advantages and disadvantages.

Method	Advantage	Disadvantage
Freezing point osmometer	(1) Rapid analysis of numerous samples (2) Can be performed on crude samples (3) Easily mastered technique	(1) Solutions are rapidly cooled which does not allow one to study variation in freezing due to relative rates of freezing. (2) Some antifreezes are sensitive to rapid supercooling giving large variations in freezing point measurements (Schrag et al., 1982; Schrag and DeVries, 1983). (3) Does not allow measurement of melting temperature and therefore precludes thermal hysteresis measurements.(A) (4) Not sensitive: Difficult to detect concentrations of antifreezes below 0.5 mg/ml. (A) (5) Solutions containing high concentrations (>6 mg/ml) of antifreeze are easily masked resulting in underestimation of antifreeze concenrations (Slaughter and Hew, 1981). (B). (6) Requires a samples volume of at least 200 μl(A)
Ice crystal observation technique	(1) Direct, eliminates variability due to supercooling (2) Both freezing and melting points are measured. Allows one to calculate thermal hysteresis.	(1) Time consuming, and requires special handling of solutions. Difficult to do large numbers of samples. (2) The technique is not easily mastered. Takes special skill.

Table 4. (continued)

Method	Advantage	Disadvantage
	(3) Small volumes (10-20 µl) are easily measured.	(3) Crude solutions sometimes mask antifreeze thermal hysteresis measurements because ice crystals are not easily seen. In this case special purification or dialysis is necessary
	(4) Very sensitive. Can detect antifreeze concentration of 0.05 mg/ml	
	(5) Antifreeze proteins can easily be identified by their unusual ice spicule formation (Scholander and Maggert, 1971). Allows one to study ice crystal growth over long periods of time.	

Modifications: (A) Slaughter and Hew (1981). More dilute concentrations of antifreeze in solutions (<0.05 mg/ml) can be detected by using a modified freezing point osmometer tube and a silver iodide ice-nucleating agent. This technique also allows detection of melting point and therefore thermal hysteresis estimations can be made. (Generally this modification requires special skill)

(B) Mulvihill et al. (1980). By using chart recorders and increasing temperature of cooling bath the masking of high concentrations of antifreeze in solution and underestimation of antifreeze activity has been overcome.

linear range of detection (Slaughter and Hew, 1981). To date, numerous teleosts have been designated as having antifreeze proteins on the basis of one of the above techniques (Table 3), but little characterization has been carried out on the nature of these antifreezes.

The osmometer measures osmolality which is defined as the osmotic concentration of osmotically active particles (units; milli Osmol/kg H_2O). To describe the freezing resistance of fish sera in temperature units, it is necessary to make the following mathematical conversion. When one mole of non-ionic charged solute is added to one killogram of water, the colligative properties of the resulting solution lowers the freezing point by 1.86°C. If one equates the freezing point to the temperature of ice formation in the serum of a fish~~, by common usage~~, there is nearly a linear relationship between osmolality and freezing point such that:

$$1\ \text{mOsmol/kg} = 1.858\ \text{millidegrees C}$$

Sequence studies and genomic organization of fish antifreezes.

Considerable effort has been invested in determining the primary sequence of AFP and the genomic organization of AFP genes. Studies of this kind will provide invaluable information on the structure and regulation of antifreeze genes. Although the primary sequences of some AFGP have been described there are no data available on nucleic acid sequences or the genomic organization of their genes. Except for some preliminary studies (Haschemeyer and Mathews, 1980) involving *in vivo* labelling, there is also a wide gap in our knowledge on the biosynthesis of these proteins. To date, the majority of our information dealing with the synthesis of fish antifreezes and the structure of their genes have involved studies using the winter flounder, Pseudopleuronectes americanus. The fact that this species is readily available and seasonally synthesizes AFP, makes this an excellent system for studying gene structure and gene regulation.

Two different laboratories (Hew and co-workers and Lin and co-workers) working independently have purified and characterized the antifreeze protein mRNA and its complementary DNA (cDNA). The cloning and sequence analysis of antifreeze protein cDNA has increased our understanding of the structure of flounder antifreeze proteins as well as produced a well-defined hybridization probe. A specific cDNA probe for AFP has proven useful in identifying AFP genes in the genome of the winter flounder (Davies et al., 1981, 1984). The following section discusses some of the recent information available on the synthesis of AFP and the sequence organization of its genes. A later section will present our current understanding of the seasonal regulation of AFP biosynthesis.

In recent years, recombinant DNA technology has been extensively used in determining the specific sequence of genes. Full-length cDNA is prepared from isolated mRNA (by avian myeloblastosis reverse transcriptase) and sequenced. A pre-requisite to this step is the isolation and purification of a given mRNA. The mRNA for the serum AFP of the winter flounder was extracted from the liver polysomes of fish caught in November and was purified by oligo-dT-cellulose chromatography and sucrose gradient centrifugation (Davies and Hew, 1980). The length of the mRNA was estimated to be 520 nucleotides from denaturing agarose gel electrophoresis and was in good agreement with the 7.5 S size measurement made from sucrose gradient centrifugation. The mRNA was identified as that coding for flounder antifreeze peptide by the seasonal nature of its appearance in winter and by cell-free translation studies involving specific incorporation analysis using alanine/arginine ratio comparisons (Davies and Hew, 1980). The identity of the 7.5 S RNA as AFP mRNA has since been confirmed by sequence analysis of both DNA and primary sequence determinations of cell-free translation products (Davies et al., 1982).

The molecular weight of the primary translation product was 11,700, estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and by gel filtration in 6 M guanidine hydrochloride (Davies and Hew, 1980; Pickett et al., 1983). This represents an interesting discrepancy since the molecular weights of mature AFP peptides in the serum are much smaller (DeVries and Lin, 1977a; Davies et al., 1982). The AFP mRNA contains sequences of nucleotides which codes for an extra length of peptides not found in the final processed form of antifreeze peptides. That is to say that flounder AFP is synthesized as a preproprotein estimated to be 8000 daltons (based on an 82 amino acid precursor protein by nucleic acid sequence determination) which is much closer to the size of the cell-free translation product. Secondly, from the amino acid sequence data (DeVries and Lin, 1977a), it is clear that both gel filtration and SDS PAGE have given overestimates for the molecular weight of these small peptides. It has been suggested that the unusually high α-helical configuration of flounder AFP has been a major factor contributing to their molecular weight overestimation (Ananthanarayanan and Hew, 1977a; Hew, 1981).

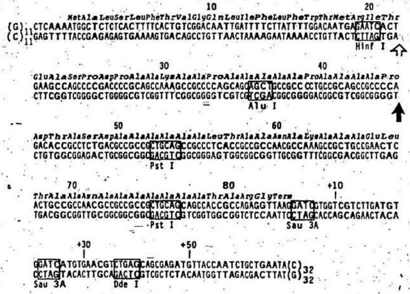
In an independent investigation, Lin and Long (1980) have isolated a 9.5 S AFP mRNA from winter flounder. When the isolated AFP mRNA was analyzed by denaturing polyacrylamide gel electrophoresis, at least two distinct bands of approximately 450 nucleotides in length were visible. It was also reported that cell-free translation products from their AFP mRNA preparation directed the synthesis of one main and two minor components of 12,000 daltons. The identity of these compounds as AFP products was verified by using specific antibodies raised against winter flounder AFP. These results were in contrast to the previous data presented by Davies and Hew (1980) and Davies et al. (1982).

At this time it is difficult to evaluate the differences in data arising from these two sets of investigations. It is not known whether these differences can be attributed to minor variations in processing (i.e. Poly (A) addition to RNA, or cleavage and post-translational modifications to the protein). Alternatively, two different groups of AFP mRNAs may have been discovered in the winter flounder, possibly due to genetic polymorphism in the population or simply multiple genes in a single individual fish. RNA excess hybridization kinetic studies, support the possibility that there are multiple mRNAs coding for the flounder AFP (Lin and Long, 1980; Pickett et al., 1983). Recent analysis of flounder genomic sequences provides further evidence for multiple AFP mRNAs and multiple AFP genes (Davies et al., 1981, 1984).

A cDNA made to purified flounder AFP mRNA was cloned in the plasmid pBR322, and its sequence determined by the method of Maxam and Gilbert (1980) (Davies et al., 1982). In the clone selected for sequence analysis (CT5), the restriction enzyme HpaII was used to cut out the cDNA insert from the CT5 along with short flanking regions of pBR322. An antifreeze cDNA sequence was obtained that had 324 base pairs (bp) along with poly (dG)-poly (dC) homopolymeric tails of 11 and 32 bp at its 5' and 3' ends,

Figure 3. Nucleotide sequence of the cloned antifreeze preproprotein cDNA. Restriction endonuclease sites are shown in boxes. The top row of amino acid sequence corresponds to the signal polypeptide, the second row to the pro- segment, and the remainder to the mature protein. The open arrow marks the putative junction between the signal polypeptide and the pro- segment. The solid arrow marks the end of the pro-segment. Term, termination.

(From Davies *et al.*, 1982)


10
20
MetAlaLeuSerLeuPheThrValGlyGlnLeuIlePheLeuPheTrpThrMetArgIleThr
(G)11CTCAAAATGGCTCTCTCACTTTTCACTGTCGGACAATTGATTTTCTTATTTTGGACAATGAGAATCACT
(C)11GAGTTTTACCGAGAGAGTGAAAAGTGACAGCCTGTTAACTAAAAGAATAAAACCTGTTACTCTTAGTGA
Hinf I
30
40
GluAlaSerProAspProAlaAlaLysAlaAlaProAlaAlaAlaAlaAlaProAlaAlaAlaAlaPro
GAAGCCAGCCCCGACCCCGCAGCCAAAGCCGCCCCAGCAGCAGCTGCCGCCCCTGCCGCAGCCGCCCCA
CTTCGGTCGGGGCTGGGGCGTCGGTTTCGGCGGGGTCGTCGTCGACGGCGGGGACGGCGTCGGCGGGGT
Alu I
50
60
AspThrAlaSerAspAlaAlaAlaAlaAlaAlaLeuThrAlaAlaAsnAlaLysAlaAlaAlaGluLeu
GACACCGCCTCTGACGCCGCCGCTGCAGCCGCCCTCACCGCCGCCAACGCCAAAGCCGCTGCCGAACTC
CTGTGGCGGAGACTGCGGCGGCGACGTCGGCGGGAGTGGCGGCGGTTGCGGTTTCGGCGACGGCTTGAG
Pst I
70
80
+10
ThrAlaAlaAsnAlaAlaAlaAlaAlaAlaAlaThrAlaArgGlyTerm
ACTGCCGCCAACGCCGCCGCCGCTGCAGCAGCCACCGCCAGAGGTTAAGGATCGTGGTCGTCTTGATGT
TGACGGCGGTTGCGGCGGCGGCGACGTCGTCGGTGGCGGTCTCCAATTCCTAGCACCAGCAGAACTACA
Pst I
Sau 3A
+30
+50
GGATCATGTGAACGTCTGAGCAGCGAGATGTTACCAATCTGCTGAATA(C)32
CCTAGTACACTTGCAGACTCGTCGCTCTACAATGGTTAGACGACTTAT(G)32
Sau 3A
Dde I

respectively. Sequence determination of the cDNA indicates that it codes for a precursor protein of 82 amino acids corresponding to a mature AFP polypeptide (38 amino acids), a signal polypeptide (21 amino acids) and a prosequence (23 amino acids) (Figure 3) (Davies et al., 1982). The AFP signal sequence is rich in hydrophobic residues which is typical of signal polypeptides (Shields and Blobel, 1978; Thibodeau et al., 1978). The composition of the prosequence was similar to that of the native protein except that it contains five prolines. The mature protein, but not the prosequence, contains three of the 11-residue repeats (Thr-Ala-X- X-Ala-Ala-X-X-Ala-Ala-X) previously observed (Lin and Gross, 1981) in two other antifreeze protein components. It is believed that this sequence repeat may play an important role in the non-colligative mechanism of antifreeze activity as described in an absorbtion-inhibition model proposed by Raymond and DeVries (1977).

Our current understanding of the biosynthesis of one of the major winter flounder AFP (component A) is outlined as follows: The antifreeze polypeptide is initially synthesized in the liver as a protein precursor, 82 residues long (Davies et al., 1982), encoded by a 7.5 S mRNA (Davies and Hew, 1980; Pickett et al., 1983). In vivo incorporation experiments support the conclusion that following the removal of the signal polypeptide the proprotein circulates in the blood where it is estimated that the pro-section is cleaved within 24-48 hours (Hew et al., 1978). Davies and co-workers (1982) have suggested that since the content of glycine in component A is well below unity as it is for AFP described by Duman and DeVries (1976), the carboxylterminal glycine residue may be lost in most of the AFP by post-translational modification. The net result is the

Table 5. Amino acid compositions of components A and B compared to the composition deduced from the cDNA sequences

Amino Acid Residue	Compositions from amino acid analysis				Composition from cDNA sequence
	Nanomoles		Ratio		
	A	B	A	B	
Asp	146	116	4.3	5.3	4*
Thr	136	88	4.0	4.0	4
Ser	38	30	1.1	1.4	1
Glu	36	4	1.1	0.2	1
Gly	4	7	0.1	0.3	1
Ala	769	494	22.6	22.5	23
Leu	71	44	2.1	2.0	2
Lys	34	22	1.0	1.0	1
Arg	34	21	1.0	1.0	1

* Asparagine is tabulated as aspartic acid

(From: Davies et al., 1982)

circulation of a mature 37 residue polypeptide (Davies *et al.*, 1982).

The cloned sequence of the mature protein reported by Davies *et al.* (1982) matches in composition and size (3300 daltons) one of the alanine-rich serum antifreeze proteins (component A) that was purified by ion-exchange and reverse phase HPLC (Table 5). The cloned cDNA sequence for component A is approximately 200 nucleotides shorter than the previously determined length of AFP mRNA (Davies and Hew, 1980). Davies *et al.* (1982) have suggested that this discrepancy could be due to the loss of the poly (A) tract and some untranslated sequences. Radioactive sequence analysis of the primary translation product from purified AFP mRNA has been used to determine the reading frame and validate the cloned AFP sequences (Davies *et al.*, 1982). Independent reports of a primary amino acid sequence of a 3300 AFP (AFP-3) (DeVries and Lin, 1977a) and an AFP cDNA sequence described by Lin and Gross (1981) are similar to the AFP cDNA sequence data reported by Davies and co-workers (1982). Substantial differences in the established reading frame of the Lin and Gross nucleotide sequence, an unusual signal sequence and the inclusion of a termination codon prior to the mature peptide-sequence, suggest this cDNA sequence may not correspond to a secretable protein. In addition, the amino acid composition derived from this DNA sequence does not correspond to amino acid compositional data of any AFP reported to date (Duman and DeVries, 1976; Hew and Yip, 1976; DeVries and Lin, 1977a; Davies *et al.*, 1982). It has been suggested (Davies *et al.*, 1982) that the Lin and Gross (1981) sequence may represent a pseudogene which is transcribed but not translated.

Analysis of flounder genomic sequences (gene library) indicate that there are at least six separate AFP gene loci that cross-hybridize extensively to the AFP cDNA plasmid CT5. One of the sequences of a genomic subclone (E3) has extensive sequence similarity to cDNA CT5 and appears to be a variant of component A isolated by Davies et al. (1982). The genomic clone E3 has one less alanine and an additional aspartic acid, as well as an intervening sequence of approximately 0.6 Kb in length (Davies et al., 1984). On the basis of cDNA sequence studies (Davies et al., 1982) and current genomic investigations (Davies et al., 1984) the AFP of winter flounder (component A) is probably encoded by a translated region of 246 nucleotides with 50 and 94 nucleotides (5' and 3', respecitvely) untranslated regions. This would represent an AFP mRNA of 390 nucleotides prior to the addition of a poly (A) tract. The organization and sequence determination of other possible AFP gene loci have not yet been investigated.

Seasonal appearance and regulation of antifreeze synthesis

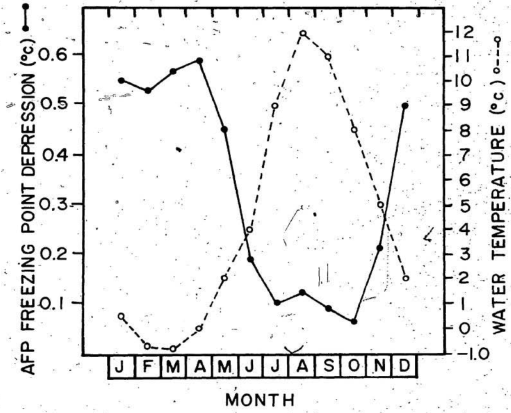
Antarctic and high Arctic waters are near the freezing point throughout the year with little variation in water temperature noted with depth or season (DeVries, 1974; DeVries and Lin, 1977b; DeVries, 1980). Consequently, in order to ensure survival, fish inhabiting these waters retain high levels of antifreeze in their blood regardless of the season. The maintenance and control of high concentrations of antifreeze in two species of Antarctic fish, Trematomus borchgrevinki and Rhigophila dearborni appears insensitive to changes in water temperature since warm water acclimation studies at 4°C for 60 days does not alter the levels of antifreeze glycopeptides found in these fish (O'Grady et al., 1982a). Antifreeze glycopeptide degradation rates (biological half life) have been estimated to be approximately four weeks in Antarctic fish maintained at

-1.5°C (Haschemeyer and Mathews, 1980). It has been suggested that fish inhabiting the long-term temperature stability of the Antarctic environment have gradually lost their ability to control antifreeze glycopeptide synthesis since synthesis of these peptides is fundamental to their survival (DeVries, 1980).

In contrast, fish inhabiting north-temperate waters are exposed to variations in temperature. Fish inhabiting these regions experience sub-zero temperatures and ice-laden seawater for a maximum of four to five months each year. As might be predicted, blood levels of antifreeze in such fish as represented by the winter flounder, shorthorn sculpin, Atlantic cod, and sea raven, correlate with the seasonal cycle of water temperatures (Duman and DeVries, 1974b; Fletcher, 1977, 1981; Fletcher and Smith, 1980; Hew *et al.*, 1980, 1981; Petzel *et al.*, 1980; Slaughter and Hew, 1982; Slaughter *et al.*, 1981). The seasonal synthesis of antifreeze proteins have attracted considerable attention as a system for studying the environmental control of gene regulation. Towards this end, our best understanding of the seasonal regulation of AFP biosynthesis comes from studies involving the winter flounder, *Pseudopleuronectes americanus*.

In winter flounder, the appearance of serum AFP in the fall and their disappearance in the summer, coincide with the seasonal variation in water temperature (Figure 4) (Hew and Yip, 1976; Fletcher, 1977, 1981; Slaughter and Hew, 1981). These proteins are produced by the liver (Hew and Yip, 1976) in large quantities in the autumn and winter, reaching a maximum concentration of 10 to 15 mg/ml (January), and are cleared from the blood in the spring (Hew *et al.*, 1978). The synthesis of AFP commences in late October as noted from *in vivo* (Hew *et al.*, 1978) and *in vitro* studies

Figure 4. Monthly changes in the freezing point depression attributable to 'antifreeze' in winter flounder plasma. These changes were calculated by subtracting the freezing point depression attributable to plasma sodium chloride from the total observed freezing point depression. Water temperatures are mean values from 1974 to 1979.
(adapted from Fletcher, 1977, 1981)


AFP FREEZING POINT DEPRESSION (°c)
0.6
0.5
0.4
0.3
0.2
0.1
WATER TEMPERATURE (°c)
12
11
10
9
8
7
6
5
4
3
2
1
0
-1.0
J
F
M
A
M
J
J
A
S
O
N
D
MONTH

(Sclater, 1979), as well as studies involving the isolation and translation of AFP mRNA (Hew and Yip, 1976; Lin, 1979; Davies and Hew, 1980; Pickett et al., 1983).

The seasonal expression of AFP mRNA has been investigated using cDNA hybridization studies and in vitro cell-free translation experiments (Hew and Yip, 1976; Lin, 1979; Lin and Gross, 1981; Pickett et al., 1983). All investigations are in agreement that AFP mRNA follows a seasonal synthesis pattern which matches closely, but slightly preceeds the rise and fall in the concentration of serum AFP. It has been estimated that in midwinter, 0.5% of the total liver RNA is AFP mRNA and in summer the AFP mRNA falls to a minimal but detectable level comprising 0.0007% of the total RNA (Pickett et al., 1983). The close parallel between seasonal availability of AFP mRNA and the synthesis of its corresponding product, supports the hypothesis that transcriptional control plays a major role in regulating AFP biosynthesis.

Recent evidence indicates that in the winter flounder the initiation of AFP synthesis in the fall is influenced by photoperiod (Fletcher, 1977, 1981) and comes under the control of the pituitary gland (Fletcher et al., 1978; Fletcher, 1979; Hew and Fletcher, 1979). Fletcher (1977, 1981) has demonstrated that fish exposed to long day length (>14 h) experience both a delay in the appearance of AFP in the serum, and a reduced accumulation of AFP during the winter. In contrast to previous studies which suggested that water temperature plays a role in the onset of AFP biosynthesis in the fall (Duman and DeVries, 1974b), the findings of Fletcher (1981) clearly demonstrated that cold water does not promote the early appearance of AFP. However, warm water temperatures does affect the rate of clearance in the spring. Fish acclimated to unseasonally warm water early in the spring, have decreased levels of serum AFP (Fletcher, 1981). Photoperiod has no effect on the disappearance of antifreeze in the spring.

Serum AFP levels are subject to regulation by the pituitary gland. Removal of the pituitary gland (hypophysectomy) in the flounder results in the accumulation of large concentrations of serum AFP regardless of the season (Fletcher et al., 1978). The effect of hypophysectomy can be reversed by pituitary implant (Fletcher, 1979). This suggests that some factor produced by the pituitary gland is affecting the repression of AFP mRNA synthesis or interfering with the clearance of AFP from the circulation. There is also some indication that the timing and length of the antifreeze biosynthetic cycle is, to some extent, endogenously controlled (Fletcher and Smith, 1980; Petzel et al., 1980; Fletcher, 1981). Strong evidence for an endogenous influence comes from experiments which demonstrated that flounder from Nova Scotia retained their antifreeze cycle characteristic of Nova Scotia when transferred to Newfoundland and maintained under Newfoundland conditions of temperature and photoperiod (Fletcher and Smith, 1980).

In the winter flounder, seasonal changes in the concentrations of AFP in the serum and AFP mRNA in the liver are correlated with water temperature and appear to be primarily controlled at the level of transcription. The interplay between the pituitary gland and environmental influence (such as photoperiod) on this regulation is not well understood. In summary, it appears that the seasonal timing of the antifreeze cycle may be endogenously controlled but the precise determination of the onset of antifreeze biosynthesis in the fall is influenced by photoperiod and comes under the control of the pituitary gland.

Statement of research problems and objectives

The coordinate expression of structural genes leading to precise patterns of differentiation and development is fundamental to life. Selective gene expression is now considered to be central to our understanding of cellular differentiation and the regulation of developmental processes (Davidson, 1976). The change in nature of, or rate at which, different genes are transcribed have been studied by numerous approaches and in different systems (For Review: Darnell, 1982; Nevins, 1983). It is generally agreed that systems involving hormonal induction of gene expression have contributed significantly to our understanding in this field. Many of these successful systems are based on similar hormones involving a different set of genes (Palmiter, 1975; Schimke et al., 1975; Deeley et al., 1977; O'Malley et al., 1977; Tata and Smith, 1979). However, novel approaches must be pursued to fully study all aspects of the regulation of gene expression. The seasonal biosynthesis of AFP in the winter flounder makes this an excellent and unique system for the study of gene structure and gene regulation in response to environmental stimuli as well as pituitary influences. The production of flounder AFP involves the rapid synthesis of large quantities of specific gene products. The fact that these polypeptides contain an unusual amino acid composition and they demonstrate unusual activities such as thermal hysteresis and freezing point depression allows for their easy identification. The availability of a well-defined flounder AFP hybridization probe enables the quantitation of AFP mRNA to be made and aids in the study of the structure and regulation of the antifreeze genes. Manipulation of environmental factors, such as photoperiod and the artificial induction of AFP biosynthesis by hypophysectomy will provide an important approach to study the detailed mechanisms involved in the environmental influence and the pituitary gland interaction on the subsequent transcription of AFP mRNA and corresponding translation of its protein product. This study has been concerned with three main objectives:

(1) A primary requirement for the study of gene regulation is precise knowledge of the protein product. As indicated in Table 6, the literature contains many inconsistencies concerning the number, size and composition of winter flounder AFP components. The initial objective of this study was to identify and characterize all AFP components in winter flounder and to determine if similarities and differences encountered in the literature are due to geographical polymorphism.

(2) In contrast to hormone induction in many systems which increases the level of translatable mRNA, the pituitary gland in winter flounder appears to have a repression effect on the synthesis of AFP. Hypophysectomy results in increased levels of AFP and pituitary transplants decreases the accumulation of these products. The second concern of this study was to determine the influence of the pituitary gland in the regulation of AFP synthesis. The technique of hypophysectomy was used as a test system to follow the synthesis and accumulation of AFP and AFP mRNA when background levels of these two products were at their lowest.

(3) Recent evidence indicates that in the winter flounder the initiation of AFP biosynthesis in the fall is influenced by photoperiod and is mediated by the pituitary. The third aspect of this study was to determine what influence photoperiod has on the synthesis and accumulation of AFP mRNA and its corresponding AFP.

All of the above studies involve identification and estimation of a specific gene product. The overall goal of this project was to develop a simple means of identifying antifreeze peptides and to develop a sensitive technique of estimating AFP mRNA concentrations in small quantities of tissue. In the final chapter, the results of these experiments are discussed with respect to current information concerning gene structure and genomic organization. A model of the regulation of AFP biosynthesis in winter flounder is presented.

Table 6. Molecular weights and number of components reported for winter flounder AFP

Reference	Sample location	Molecular weight	Number of Components	Technique involved in estimation of molecular weight and number of components
Duman and DeVries (1976)	Nova Scotia	6,000 8,000 12,000	(3)	SDS PAGE (Weber and Osborn, 1969)
		34,000	(1)	Gel filtration chromatography, Sephadex G-100
Hew and Yip (1976)	Newfoundland	10,000	(1)	Gil filtration chromatography, Sephadex G-75 and SDS PAGE (Weber and Osborn, 1969) of cell free translation products from Xenopus oocytes system
DeVries and Lin (1977a)	Nova Scotia	smallest AFP 3300	(3)	Low speed sedimentation equilibrium method
		smallest AFP 3790		Minimum molecular weight calculation from N-terminal sequence
Hew et al. (1978)	Newfoundland	precursor 15,000	(1)	Gel filtration chromatography, Sephadex G-75
		mature polypeptide 10,000	(1)	
Lin (1979)	New York	16,000	(3)	SDS PAGE
		smallest 3,300		Low speed sedimentation equilibrium method
		precursor 12,000		Primary cell-free translation product from wheat germ system. Estimated by SDS urea PAGE
Davies and Hew (1980)	Newfoundland	precursor 12,000	(1)	Primary translation product from reticulocyte lysate system. Estimated by SDS gradient PAGE (Laemmli, 1970)
DeVries (1980) (Review)	Nova Scotia	3,000 to 8,000	(3)	No technique given
		smallest component 4,664		Minimum molecular weight estimation from N-terminal sequence

Table 6. (continued).

Reference	Sample location	Molecular weight	Number of Components	Technique involved in estimation of molecular weight and number of components
Lin and Long (1980)	New York	12,000	(2)	Primary translation products from wheat germ or reticulocyte lysate systems. Estimated by SDS urea PAGE
DeVries (1982) (Review)	Nova Scotia	12,000 8,000 3,200	(3)	No technique given. Correction for overestimation not discussed
		corrected to 8,000 5,000 3,200		
Davies et al. (1982)	Newfoundland	3,200 (both)	(2)	Minimum molecular weight estimate from AFP cDNA sequence and amino acid composition of isolated peptides
				Previous report of 10,000 daltons, regarded as overestimation
Pickett et al. (1983)	Newfoundland	precursor 12,000	(1)	Primary translation product from reticulocyte lysate system. Estimated by SDS PAGE (Laemmli, 1970)

CHAPTER 2

WINTER FLOUNDER ANTIFREEZE POLYPEPTIDES

Introduction

The Newfoundland seawater temperatures fluctuate from 12°C in the summer to a low value of -1.4°C during the winter (Fletcher, 1977). The body fluids of most temperate marine teleosts freeze at temperatures between -0.5 and -0.9°C (Holmes and Donaldson, 1969). To avoid freezing, the winter flounder, Pseudopleuronectes americanus which inhabits these coastal waters produces antifreeze polypeptides (AFP) in the winter (Duman and DeVries, 1974a, 1976; Hew and Yip, 1976). These polypeptides are synthesized by the liver and are circulated in the blood plasma (Hew et al., 1978; Davies and Hew, 1980). Flounder AFP lower the freezing temperature of the serum in an non-colligative manner and are essential to the survival of the winter flounder in ice-laden seawater (Fletcher, 1977; Feeney and Yeh, 1978; Hew et al., 1978; Davies and Hew, 1980; DeVries, 1982).

Although winter flounder AFP have been studied extensively, there is still some controversy over the number, size and amino acid sequence of these polypeptides (Duman and DeVries, 1976; Hew and Yip, 1976; Lin and Gross, 1981; Davies et al., 1982). Working with plasma from more southerly populations of flounder (Nova Scotia), Duman and DeVries (1976) have reported the presence of three separate components of molecular weights 6000, 8000 and 12,000 which were later corrected to 3200, 5000 and 8000 (DeVries, 1982). In contrast, by gel filtration chromatography, the presence of a major component of apparent molecular weight 10,000 was demonstrated (Hew and Yip, 1976) which could be further fractionated into two distinct components of molecular weight 3300 using reverse phase high performance liquid chromatography (Davies et al., 1982). This was confirmed by estimating the size of the peptide from cDNA sequence analysis (Davies et al., 1982). In addition, Lin and Gross (1981) reported the cDNA sequence for an antifreeze polypeptide of approximately 6000 daltons. However, neither the amino acid composition nor the sequence of this putative antifreeze polypeptide resembles those of any of the winter flounder antifreeze polypeptides reported to date (Duman and DeVries, 1976; DeVries and Lin, 1977a; Davies et al., 1982).

Flounder AFP stain poorly with protein staining solutions such as Coomassie Brilliant Blue and Amido Black and this has made it difficult to examine their homogeneity by conventional electrophoretic procedures (Sclater, 1979). However, reverse phase high performance liquid chromatography (HPLC), because of its superior resolving power appears to be well suited for the analysis of the heterogeneity of these polypeptides.

Differences between the AFP reported for Newfoundland winter flounder (Davies et al., 1982) and the AFP described by Lin and Gross (1981) and DeVries (1982) raised the possibility that geographically distinct populations of winter flounder may produce different antifreeze polypeptides. One means of answering this question is by comparing the antifreeze polypeptides isolated from the plasma of winter flounder collected from selected sites throughout most of its geographical range, namely Newfoundland, Nova Scotia, New Brunswick and Long Island (New York) (Leim and Scott, 1966).

The present study was carried out to characterize winter flounder AFP further, to report any microheterogeneity which may exist, and to establish whether geographical polymorphism can account for any of the discrepancies concerning size, number and sequence which have been reported in the literature.

Materials and Methods

Collection of experimental materials

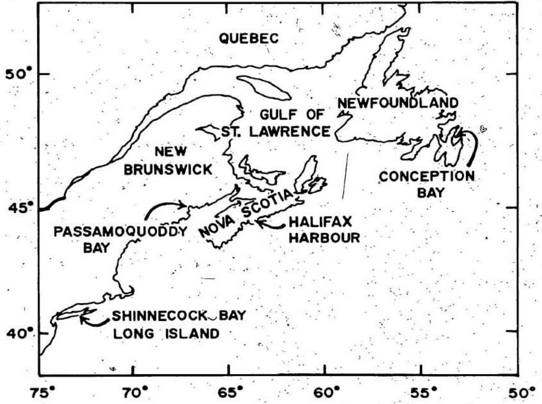
Winter flounder (Pseudopleuronectes americanus) (400-600 g, 30-40 cm long) were collected from four geographical locations: Chapel's Cove, Newfoundland; Halifax Harbour, Nova Scotia; Passamaquoddy Bay, New Brunswick; and Shinnecock Bay, Long Island, New York (Fig. 5). In Newfoundland the fish were caught by divers equipped with SCUBA, while in the other locations the fish were caught using small otter trawls. Blood was obtained from a caudal blood vessel using 21 to 23 gauge syringe needles and stored in heparinized test tubes. Plasma was separated from the red cells by low speed centrifugation (~4000 x g) and stored at -20°C prior to analysis. Fish caught in Newfoundland waters were either bled immediately or maintained in 250-L aquaria supplied with flowing seawater (31-33 ⁰/oo) at seasonally ambient temperature and photoperiod (Fletcher, 1977). Fish kept in the lab were monitored for disease and stress and only healthy unstressed fish were used. Fish maintained for extended periods of time were fed capelin during the normal feeding cycle which is from April to October (Fletcher and King, 1978).

In some cases, Newfoundland flounder serum was collected without clotting agents, centrifuged at ~4000 x g, for 10 min and used directly without storage. No difference was encountered between AFP components isolated from serum or plasma preparations.

Isolation and product analysis of flounder antifreeze polypeptides

Flounder serum or plasma (2 ml) was applied directly on a Sephadex G-75 column (1.5 x 84 cm) and eluted with 0.1 M NH_4HCO_3 at 4°C. Fractions were monitored at 230 nm (A_{230}) and antifreeze activity

Figure 5. Geographical range where winter flounder, Pseudopleuronectes americanus is found in abundance. The locations where the animals were sampled are indicated.


QUEBEC
50°
NEWFOUNDLAND
GULF OF
ST. LAWRENCE
NEW
BRUNSWICK
CONCEPTION
BAY
45°
NOVA SCOTIA
HALIFAX
HARBOUR
PASSAMOQUODDY
BAY
SHINNECOCK BAY
LONG ISLAND
40°
75°
70°
65°
60°
55°
50°

was measured using a freezing point osmometer (Model 3R, Advanced Instruments, Needham Height, M.A., USA), as described by Hew and Yip (1976). Active fractions (exhibiting freezing point depression) were pooled, lyophilized and rechromatographed on the same Sephadex column. After lyophilization, individual fractions and pooled samples representing the active Sephadex fractions were dissolved in 300 μl of 5% formic acid, further fractionated by reverse phase HPLC using an Altex Ultrasphere ODS (C_{18}) column (particle size 5μ, 4.6 mm x 25 cm, Beckman, Toronto) in 0.02 M triethylamine phosphate buffer, pH 3.0 and eluted with an acetonitrile gradient at room temperature (Seidah et al., 1980). Following chromatography, the different elution peaks were pooled, lyophilized, reequilibrated with 1 ml of 0.1 M NH_4HCO_3 and desalted on a Sephadex G-25 column (1.5 x 30 cm) using the equilibration buffer. The HPLC components were chromatographed to single peak homogeneity by repetition of HPLC.

The proportions of the different antifreeze components were determined from peak area integration of the elution profiles using an HPLC equipped with an integrator (Altex, Model C-RIA, Beckman). The relative proportion of each component in the AFP from each geographical sample was calculated from the elution profiles of at least three separate trials. Each trial represented a different fish with the exception of the Nova Scotia sample which was a pooled sample from five fish. The recovery of AFP from HPLC was estimated by measuring the A_{230} before and after the sample was chromatographed.

Polyacrylamide gel analysis of serum components

Following chromatography on Sephadex G-75 and reverse phase HPLC, blood serum components were further examined by labelling the components with dansyl chloride (5-dimethylamino-naphthalene-1-sulfonylchloride, Pierce Chemical, Rockford, Illinois, U.S.A.) as described by Gray (1967). The fluorescently labelled peptides were desalted on a Sephadex G-25 column (1.0 x 30 cm) using 0.1 M ammonium hydroxide and analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). The separating gel was either a 9-22.5% concave exponential polyacrylamide gradient or a 15% polyacrylamide gel. The gels were either stained with 0.1% Coomassie Brilliant Blue (BioRad) (Paul et al., 1972) or visualized directly and photographed by transilluminated short wave ultraviolet light using a red 25A filter with Polaroid type PN, 665 film. Gels stained with Coomassie Brilliant Blue were photographed through a yellow (Y48) filter using either Polaroid type PN, 665 film or a fine grain 35 mm black and white film.

Molecular weights of constituent polypeptides were obtained by comparison of electrophoretic mobilities with known standards (BioRad, Pharmacia or Sigma) in SDS polyacrylamide gels according to the method of Weber and Osborn (1969).

Specific polypeptide bands separated by 15% polyacrylamide SDS gel electrophoresis (determined from a corresponding gel lane containing a fluorescently labelled sample) were excised and eluted overnight at 37° C in 0.01 M NH_4HCO_3 containing 0.05% SDS. The eluted fractions were either desalted as described above or lyophilized directly and subjected to amino acid analysis.

Measurements of thermal hysteresis and amino acid analysis

Fractions purified from HPLC were desalted on a Sephadex G-25 column and lyophilized as described above. After lyophilization, they were dissolved in 10 to 100 μl of 0.01 M NH_4HCO_3. Antifreeze activities of the individual components were determined from thermal hysteresis measurements (the difference between freezing and melting temperatures) made using a nanoliter osmometer (Clifton Technical Physics, Hartford, N.Y., U.S.A.). In this method the temperature at which ice crystals grow is the freezing temperature and the melting temperature is when the crystal shrinks.

Amino acid analyses were conducted on freeze dried protein samples (from chromatography preparations or samples eluted from PAGE) which were hydrolyzed in vacuo for 24 h at 110°C in 6 N HCl. The hydrolysate was analyzed on a Beckman 121C amino acid analyzer.

Results

Analysis of flounder AFP by reverse phase HPLC

Consistent with observations reported earlier (Hew and Yip, 1976), Sephadex G-75 chromatography of the flounder's winter serum revealed the presence of only a single macromolecular antifreeze of approximately 10,000 daltons (Fig. 6). Analysis of this fraction (represents pooled fractions 36-62) on reverse phase HPLC indicated that flounder AFP occurs as a complex mixture. At least nine distinct components could be resolved by an extended acetonitrile gradient (15-60%, Fig. 7) but superior resolution and better separation of the minor components were attained using a shallow acetonitrile gradient (15-40%, Fig. 8). Components 6 and 8 were the two major species corresponding to components A and B reported by Davies *et al.* (1982).

When the pooled samples (36-62) were re-chromatographed on Sephadex G-75 (Fig. 6) and the individual fractions from the column analyzed systematically by reverse phase HPLC using a more extended gradient, it was apparent that these Sephadex fractions were heterogeneous (Fig. 9). Component 9 (elution time of 45 min), which was still retained in the column under the conditions used in Figure 8, was the major component in Sephadex fraction 42. Components 3 to 8 were the major species in Sephadex fractions 46 to 50. Finally, the predominant peptides in Sephadex fraction 56 had elution times of 13 to 16 minutes and would correspond to components 1 and 2 in Figures 7 and 8. When a pooled sample was analyzed (representing Sephadex fractions 36-62), the relative proportions of these different components from 1 to 9 were 13:11:10:5:2:27:8:20:4, respectively. No significant or distinct polypeptides were

Figure 6. Fractionation of winter flounder AFP by Sephadex G-75 chromatography. Flounder serum (2 ml) was chromatographed directly on a column (1.5 x 84 cm) in 0.1 M NH_4HCO_3, pH 8.0 and 2.3 ml fractions were collected. A) open symbols represent the first application of the serum; B) closed symbols represent the reapplication of fractions 36-62 from A) on the same column. Individual fractions were monitored for antifreeze activity as indicated by freezing point depression measurements (▨). The active fractions were lyophilized and used for HPLC analysis. 1) Void volume peak, 2) Antifreeze active peak.

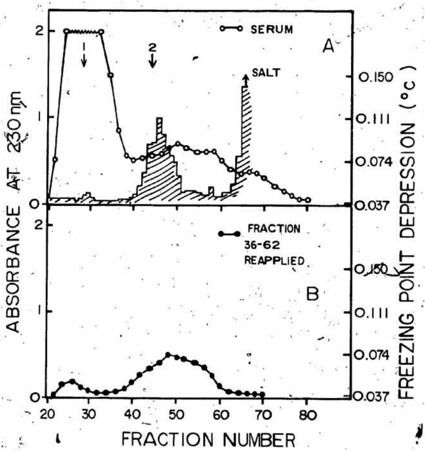

SERUM
A
1
2
SALT
FRACTION
36-62
REAPPLIED
B
ABSORBANCE AT 230 nm
FREEZING POINT DEPRESSION (°C)
0
1
2
0.037
0.074
0.111
0.150
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 7. Analysis and isolation of flounder AFP by reverse phase HPLC. Flounder AFP from Sephadex G-75 chromatography (fractions 36-62, Fig. 6), 0.5 mg of lyophilized material was dissolved in 100 µl of 5% formic acid and applied to an Altex Ultrasphere ODS column at 22°C. Fractionation was achieved using an acetonitrile gradient (15-60%) in a 0.02 M triethylamine phosphate buffer, pH 3.0 with a flowrate of 1 ml per min.

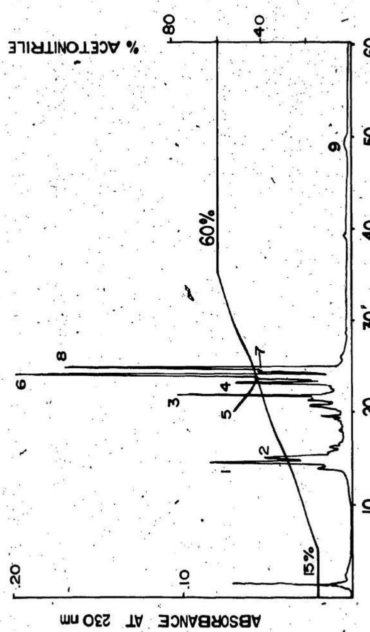
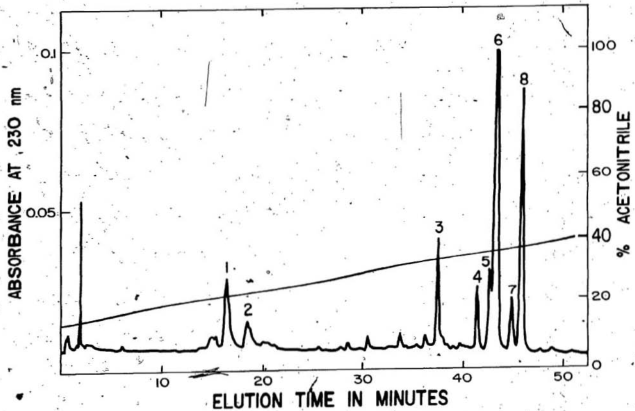

% ACETONITRILE
80
40
ABSORBANCE AT 230 nm
.20
.10
15%
60%
1
2
3
4
5
6
7
8
9
ELUTION TIME IN MIN
10
20
30
40
50
60

Figure 8. Analysis and isolation of flounder AFP by reverse phase HPLC using a shallower acetonitrile gradient (15-40%) than in Figure 7. Note, that although component 9 was not eluted under these conditions, the resolution of the other components was much improved. Sample preparation and chromatography was as described in the legend of Figure 7.


ABSORBANCE AT 230 nm
0.1
0.05
% ACETONITRILE
100
80
60
40
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0
1
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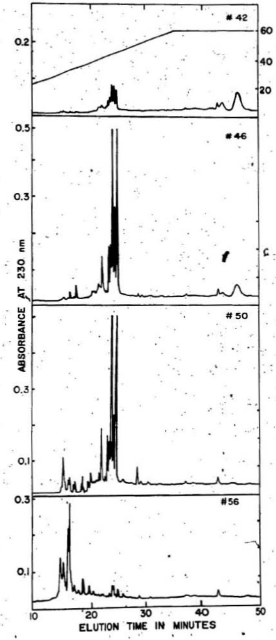
6
7
8
10
20
30
40
50
ELUTION TIME IN MINUTES

Figure 9. Analysis of winter flounder AFP fractions by reverse phase HPLC. Individual fractions from Sephadex G-75 rechromatography (Fig. 6) were separately dissolved in 300 µl of 5% formic acid, and 50 µl aliquots were analysed on an Altex Ultrasphere ODS reverse phase column using an acetonitrile gradient (15-60%) in 0.02 M triethylamine phosphate buffer, pH 3.0 at 22°C. The flow rate was 1 ml per min.


#42
#46
#50
#56
0.2
0.5
0.3
0.3
0.1
0.3
0.1
60
40
20
% ACETONITRILE
ABSORBANCE AT 230 nm
10
20
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40
50
ELUTION TIME IN MINUTES

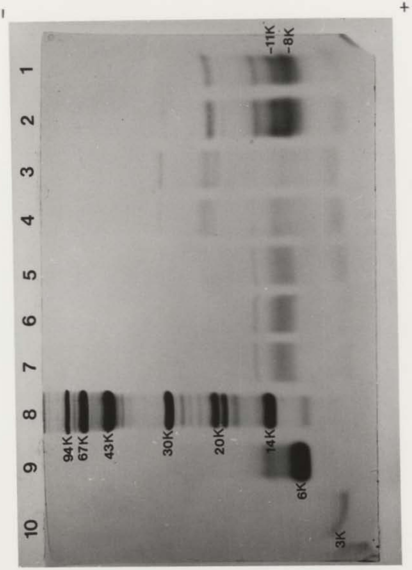
eluted from the reverse phase column when the concentration of acetonitrile was increased to 80%. The recovery of total proteins applied to the column, based on optical density at 230 nm, was 85-90%. To avoid the possibility that some larger polypeptides were absorbed on the C_{18} reverse phase column, the Sephadex AFP preparation was analyzed on a less hydrophobic C_3 column (Ultrapore RPSC) in 0.1% trifluoroacetic acid-acetonitrile gradient. The AFP elution profile from the C_3 column was similar to the elution profile reported above indicating that no larger polypeptides were selectively absorbed to the C_{18} reverse phase column. The AFP resolution on the C_3 column was inferior to that obtained on the C_{18} column.

In agreement with the position of the activity on the Sephadex column, amino acid analysis showed that Sephadex fractions 44 to 52 contained a large amount of alanine (approximately 52 mole %), a feature characteristic of flounder AFP (60 mole %) (Hew and Yip, 1976). On the other hand, Sephadex fraction #56 contained considerably less alanine (33 mole %) and was devoid of antifreeze activity.

SDS polyacrylamide gel electrophoresis of flounder serum polypeptides

When the flounder AFP pooled fraction (36-62) (Fig. 6) and individual fractions from Sephadex G-75 chromatography were analyzed by SDS PAGE and stained directly with Coomassie Brilliant Blue, two major bands of approximately 8000 and 11,000 daltons were apparent (Figs. 10 and 11). These two bands predominated in Sephadex G-75 fractions #54 to 58 and therefore, corresponded to components 1 and 2 resolved on HPLC (Figs. 7 and 8). These peptides were eluted from PAGE and subjected to acid hydrolysis.

Figure 10. Coomassie Brilliant Blue staining of flounder AFP peptides following SDS PAGE (15%). Lane 1, AFP (pooled Sephadex G-75 fractions 36-62) 28 μg; Lane 2, AFP (pooled Sephadex G-75 fractions 36-62) 35 μg. The following samples represent individual Sephadex G-75 fractions as indicated: Lane 3, Fraction 43; Lane 4, Fraction 47; Lane 5, Fraction 51; Lane 6, Fraction 55; Lane 7, Fraction 57; Lane 8, Pharmacia protein standards; phosphorylase b (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soya bean trypsin inhibitor (20K), α-Lactalbumin (14K); Lane 9, aprotinin (6.5K) (from Boehringer Mannheim) 35 μg; Lane 10, insulin A chain (~3K) (from Sigma) 25 μg. Equal volumes of sample were loaded from each Sephadex G-75 fraction.


−
+
1
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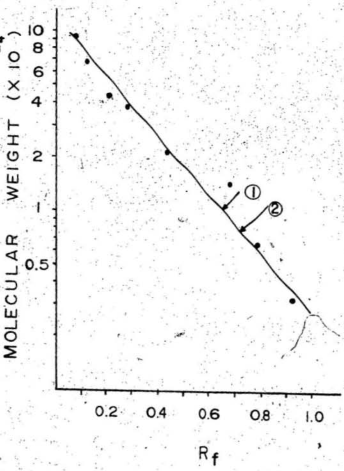
3
4
5
6
7
8
9
10
-11K
-8K
94K
67K
43K
30K
20K
14K
6K
3K

Figure 11. Molecular weight estimation of serum components 1 and 2. Molecular weight estimations were made according to the method of Weber and Osborn (1969). Each R_f value represents the average of at least three determinations made by SDS 15% PAGE. The molecular weights of the major Coomassie Brilliant Blue staining components isolated from serum fractionation by Sephadex G-75 chromatography are 11,000 and 8000. These components correspond to components 1 and 2 (Figs. 7 and 8) following serum fractionation by HPLC.


MOLECULAR WEIGHT (X 10^{-4})
10
8
6
4
2
1
0.5
①
②
0.2
0.4
0.6
0.8
1.0
R_f

The amino acid compositions of these two bands were distinctly different in content from the amino acid compositions of known AFP components (Table 7). The molecular weights of these two components from gel electrophoresis (Fig. 11) are in good agreement with those observed using gel filtration chromatography.

Flounder AFP stain poorly with Coomassie Brilliant Blue. The above staining result is misleading in that it overestimates the contribution of these larger peptides (components 1 and 2) in comparison to flounder AFP. The results using dansylated labelled materials overcame this difficulty. On SDS PAGE, dansylated labelled materials showed a predominant band of approximately 3300 daltons (Fig. 12, Track 10). The larger bands, which stained with Coomassie Brilliant Blue, were minor in contribution when compared to AFP after dansylation analysis.

To further characterize the AFP, these peptides were purified by reverse phase HPLC. SDS gel electrophoresis of these dansylated peptides demonstrated that component 9 was larger than component 6 or 8 (Fig. 12). The molecular weight of component 9 was estimated to be 4500 whereas components 6 and 8 were smaller, approximately 3300 (Fig. 13). PAGE of dansylated AFP components 3, 4 and 5 indicated that these components had equivalent molecular weights of approximately 3300 whereas component 7 was judged to be 4500 and therefore similar in molecular weight to component 9. The molecular weight estimations by PAGE agreed with their minimum molecular weight values calculated from amino acid compositions (Table 8).

Antifreeze activity and amino acid analysis

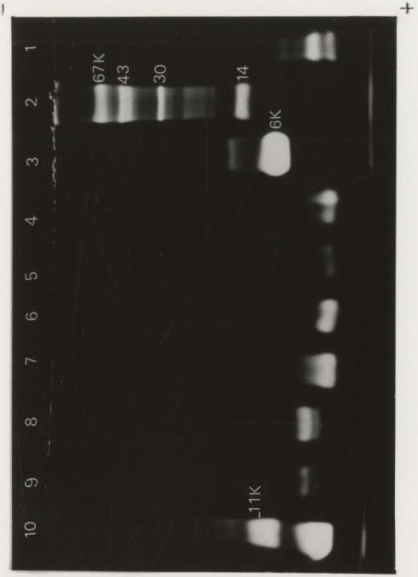
To further characterize the AFP, amino acid analyses and antifreeze

Table 7. Comparison of amino acid composition of polypeptides 1 and 2 with AFP extracted from SDS gels

(Mol %) Polypeptide #	1	2	AFP	6*	8*
Cysteic acid	3.6	1.1	0	0	0
Asx	11.5	11.5	11.8	10.6	13.6
Thr	9.2	10.5	11.6	10.1	9.0
Ser	16.1	7.3	3.8	2.5	3.1
Pro	1.6	0	0	0	0
Glx	7.8	6.1	1.5	2.8	0.4
Gly[a]	-	-	-	0.5	1.0
Ala	20.4	32.6	59.5	62.8	62.5
1/2Cys	1.5	1.0	0	0	0
Val	1.2	7.7	5	0	0
Met	0	0.7	0	0	0
Ile	2.8	1.4	0	0	0
Leu	7.0	4.2	6	5.7	5.2
Tyr	1.9	1.3	0	0	0
Phe	4.1	1.4	0	0	0
Lys	5.5	4.6	2.8	2.7	2.9
His	2.9	4.9	0.4	0	0
Arg	2.9	3.6	3.1	2.3	2.3

*Antifreeze components purified on HPLC. AFP corresponds to peptides eluted from the 3300 molecular weight region of the SDS polyacrylamide gel and represents the average of two amino acid composition determinations. PAGE and elution were conducted as described in Materials and Methods. [a]Gly content disregarded due to contamination of electrophoresis buffer.

Figure 12. SDS PAGE of dansylated peptides. Acrylamide concentration, 15%. See Materials and Methods for dansylation procedure. Lane 1, reduced insulin, 5 μg (from Sigma); Lane 2, Pharmacia protein standards; Lane 3, aprotinin, 5 μg (from Boehringer Mannheim); Lane 4, HPLC component 6, 3 μg; Lane 5, HPLC component 6, 2 μg; Lane 6, HPLC component 8, 4 μg; Lane 7, HPLC component 8, 5 μg; Lane 8, HPLC component 9, 4 μg; Lane 9, HPLC component 9, 3 μg and Lane 10, Sephadex G-75 AFP (pooled fractions 36-62), 12 μg.


-
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67K
43
30
14
6K
11K

Figure 13. Molecular weight determination of HPLC components 6 and 9 from Figs. 7 and 8. Molecular weight estimations were made on dansylated samples run on SDS 15% polyacrylamide gels according to the method of Weber and Osborn (1969). Each R_f value represents the average of at least three separate determinations. The molecular weights for AFP components 6 and 9 are 3300 and 4500, respectively. As noted in Figure 12, component 8 has the same electrophoretic mobility as component 6 and therefore was considered to have the same molecular weight.

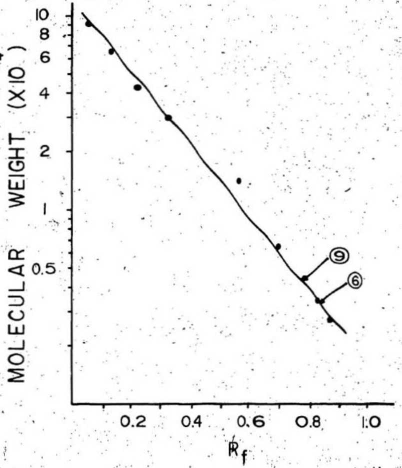

MOLECULAR WEIGHT (X10^{-4})
10
8
6
4
2
1
0.5
0.2
0.4
0.6
0.8
1.0
R_f
⑨
⑥

Table 8. Amino acid compositions of winter flounder AFP components isolated from reverse phase HPLC

	1	2	3	4	5	6	7	8	9	AFP-3	X-AFP	D-AFP
Asx	11	37	115 (4)	38 (4)	35 (4)	108 (4)	133 (7)	95 (5)	158 (4)	(5)	(3)	(4)
Thr	3	19	108 (4)	35 (4)	34 (4)	102 (4)	109 (5)	63 (4)	244 (7)	(4)	(9)	(4)
Ser	6	52	27 (1)	11 (1)	8 (1)	25 (1)	62 (3)	22 (1)	57 (2)	(1)	(1)	(1)
Pro	-	3	-	-	-	-	5	-	-	-	-	-
Glx	4	15	30 (1)	10 (1)	8 (1)	28 (1)	40 (2)	3 (0)	31 (1)	-	-	(1)
Gly	5	38	6 (0)	6 (0)	5 (0)	5 (0)	72 (4)	7 (0)	21 (1)	-	(1)	(1)
Ala	25	24	665 (22)	217 (22)	212 (23)	636 (23)	620 (31)	437 (23)	1219 (34)	(24)	(47)	(23)
Val	-	6	-	-	-	-	11 (1)	-	68 (2)	-	-	-
Ile	-	5	-	-	-	-	-	-	-	-	-	-
Leu	4	16	60 (2)	19 (2)	19 (2)	58 (2)	69 (3)	36 (2)	53 (2)	(2)	(1)	(2)
Tyr	-	10	-	-	-	-	-	-	-	-	-	-
Phe	-	-	-	-	-	-	-	-	-	-	-	-
Lys	5	12	57 (2)	12 (1)	9 (1)	27 (1)	37 (2)	20 (1)	40 (1)	(1)	(1)	(1)
Arg	2	3	19 (1)	8 (1)	1 (0)	23 (1)	20 (1)	16 (1)	36 (1)	-	(1)	(1)
Minimum number of amino acids	-	-	37	36	36	37	59	37	55	37	64	38
Thermal hysteresis	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-	-	-

Amino acid composition of AFP components isolated from reverse phase HPLC. The yield is presented in nanomoles. Numbers in brackets represent the number of residues. Thermal hysteresis was measured using a nonoliter osmometer. AFP-3, from DeVries and Lin (1977a). X-AFP, cDNA sequence for an AFP from Lin and Gross, 1981. D-AFP, cDNA sequence data for an AFP (believed to be component 6) from Davies et al., 1982. Asx and Glx, asparagine and glutamine tabulated as aspartic acid and glutamic acid respectively.

activity measurements (thermal hysteresis) were made on the peptides purified by reverse phase HPLC (Table 8). Components 1 and 2 (which were the major components in fraction #56 from the Sephadex G-75 column) were devoid of antifreeze activity and lacked the amino acid composition characteristic of flounder AFP. AFP components 3, 4, 5, 6, 7, 8 and 9 exhibited thermal hysteresis, contained an abundance of alanine and demonstrated an amino acid composition which is characteristic of flounder AFP components (Table 8).

Flounder summer serum analysis

Control flounder serum obtained in July was chromatographed, pooled and rechromatographed on Sephadex G-75 (Fig. 14) and analyzed by reverse phase HPLC (Fig. 15). No freezing point depression was detected in any of the fractions and the HPLC profile of the pooled area corresponding to AFP in flounder serum sampled during the winter months, lacked the characteristic HPLC components associated with AFP. The summer serum fractionated by HPLC contained two prominent components which eluted at approximately twenty-two minutes. These components were not evident in the HPLC analysis from flounder winter serum.

Analysis of flounder plasma from Nova Scotia, New Brunswick and Long Island (New York)

Flounder plasma from Nova Scotia, New Brunswick and Long Island (New York) chromatographed on Sephadex G-75 had similar elution profiles of antifreeze activity showing a single peak with an apparent molecular weight of 10,000 (Fig. 16). Measurements of the freezing point activity indicated that the AFP in all the samples were primarily located in the initial fractions of elution peak (2) (Fig. 16). These observations were

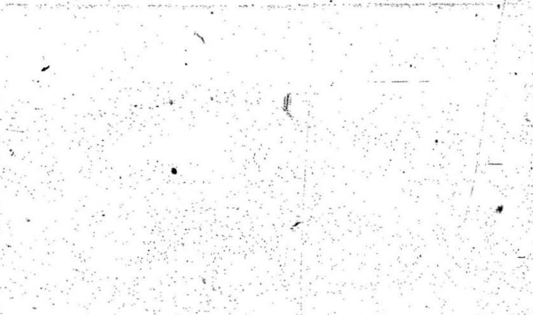
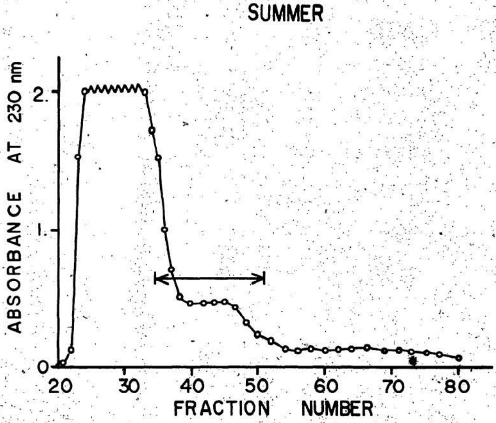


Figure 14. Fractionation by Sephadex G-75 chromatography of winter flounder serum taken from fish in July. Flounder serum (2 ml) was chromatographed as described in the legend of Figure 6. Fractions 34 to 52 were pooled, lyophilized and used for HPLC analysis.


SUMMER
ABSORBANCE AT 230 nm
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80
FRACTION NUMBER

Figure 15. Analysis and isolation of flounder summer serum by reverse phase HPLC. Approximately 0.5 mg (dry weight) of Sephadex G-75 material (pooled fractions 34-52, Figure 14) was dissolved in 100 µl of 5% formic acid and fractionated by reverse phase HPLC as described in the legend of Figure 7.

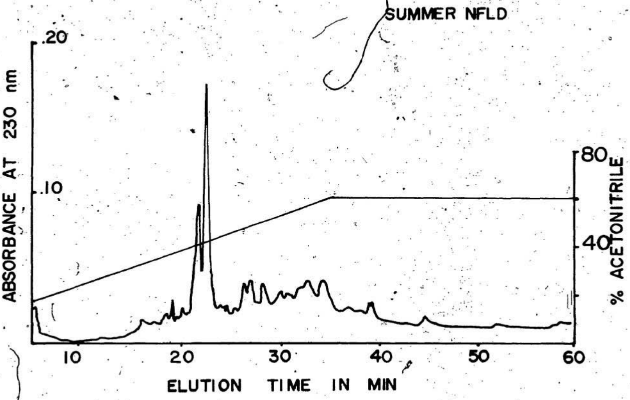
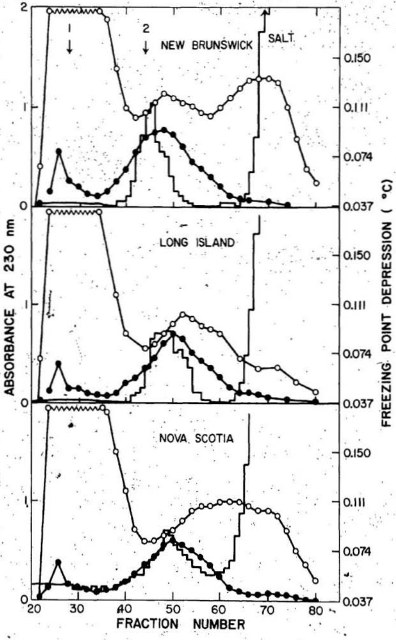

SUMMER NFLD
ABSORBANCE AT 230 nm
.20
.10
% ACETONITRILE
80
40
10
20
30
40
50
60
ELUTION TIME IN MIN

Figure 16. Fractionation by Sephadex G-75 chromatography of winter flounder AFP from New Brunswick, Long Island and Nova Scotia fish. Flounder serum (2 ml) was chromatographed and individual fractions monitored for antifreeze activity as described in the legend of Figure 6 and Materials and Methods. (a) New Brunswick, (b) Long Island, (c) Nova Scotia; (1) Void volume peak, (2) Antifreeze peak. o--o Initial chromatography run of flounder serum; ●--● Fractions 36-62 were pooled and rechromatographed; The symbol (⎍) denotes freezing point depression.


NEW BRUNSWICK
SALT
1
2
LONG ISLAND
NOVA SCOTIA
ABSORBANCE AT 230 nm
FREEZING POINT DEPRESSION (°C)
FRACTION NUMBER
0
1
2
0.037
0.074
0.111
0.150
20
30
40
50
60
70
80

the same as those described above for the analysis of AFP from Newfoundland winter flounder.

The AFP samples from the Sephadex G-75 column were separated into multiple components by reverse phase HPLC (Fig. 17). The elution profiles were remarkably similar regardless of the source of the AFP sample. The major components had identical elution times which agreed with the HPLC elution profile of Newfoundland flounder AFP (Fig. 7). The relative concentrations of the major AFP components from all four populations of flounder were similar with components 6 and 8 being the major fractions (Fig. 18). One exception was noted in a single New Brunswick fish where component 8 represented a smaller proportion of the total AFP present (HPLC profile shown in Figure 17). Two other New Brunswick samples had an AFP HPLC profile with component proportions similar to the Newfoundland, Nova Scotia and Long Island samples (Fig. 19). It was also noted that component 3 contributed less to the overall AFP profile in samples from New Brunswick and Nova Scotia and that components 4, 7 and 9 were considerably more variable than the other AFP components.

The AFP components were repurified on the HPLC to single peak homogeneity. Mixing experiments with the same components from the various sources gave single homogeneous peaks on the HPLC. Figure 20 is representative of these results. The shared elution identity of the AFP components from flounder samples is best illustrated in cruder preparations of AFP run on HPLC. Sephadex G-75 prepared AFP samples from Newfoundland and New Brunswick were chromatographed together on reverse phase HPLC (Fig. 21) and all major AFP peaks corresponded to the elution profiles established previously on individual samples run separately.

Figure 17. Reverse phase HPLC analysis of AFP containing samples from Sephadex G-75 chromatography (Figure 16). Sample preparation and HPLC fractionation was conducted as described in the legend of Figure 7. AFP components are numbered 3 to 9. Components 1 and 2 lack thermal hysteresis and are not considered to be AFP. Equivalent sample loads were fractionated in each chromatography run.

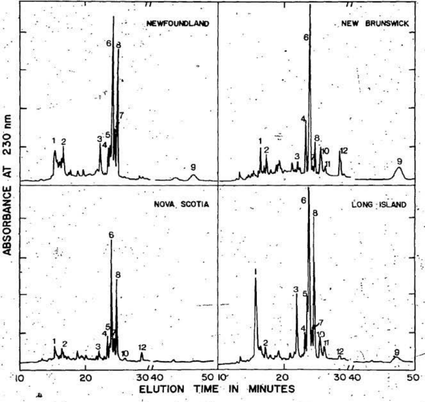

NEWFOUNDLAND
NEW BRUNSWICK
NOVA SCOTIA
LONG ISLAND
ABSORBANCE AT 230 nm
ELUTION TIME IN MINUTES
10
20
30
40
50
1
2
3
4
5
6
7
8
9
10
11
12

Figure 18. Relative proportions of the AFP components fractionated by reverse phase HPLC. NFLD; Newfoundland N = 5, NB; New Brunswick N = 3, NS; Nova Scotia N = 5, LI; Long Island (New York) N = 3. N = number of individual samples taken from each location and separately chromatographed except NS, where N = 5 refers to a pooled sample of 5 fish. Numbers on the abscissa refer to the individual HPLC components. I = ± standard deviation where applicable.

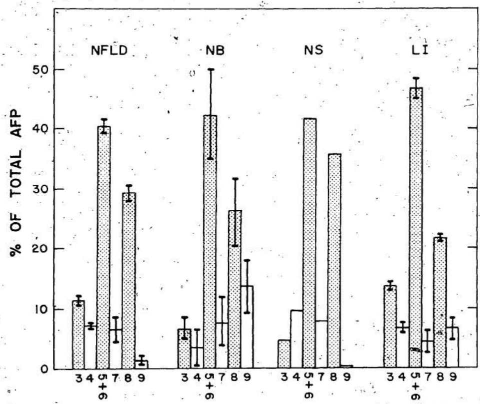

NFLD
NB
NS
LI
% OF TOTAL AFP
0
10
20
30
40
50
3
4
5+6
7
8
9

Figure 19. Analysis of New Brunswick flounder AFP by reverse phase HPLC. Repurified flounder AFP (approximately 0.4 mg, dry weight) from Sephadex G-75 chromatography was fractionated by reverse phase HPLC as described in the legend of Figure 7.

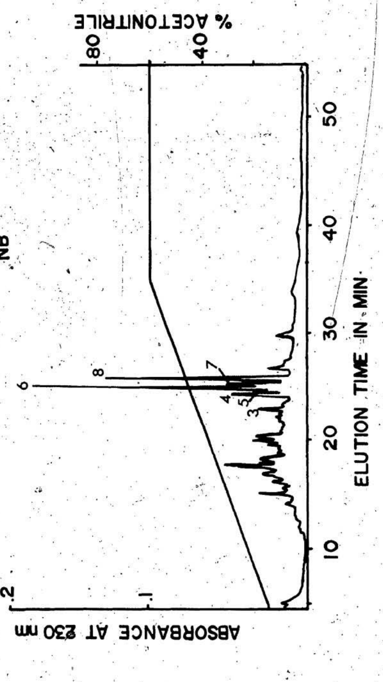

ABSORBANCE AT 230 nm
.2
.1
% ACETONITRILE
80
40
ELUTION TIME IN MIN
10
20
30
40
50
3
4
5
6
7
8
NB

Figure 20. Analysis of flounder AFP component 6 by reverse phase HPLC. Equivalent amounts of HPLC purified component 6 (approximately 40 μg) from Newfoundland, New Brunswick and Long Island flounder were dissolved in 50 μl of 5% formic acid and fractionated by reverse phase HPLC as described in the legend of Figure 7.

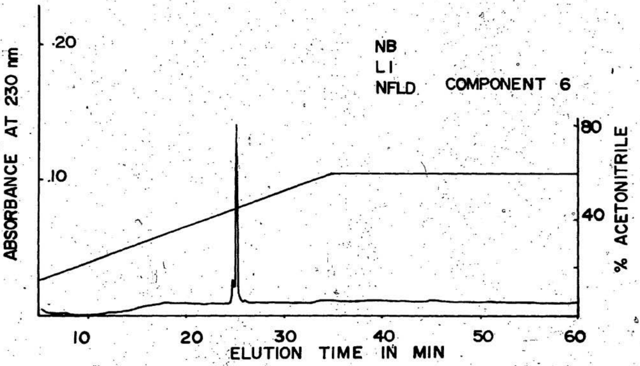
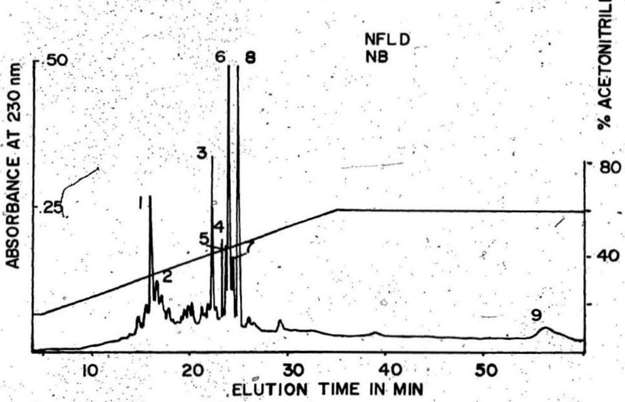

NB
L I
NFLD
COMPONENT 6
ABSORBANCE AT 230 nm
.20
.10
% ACETONITRILE
80
40
10
20
30
40
50
60
ELUTION TIME IN MIN

Figure 21. Reverse phase HPLC analysis of co-injected AFP from Newfoundland and New Brunswick fish. Equivalent amounts of Newfoundland and New Brunswick repurified flounder AFP from Sephadex G-75 chromatography (pooled fractions 36-62) were dissolved in 100 μl of 5% formic acid and fractionated by the reverse phase HPLC as described in the legend of Figure 7.


NFLD
NB
ABSORBANCE AT 230 nm
.50
.25
% ACETONITRILE
80
40
1
2
3
4
5
6
7
8
9
10
20
30
40
50
ELUTION TIME IN MIN

It should be noted that although HPLC separation and amino acid analysis were carried out on Nova Scotia samples, the limited amount of Nova Scotia material precluded the possibility of carrying out mixing experiments. However, the elution profile indicated in Figure 17 suggests that close elution identity exists with Nova Scotia AFP components and those of other flounder AFP components separated from New Brunswick, Long Island and Newfoundland samples.

Amino acid analysis and thermal hysteresis measurements of AFP components from New Brunswick, Long Island and Nova Scotia flounder

The amino acid compositions of the major components are tabulated in Table 9. The amino acid composition of Newfoundland AFP is included for comparison. There is a striking similarity in amino acid composition between the same components obtained from different geographical locations. Alanine is the major amino acid representing approximately 60% of the total, and aspartate (aspartate plus asparagine) and threonine are second and third in abundance. One major difference was noted in that component 8 from all of the New Brunswick samples contained a significant amount of valine. Trace amounts of valine were also observed in component 3 from New Brunswick and Long Island. Component 6, the major AFP in all locations had no valine present. Valine was present in component 9 from Newfoundland, New Brunswick and Long Island. Insufficient material was isolated from the Nova Scotia samples to carry out an amino acid analysis on this component.

AFP components 3, 4, 5+6, 7, 8 and 9 from Long Island and New Brunswick demonstrated thermal hysteresis (Table 10) and agreed with the earlier findings for the similar components isolated from the

Table 9. Comparison of amino acid compositions of major AFP from winter flounder inhabiting different geographical areas

	COMPONENT #3				COMPONENT #6				COMPONENT #8				COMPONENT #9			
	NFLD	NS	NB	LI	NFLD	NS	NB	LI	NFLD	NS	NB	LI	NFLD	NS	NB	LI
(mol %)																
Asx	10.6	14.3	10.6	10.3	10.6	11.8	12.3	11.4	13.6	15.1	13.5	14.6	8.2	-ND-	6.4	5.8
Thr	9.9	10.1	9.8	10.9	10.1	10.1	9.5	10.3	9.0	10.9	8.8	10.7	12.7		12.8	12.8
Ser	2.5	4.3	3.0	3.5	2.5	3.8	3.1	3.0	3.1	3.7	3.0	2.8	2.9		3.1	2.7
Glx	2.8	1.1	3.7	3.6	2.8	2.9	1.2	3.0	0.4	1.3	1.4	0.4	1.6		1.4	0.7
Gly	0.6	1.9	0.9	0.6	0.5	1.4	2.0	0.9	1.0	1.6	1.0	0.5	1.1		1.5	1.2
Ala	61.2	57.7	59.0	57.1	62.8	58.8	57.4	58.8	62.5	58.4	58.8	60.0	63.3		61.3	68.0
Val	0	0	0.9	0.4	0	0	0	0	0	0	2.8	0	3.5		3.5	1.7
Leu	5.5	5.5	6.0	5.1	5.7	5.5	8.5	7.2	5.2	5.2	5.7	5.7	2.8		4.6	3.5
Lys	5.2	2.5	3.1	6.1	2.7	2.7	3.4	2.9	2.9	3.1	2.2	2.6	2.1		3.3	1.7
Arg	1.7	2.8	3.0	1.8	2.3	3.0	2.6	2.5	2.3	0.7	2.6	2.8	1.9		2.0	1.8
Pro	0	0	0	0.7	0	0	0	0	0	0	0	0	0		0	0

Samples were hydrolyzed in 6 N HCl at 110°C for 24 h. NFLD; Newfoundland, NS: Nova Scotia, NB; New Brunswick, LI; Long Island New York.

Table 10. Antifreeze activity of AFP components from Newfoundland, New Brunswick and Long Island (New York) winter flounder

Purified HPLC components:	1	2	3	4	5	6	7	8	9	Control
Sample origin :	(Thermal hysteresis in °C)									
Newfoundland	.05	0.05	0.30	0.22	0.30	0.48	0.32	0.52	0.35	0.04
New Brunswick	ND	ND	0.30	0.37	ND	0.56	0.22	0.58	>0.37	
Long Island	*	*	0.30	0.47	ND	0.65	0.24	0.63	0.32	

Thermal hysteresis measurements were made using a Clifton nanoliter Osmometer as described in the Materials and Methods. Evaluations were conducted on equivalent volumes of samples (3 µl) previously purified by HPLC. Sample concentration was 2 mg/ml and should be considered approximate since estimations were based on each HPLC component having an equivalent E_{230} (1.8 OD/ml = 1 mg/ml). Each data point represents the average of at least three determinations. ND - not determined. *, pooled samples containing components 1 and 2 were measured together and exhibited no thermal hysteresis. Control, 5 µg of 0.01 M NH_4HCO_3.

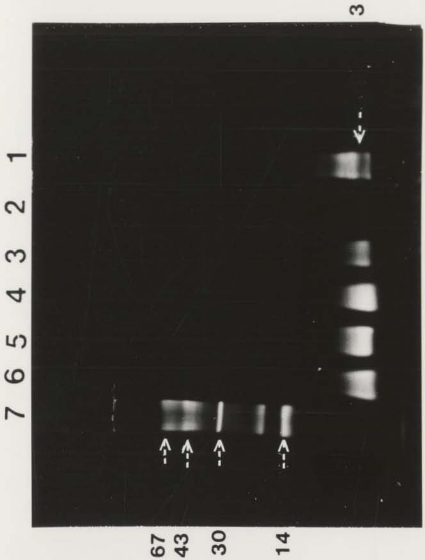
Newfoundland flounder plasma. Components 1 and 2 had no thermal hysteresis and are not considered to be AFP. Those components which have thermal hysteresis also exhibited the growth formation of long thin ice spicules which was first described by Scholander and Maggert (1971) and is highly characteristic of glycopeptides and peptide antifreezes (DeVries, 1983; Raymond and DeVries, 1977).

Although there was not enough HPLC purified material to do freezing point activity measurements on the individual Nova Scotia components, collectively these components were indistinguishable in thermal hysteresis when compared to Newfoundland, Long Island and New Brunswick sample analysis conducted on Sephadex G-75 material (Fig. 16). No comparison was made between thermal hysteresis of different AFP components since an absolute measurement of the concentration for the various components was lacking.

SDS gel electrophoresis and dansylation analysis of New Brunswick AFP components

Components 6 and 8 from New Brunswick HPLC fractionation demonstrated electrophoretic identity with Newfoundland component 6 and 8 on SDS polyacrylamide gels (Fig. 22). Since earlier analysis indicated that Newfoundland components 6 and 8 had molecular weights of 3300 it was apparent that these components from New Brunswick samples had similar molecular weights.

Figure 22. Polyacrylamide gel electrophoresis of Dansylated AFP purified from Newfoundland and New Brunswick winter flounder by HPLC. Peptides were electrophoresed on a 15% polyacrylamide gel (as described in Materials and Methods). Lane 1, reduced insulin; 5 µg (from Sigma); Lane 2, component 6 from Newfoundland 1 µg; Lane 3, components 6 and 8 from Newfoundland; Lane 4, components 6 and 8 from New Brunswick; Lane 5, components 6 and 8 from Newfoundland; Lane 6, components 6 and 8 from New Brunswick; Lane 7, Pharmacia standards. Each lane contained approximately 10 µg of dansylated material unless otherwise noted. Approximate molecular weights are indicated by arrows.


1
2
3
4
5
6
7
67
43
30
14
3

Discussion

Considerable discrepancy exists in the literature concerning the number, size and amino acid sequence of flounder AFP.

DeVries and co-workers had initially reported the presence of three polypeptides of 6000, 8000 and 12,000 (Duman and DeVries, 1976). Recently these molecular weight values have been corrected to 3200, 5000 and 8000 (DeVries, 1982). In contrast, Hew and Yip (1976) reported the presence of a 10,000 dalton species as estimated by gel filtration chromatography. It has been suggested that the high α-helical content and rodlike structure of AFP results in an overestimation of its molecular weight by gel filtration chromatography (Ananthanarayanan and Hew, 1977a). Further studies to characterize flounder AFP utilized reverse phase HPLC to fractionate the major fractions isolated from both Sephadex G-75 and QAE Sephadex ion-exchange chromatogrpahy into two distinct, yet similar, components A and B of 3300 daltons (6 and 8 in this chapter) (Davies et al., 1982). The primary structure of one of the components (component A) has been elucidated by recombinant DNA technology to be a 38 amino acid polypeptide with a corresponding proAFP (61 amino acids) and preproAFP (82 amino acids) sequences (Davies et al., 1982). Independently, DeVries and Lin (1977a) have reported the primary structure of a 3300 dalton polypeptide (AFP-3) by protein sequencing, and Lin and Gross (1981) have deduced an antifreeze peptide structure of 64 amino acids from a cDNA clone. Although the above three sequences are similar, they are not identical. As pointed out by Davies et al. (1982), the AFP structure deduced by Lin and Gross (1981) from the nucleotide sequence might not correspond to a secretable protein. In addition, the amino acid composition of such a protein does not match the composition of any of the AFP published by these workers.

The high solubility of flounder AFP in most aqueous solutions (AFP are known to be soluble in 10% TCA) and their poor staining by conventional protein stains have made their direct visualization and characterization by PAGE a difficult task. Differences in flounder AFP, noted in the literature, could be due to several factors: 1) the timing of sample collection; 2) difficulties in fractionation and characterization; or 3) population variations.

Initial attempts to fractionate flounder AFP by reverse phase HPLC (Davies et al., 1982) had utilized material which had been previously chromatographed by Sephadex G-75 followed by QAE-Sephadex ion-exchange chromatography. The major ion-exchange peak was fractionated into two major components A and B (6 and 8, respectively in this paper) but a few minor peaks were also noted. The protocol developed in this study utilizing Sephadex G-75 chromatography and two different acetonitrile gradients on reverse phase HPLC has superior resolving capabilities for AFP components and eliminates the need for ion-exchange chromatography.

The fractionation pattern of flounder AFP is highly reproducible. It was noted that none of the summer flounder serum components separated by Sephadex G-75 chromatography and reverse phase HPLC coeluted with the known AFP. Flounder AFP repurified by Sephadex G-75 chromatography can be resolved into at least nine components by HPLC. However, only seven of these HPLC components are considered to be active; exhibiting the amino acid composition and thermal hysteresis characteristics of flounder AFP. Components 1 and 2, which represent the Coomassie Blue staining bands on SDS gel electrophoresis, lack antifreeze activity and do not show any significant amount of alanine in their amino acid composition. Components 6 and 8, are the two major antifreeze peptides. These two components differ in only one amino acid, where glutamic acid in component 6 is replaced by aspartic acid or aparagine in component 8. Except for the presence of an arginine and one less alanine, component 8 has the same amino acid composition as AFP-3 reported by DeVries and Lin (1977a). Components 3, 4, 5, 6 and 8 are small (3300 daltons, 36-37 amino acids) and more homologous to each other in comparison to components 7 and 9. The amino acid sequence of component 6 (component A, Davies et al., 1982) deduced from the cDNA sequence showed the presence of glycine as its C-terminal amino acid which was absent in the mature peptide. This post-translational cleavage presumably occurs after the nascent peptide has been synthesized. Component 5, except for the absence of arginine, is identical to component 6. It would appear that the terminal arginine is removed subsequent to the removal of glycine (---ArgGly-COOH). This cleavage mechanism could also account for the absence of arginine and glycine in the AFP-3 reported by DeVries and Lin (1977a). However, the presence of one additional alanine in AFP-3, compared to component 8, is difficult at present to explain. Component 4 is identical to component 6 with one less alanine. Whether these differences represent genuine deletions or additions or some error in amino acid analysis remains to be established.

Components 7 and 9 are unusual in that they are larger than the other AFP components (approximately 4500 daltons and 56-59 amino acids) and they contain valine. The amino acid composition of these components are similar to the composition deduced from the cDNA clone reported by Lin and Gross (1981) with some differences, notably the presence of valine in both components 7 and 9. Recently a valine-containing AFP gene has been detected from the winter flounder gene library (Davies and Hew, unpublished results). It will be interesting to compare the amino acid sequences of these components in order to determine whether or not they share any structural homology that might be absent in the smaller AFP.

In order to determine if differences in flounder AFP noted in the literature could be attributable to geographical polymorphism in different flounder populations, the remaining part of this study was concerned with the characterization of flounder AFP isolated from various populations throughout its range. Samples were taken during the same winter months to eliminate any possible variation due to sampling time during the seasonal synthesis. The superior resolution of reverse phase HPLC was utilized to purify and characterize the samples.

AFP isolated from winter flounder inhabiting the coastal waters of Newfoundland, New Brunswick, Nova Scotia and Long Island (New York) are very similar, if not identical. These AFP share common Sephadex G-75 chromatography and HPLC profiles. There are at least seven active components isolated in similar proportions in all samples and the major components have similar amino acid compositions. There appears to be little geographical polymorphism in flounder. However, a few interesting minor variations are noted.

The presence of valine in component 8 and possibly component 3 from New Brunswick suggests that minor variations in flounder AFP do exist in this population. Valine is a hydrophobic amino acid and its presence in some samples may reflect a conservative substitution, since flounder AFP in general contain a large concentration of hydrophobic amino acids. If this is the case, one would expect little change in biochemical or physical properties of the AFP with this substitution. However, this may be too simplistic an interpretation since the substituion of valine for another amino acid in some proteins may result in major biochemical changes. The replacement of glumatic acid by valine in hemoglobin results in the manifestation of sickle cell anemia (Ingram, 1957) and more recently it has been shown that a single point mutation resulting in the replacement of valine for glycine in an oncogene product was responsible for the acquisition of transforming properties by the EJ and T24 human bladder carcinoma gene (Reddy et al., 1982 and Tobin et al., 1982). The significance of the valine variation in the New Brunswick AFP commponent 8 is unknown, except that antifreeze activity is present and thus appears to be unaffected by this alteration. The presence of valine in one of the major AFP components does reflect a molecular change at the gene level and would suggest that some genetic polymorphism exists within the New Brunswick population.

Component 9 from New Brunswick and Long Island, like the Newfoundland sample, also contains valine. In the case of the Newfoundland samples, component 9 and component 7, which both contain valine, are approximately 4500 daltons and have 56-59 amino acids, whereas the other AFP components are smaller, approximately 3300 daltons, and have 36-37 amino acids. This may suggest that component 9 in New Brunswick and Long Island samples may also be a larger AFP. The significance of the absence of a component 9 in Nova Scotia samples and the structural relationships of a larger group of AFP remains to be investigated.

The proportions of the major AFP components from the different samples were similar but a slight variation was observed in one of the New Brunswick samples in that component 8 occurred in a smaller concentration. Samples from other individual New Brunswick animals gave profiles similar to that of Newfoundland AFP. Our experience with the limited number of samples from New Brunswick indicated that minor variations occurred in the ratio of AFP and amino acid composition (i.e. presence of valine) as compared to other locations. Once again, this may suggest that some genetic polymorphism exists in the New Brunswick population.

It appears that geographical polymorphism cannot account for the differences in the number and size of AFP reported in the literature between the Newfoundland winter flounder AFP (Davies <u>et al</u>., 1982) and the AFP isolated from winter flounder inhabiting more southerly coastal waters (Lin and Gross, 1981; DeVries, 1982). Although genetic polymorphism may be present this variation appears to be minimal, restricted to a single population of flounder and may reflect a conservative amino acid substitution. Winter flounder share a remarkable constancy and identity in their AFP regardless of their habitat. A previous study conducted on shorthorn sculpin populations from Grise Fiord (Southern Ellesmere Island, arctic Canada) and Newfoundland indicated that the AFP were essentially identical, with respect to molecular weight, number of components and amino acid composition (Fletcher <u>et al</u>., 1982a). This suggests that survival in freezing coastal waters requires a closely defined set of antifreeze polypeptides, all of which are similar and can only tolerate minor variation or modification within a species.

The presence of multiple AFP in the winter flounder is consistent with recent observations on the large number of AFP seen in ocean pout (Hew et al., 1984) and shorthorn sculpin (Hew et al., 1980; Fletcher et al., 1982a). The presence of multiple antifreeze glycopeptides in the Antarctic notothenids and Northern gadoids has also been well documented (Feeney and Yeh, 1978; Hew et al., 1981; DeVries, 1982; Fletcher, 1982b). Thus, it may be that all fish require a multiple component antifreeze system.

CHAPTER 3

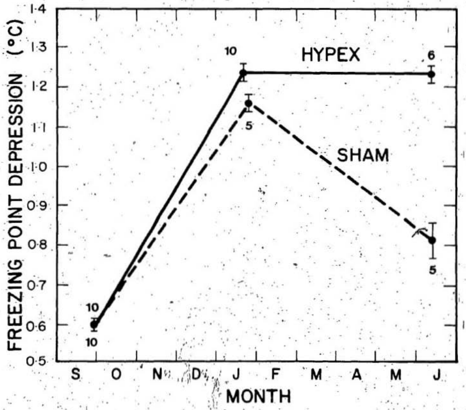
ACCUMULATION OF WINTER FLOUNDER ANTIFREEZE mRNA AFTER HYPOPHYSECTOMY

Introduction

The survival of the winter flounder (Pseudopleuronectes americanus) in the freezing coastal waters of Newfoundland during the winter months is primarily due to the seasonal synthesis and accumulation of AFP (Duman and DeVries, 1974a, 1976; Fletcher, 1977, 1981; Fletcher and Smith, 1980). AFP concentrations are high (10-15 mg/ml) in the winter and are absent in the summer (Hew and Yip, 1976; Fletcher, 1977; Slaughter and Hew, 1982). In addition, AFP mRNAs also follow a pattern of seasonal synthesis (Hew and Yip, 1976; Lin, 1979; Lin and Long, 1980; Pickett et al., 1983). In a detailed study, Pickett and co-workers (1983) have demonstrated that AFP mRNAs constitute 0.5% of the total liver RNA in winter and only 0.0007% in the summer. The seasonal change in AFP mRNA concentrations and their corresponding protein products suggests that transcriptional control plays a critical role in the seasonal regulation of AFP protein genes.

The seasonal synthesis of AFP and their mRNAs is apparently influenced by environmental factors (Fletcher, 1981) and the pituitary gland (Fletcher et al., 1978; Hew and Fletcher, 1979) but the molecular mechanisms underlying this influence are unknown. Fletcher et al. (1978) have demonstrated that in flounder hypophysectomized in September, high concentrations of AFP appeared in the plasma during the winter but the AFP did not disappear at the normal time in the spring (Fig. 23). Similar results were obtained with flounder hypophysectomized in April, near the end of the normal seasonal synthesis pattern of AFP (Hew and Fletcher, 1979). These results suggest that the pituitary gland operates in a

Figure 23. Seasonal changes in plasma freezing point depression of hypophysectomized (hypex) and sham-operated (sham) winter flounder. Values are plotted as means ± 1 SE. Numbers at each point represent the number of fish sampled. The initial values for September are concentrations before operation. Fish were hypophysectomized in late September and held under ambient temperature and photoperiod. Figure adapted from Fletcher *et al*. (1978).


FREEZING POINT DEPRESSION (°C)
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
S
O
N
D
J
F
M
A
M
J
MONTH
HYPEX
SHAM
10
10
10
5
6
5

negative fashion for upon its removal, flounder retain high concentrations of AFP regardless of summer photoperiod and water temperatures. It is unlikely that the maintenance of high concentrations of AFP in hypophysectomized fish is due to changes in clearance. Preliminary results indicate that the clearance of AFP from hypophysectomized fish is similar to that of controls (Hew and Fletcher, 1979). The elevated levels of AFP in hypophysectomized fish appear to be due to continued synthesis of AFP.

These results imply that the concentration of AFP in the winter flounder may be under transcriptional control. This chapter describes experiments designed to understand further the role of the pituitary in the regulation of AFP synthesis.

Materials and Methods

Collection of experimental materials

Winter flounder (*Pseudopleuronectes americanus*) (400-600 g) were collected from Conception Bay, Newfoundland and maintained in 250-L aquaria at seasonally ambient temperature and photoperiod (Fletcher, 1977). Hypophysectomy and sham operations were conducted in June as described earlier (Campbell and Idler, 1976). The biosynthesis of AFP and their corresponding mRNA was studied in July and August (water temp. 8 to 10° C) when all traces of plasma antifreeze had disappeared from control animals. Experimental as well as control animals were starved throughout the experimental period since it has been observed that hypophysectomized animals do not eat following surgery (Fletcher and King, 1978).

Blood samples (0.6 ml) were collected from the caudal vein using 3cc syringes fitted with 25-gauge needles and stored in heparin coated tubes. After low speed centrifugation, the plasma was stored at -20° C. Freezing points were determined using a freezing point osmometer (Advanced Instruments, model 3D, Needham Height, MA) as described earlier (Hew and Yip, 1976).

Livers from winter (November), summer (July or August), hypophysectomized (hypex) and sham operated animals were removed from the animals and were either processed immediately or frozen in liquid nitrogen and stored at -70° C.

Plasma from hypex animals was chromatographed on Sephadex G-75 and fractionated by reverse phase HPLC as described in Materials and Methods, Chapter 2.

RNA isolation

Total cellular RNA from 7-12 gm of liver from individual fish was isolated by phenol chloroform extraction, followed by a proteinase K digestion as described by Davies and Hew (1980).

Antifreeze mRNA was purified by chromatography on oligo-(dT)-cellulose (Aviv and Leder, 1972) and sedimentation on a 15-30% linear sucrose gradient following a dimethyl sulfoxide disaggregation treatment (Schleif and Wensink, 1981). RNA isolated from the sucrose gradient was further separated on 1.5% agarose gels containing the denaturant methyl mercury hydroxide (Bailey and Davidson, 1976). Agarose gel slices containing specific RNAs were excised and extracted in 2 volumes of 50% formamide, containing 50 mM tris, pH 7.4, 1 mM EDTA and 1.0 M NaCl. The RNA was left overnight in the above buffer and re-extracted with an equal volume of chloroform: isoamyl alcohol (24:1, v:v). The purified RNA was recovered by ethanol precipitation at -20°C overnight. The integrity of RNA preparations was checked by agarose gel electrophoresis in the presence of methyl mercury hydroxide followed by ethidium bromide staining (Bailey and Davidson, 1976).

Cell-free translation

A mRNA - dependent rabbit reticulocyte lysate kit (Pelham and Jackson, 1976) was obtained from Bethesda Research Laboratories, MD, USA. Directions according to the kit protocol were followed but concentrations of potassium acetate and magnesium acetate were optimized in trial experiments. Typically the incubation mixtures for cell-free translations contained the reaction mixture from the kit, 10 μl of nuclease-treated lysate, 16 to 20 μg of total RNA or 0.5 to 1 μg of purified antifreeze mRNA, and 2.5 μl of undiluted translation grade (^{35}S) methionine (specific activity 1226.5 Ci/mmol, New England Nuclear, Montreal, Canada). Samples were incubated at 37° for 90 min during which time, incorporation of radioactivity was almost linear.

Product analysis

Incorporation of radioactively-labelled amino acid into protein was measured by acid precipitation followed by collection of the precipitate on glassfiber filters and scintillation counting in a toluene based liquid scintillation fluid (Pelham and Jackson, 1976). Radioactively-labelled proteins were analysed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Laemmli, 1970). The separating gel was a 9-22.5% concave exponential polyacrylamide gradient or a 15% SDS gel. After electrophoresis, gels were either stained in a 0.1% Coomassie Brilliant Blue solution (Paul et al., 1972) or treated directly for fluorography (Bonner and Laskey, 1974). Dried gels were autofluorographed at -70°C for 48 h using Kodak X-OMAT XAR-5 X-ray film.

Transfer of RNA to nitrocellulose and hybridization to a radioactively-labelled antifreeze cDNA.

RNA samples from flounder liver were initially electrophoresed on a 1.5% agarose gel as described above. After staining with ethidium bromide and photography, the gel was prepared for Northern blot transfer by a modification of the procedure of Thomas (1980) and Alwine et al. (1977). The gel was washed in 2 x gel volume of 50 mM NaOH containing 5 mM 2-mercaptoethanol for 30 min and then washed for 20 min in 2 x gel volume of 200 mM potassium phosphate pH 6.4 containing 7 mM iodoacetic acid. The gel was finally equilibrated in 2 x gel volume of 20 x SSC buffer (0.15 M

NaCl/0.015 M Trisodium citrate) for 20 min with gentle shaking. Nitrocellulose (BA 85, Schleicher and Schuell) was prepared by washing in 200 ml of diethylpyrocarbonate treated sterile water for 10 min and the transfer was carried out according to Thomas (1980). All of the above steps were carried out at room temperature.

After transfer, the nitrocellulose was baked at 80°C for 2 h in vacuo to fix the RNA to the filter. The nitrocellulose was pretreated and hybridized as described by Thomas (1980) except that the carrier DNA was from calf thymus. The RNA bound to the nitrocellulose was hybridized to a nick-translated CT5 probe which contains 324 bp of antifreeze protein cDNA cloned into the Pst 1 site of pBR322 (Davies et al., 1982). The cDNA sequence in CT5 codes for an 82-residue antifreeze preproprotein flanked by untranslated regions. The CT5 plasmid was labelled to a specific activity of 1.8×10^8 cpm/μg by nick-translation (Maniatis et al., 1975) with [α^{32}P] dCTP using a New England Nuclear nick-translation kit (New England Nuclear, Montreal, Canada). Prehybridization and hybridization was carried out at 42°C for 24 h with gentle agitation. Washing "stringency" was conducted according to the following procedure: washed twice for 20 min each, with 1 ml of 2 x SSC, 0.1% SDS per cm^2 of nitrocellulose at room temperature and twice for 20 min each, with 0.1 x SSC, 0.1% SDS at 50°C (1 ml/cm^2 of nitrocellulose). Autoradiography was carried out at -70°C with Kodak X-OMAT, XAR-5 X-ray film and an intensifying screen (Dupont Cronex; Swanstrom and Shank, 1978).

Cytoplasmic dot hybridization

Dot hybridization analyses of cellular cytoplasmic preparations were carried out according to a procedure modified from White and Bancroft

(1982). Liver fractions (0.2 g) were homogenized in a loose-fitting 100-1000 μl capacity glass homogenizer (Thomas Co.) containing 550 μl of 10 mM Tris, pH 7.0, 1 mM EDTA, 0.5% Nonidet P-40 with 10 revolutions of the pestle. The contents were transferred to a sterile 1.5 ml polypropylene tube and incubated for at least 2 min on ice. The nuclei were pelleted by centrifugation (15,000 x g, 2.5 min). The cytoplasm was carefully transferred to another sterile 1.5 ml tube and centrifuged a second time (15,000 x g, 2.5 min). Following the second centrifugation, 500 μl of the supernatant was transferred to a 1.5 ml tube containing 300 μl of 20 x SSC, plus 200 μl of 37% formaldehyde (Fisher No. F-79). The mixture was then incubated at 60°C for 15 min, and either stored at -70°C or analyzed immediately. For analysis, 20 μl of each sample were initially diluted to 400 μl with 15 x SSC and then 200 μl was serially diluted with 15 x SSC to yield a final volume of 400 μl and 150 μl of each dilution was applied with suction to a 4 mm diameter spot on a nitrocellulose sheet (BA 45, 0.45 m Schleicher and Schuell) employing a 96-hole dot blot manifold apparatus (Bethesda Research Labs Inc., Maryland). The nitrocellulose sheet was then baked at 80°C for 2 h and the RNA was prehybridized, hybridized to a nick-translated CT5 antifreeze cDNA clone, washed and autoradiographed as described in the above section. To ensure linear dependence on radioactive label the X-ray film was preflashed according to the procedure of Laskey and Mills (1975). Scanning and quantitation of the autoradiograph was performed on a Corning 750 integration densitomer (Medford, Mass.).

Rate of accumulation of AFP mRNA

The rate of accumulation of antifreeze mRNA was followed in two hypophysectomized fish by sampling the livers (0.2-0.3 g) through an abdominal incision. The levels of AFP mRNA in the samples were monitored by the cytoplasmic dot hybridization procedure as described above.

Results

Effect of hypophysectomy on plasma freezing temperature, liver weight and poly (A)$^+$ RNA content

Hypophysectomy was performed in early summer (June and July). Plasma samples taken prior to surgery had freezing temperatures of -0.68°C indicating little or no AFP were present. When these hypophysectomized (hypex) fish were kept for 2 to 4 weeks under ambient conditions of seawater temperature and photoperiod (8-10°C) (Fletcher, 1977), the freezing temperatures of their plasma were lowered to -1.02°C which is comparable to the freezing temperatures of plasma found in fish actively synthesizing AFP during the winter months (Table 11). In contrast, the unoperated summer animals as well as the sham operated animals, had plasma freezing temperatures of -0.6°C to -0.7°C. Previous studies have shown that the lower freezing temperatures observed in the plasma of hypex animals were due to an increase in the concentration of AFP (Fletcher et al., 1978). Changes in the electrolyte contribution to freezing point depression are small relative to the antifreeze contribution. Hypex flounder, therefore, appear to contain a high level of AFP regardless of seawater temperature and photoperiod.

Although both the sham operated and the hypex animals were kept under identical conditions of starvation, the weights of their livers differed significantly. The livers in sham operated animals regressed and accounted for only 0.6% of total body weight whereas those from the hypex animal were larger and morphologically indistinguishable from the livers of normal, winter animals actively synthesizing AFP (Table 11). In addition, livers of hypex animals contained at least twice as much poly (A)$^+$ RNA

Table 11. Effect of hypophysectomy on flounder serum freezing temperature, liver weight and poly $(A)^+$ RNA content

	N	freezing$^{(a)}$ temperature	liver weight % body weight	Poly (A) RNA$^{(b)}$ % total RNA
Nonoperated (July)	4	-0.62° C	0.90±0.073	0.49-0.52
Sham operated (July)	2	-0.73° C	0.60±0.087	0.40
hypophysectomized (July)	5	-1.02° C	1.67±0.108	1.36-2.30
Nonoperated (November)	6	-1.01° C	1.65±0.16	1.01-1.50

(a) determined on pooled serum samples, (b) denotes range, estimated from A_{260} values of material bound to oligo-dT-cellulose. N = number of samples; values expressed as means ± standard error where applicable.

compared to those from the sham controls (Table 11).

Comparison of plasma AFP from winter control versus hypex flounder

AFP isolated from the hypex animals were analysed by reverse phase HPLC as described in the preceeding chapter (see Chapter 2; Materials and Methods and Fig. 7). The plasma from hypex animals contained the same AFP (components 3-8) as those of winter control animals (Fig. 24). Components 6 and 8 were the major components in both cases. Component 9, although present in both hypex and control animals at approximately 50 min elution time, has not been indicated in Figure 24. Components 1 and 2 appear to be missing from the hypex flounder plasma HPLC profile. However, freezing point depression studies and amino acid analysis have indicated that components 1 and 2 are not AFP (Chapter 2). The structure and function of these two polypeptides are unknown.

Isolation of AFP mRNA and cell-free translation studies

When poly $(A)^+$ mRNA isolated from hypex flounder was analyzed in 1.5% agarose gels in the presence of methyl mercury hydroxide, three major components were observed which corresponded to the 18 S and 28 S ribosomal RNA and a 7.5 S RNA (Fig. 25). This latter species of RNA had the same electrophoretic migration as AFP mRNA extracted from fish actively synthesizing AFP during the winter. The 7.5 S mRNA from hypex flounder is considered to be AFP mRNA by cell-free translation and Northern blot hybridization and was indistinguishable from the previously identified and isolated AFP mRNA from winter fish actively synthesizing AFP (Davies and Hew, 1980; Davies et al., 1982; Pickett et al., 1983). The mRNA-directed cell-free translation products from a reticulocyte lysate system were analysed by SDS PAGE (Fig. 26). The pure AFP mRNA synthesized cell-free

Figure 24. Analysis of AFP from hypophysectomized flounder by reverse phase HPLC. (A) AFP from winter control animals (B) AFP from hypex animals. Samples were dissolved in 5% formic acid and analyzed with an Altex Ultrasphere ODS column, 0.02 M triethylamine phosphate buffer, pH 3.0 with an acetonitrile gradient, flow rate 1 ml/min (as described in Fig. 7, Chapter 1). As outlined in Chapter 2, components 1 and 2 are not AFP.

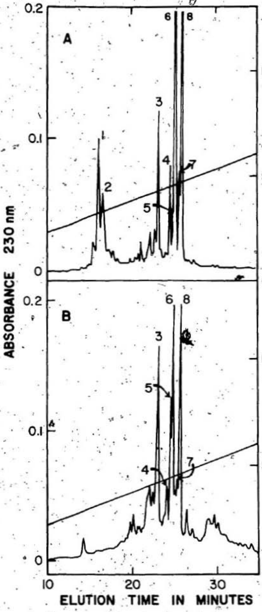

A
B
1
2
3
4
5
6
7
8
0.2
0.1
0
100
50
0
10
20
30
ABSORBANCE 230 nm
% ACETONITRILE
ELUTION TIME IN MINUTES

Figure 25. Analysis of flounder RNA extracted from hypophysectomized, sham and control fish. RNA samples were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide as described in Materials and Methods. Gel lanes contain the following RNA: (1) Dec RNA 3.0 μg, (2) Hypex RNA (from July fish) 2 μg, (3) QX 174 DNA Hae III restriction fragment markers (from Bethesda Research, Maryland), fragment size indicated in left margin, (4) Dec RNA 10 μg, (5) Dec RNA 4 μg, (6) Control summer RNA (July) 5 μg, (7) Hypex RNA 4 μg. All samples had been chromatographed once through an oligo-(dT)-cellulose column. The fraction which bound to the column was used in these studies. Ribosomal 28 S and 18 S and the 7.5 S AFP mRNA are indicated in the right margin.

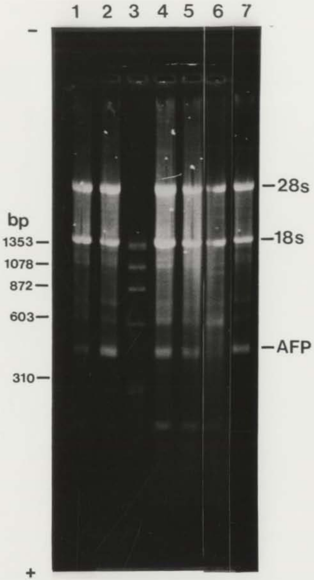
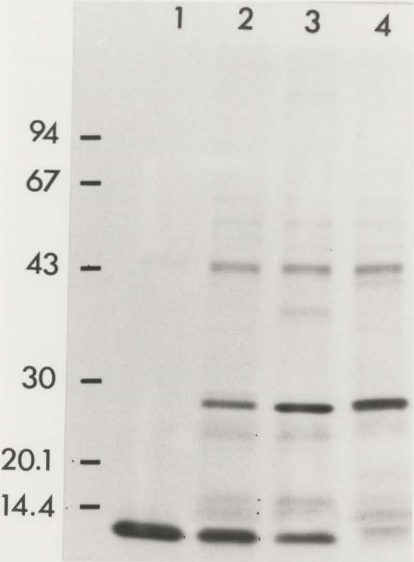

1
2
3
4
5
6
7
–
+
bp
1353
1078
872
603
310
28s
18s
AFP

Figure 26. Analysis of cell-free translation products of flounder liver mRNA by SDS PAGE. Acrylamide gel concentration 9 to 22.5%. 15,000 cpm of labelled proteins were loaded on each lane and were translated from the following concentrations of RNA: Lane 1, pure AFP mRNA 2.4 μg, Lane 2, total RNA from hypex animals, 16 μg. Lane 3, total RNA from December animals, 16 μg. Lane 4 total RNA from sham-operated (summer) animals, 16 μg.


1
2
3
4
94
67
43
30
20.1
14.4

translation products of 12,000 daltons. These products were present only in RNA preparations from the winter and the hypex animals. The identity of the AFP mRNA and its translation product from winter animals has been well characterized from automatic protein sequencing and cDNA sequence analysis (Davies et al., 1982).

AFP mRNA was purified from fish sampled during the winter months (Fig. 27). The extraction of 10 g of liver yielded approximately 1600 A_{260} units of total RNA and 1 to 2 A_{260} units of purified APP mRNA following the final purification step. The criteria of a single ethidium bromide-stained band on an agarose RNA gel and a single cell-free translation product with this mRNA was considered to be evidence that the flounder AFP mRNA was greater than 90% pure.

RNA analysed by the Northern blot technique with a ^{32}P-labelled, nick-translated antifreeze cDNA clone (CT5), showed a high degree of hybridization to both poly $(A)^+$ and total liver RNA from hypex and the November animals. No hybridization was detected in either the sham operated or summer animals (Fig. 28). However, with longer exposure and higher RNA concentrations, a weak hybridization could be observed for these two latter samples. This is consistent with the recent observation that there is a very small but detectable level of AFP mRNA in the summer (Pickett et al., 1983).

Cytoplasmic dot hybridization analysis

Hypophysectomy and sham operations were carried out during August and the animals were sacrificed in the first week of September. Individual cytoplasmic preparations from hypex, sham-operated, summer (July) and winter (December) fish were serially diluted and dotted on a single nitro-

Figure 27. Analysis of purified flounder AFP mRNA by agarose gel electrophoresis. RNA samples were electrophoresed in a 1.5% agarose gel and stained by ethidium bromide as described in Materials and Methods. Lanes 1 to 10 contain equal volumes of RNA sample from sucrose gradient fractions 2 to 11 respectively. Lane 11 contains purified AFP mRNA eluted from an 1.5% agarose gel. Lane 12 contains a purified mRNA of unknown identity eluted from an 1.5% agarose gel. Lane 13 contains 20 μg of flounder RNA passed through an oligo-(dT)-cellulose column once and represents a sample of the initial material loaded onto the sucrose gradient. Ribosomal 28 S and 18 S RNAs and the 7.5 S AFP mRNA are indicated by arrows.

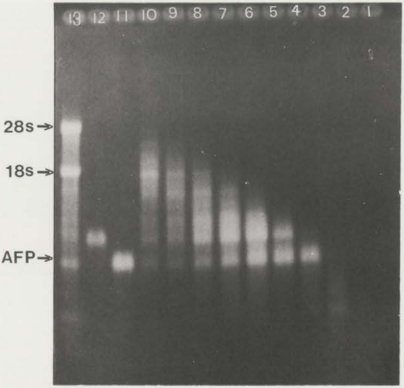
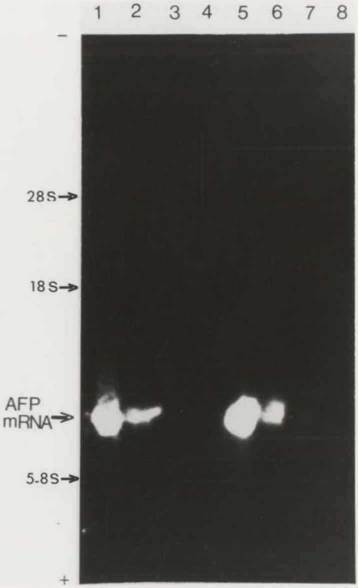

13
12
11
10
9
8
7
6
5
4
3
2
1
28s
18s
AFP

Figure 28. Analysis of flounder RNA by Northern blot hybridization. RNA samples were electrophoresed in 1.5% agarose gel, transferred to nitrocellulose paper, hybridized to nick-translated plasma CT5 as described under Materials and Methods. Gel lanes contain the following RNA: (1) hypex poly $(A)^+$ RNA, 3.0 μg, (2) hypex, total RNA, 28 μg, (3) sham-operated poly $(A)^+$ RNA, 11.0 μg, (4) sham operated total RNA, 26 μg (5) November poly $(A)^+$; 12 μg, (6) November total RNA, 20 μg, (7) control nonoperated (July) poly $(A)^+$ RNA, 9.0 μg, (8) control nonoperated (July) total RNA, 22.0 μg. Flounder 28 S, 18 S and 5.8 S ribosomal RNAs are indicated by the arrows and were determined from a photograph of the ethidium bromide stained gel. The (-) and (+) indicate the sample well position and bottom of the gel respectively and the direction of electrophoresis.


1
2
3
4
5
6
7
8
–
28S
18S
AFP mRNA
5.8S
+

cellulose sheet and hybridized with nick-translated CT5. The results of dot hybridization analysis are shown in Figure 29. It was evident that the liver from both December and hypex animals in both sexes showed a high level of AFP mRNA. Scanning densitometry indicated that hypex and November samples contained approximately equal concentrations of AFP mRNA. Based on earlier studies (Lin and Long, 1980; Pickett et al., 1983) where it was shown that AFP mRNA comprised 0.5% of the total RNA, it was apparent that the hypex animals contained a similar concentration of AFP mRNA. Cytoplasmic dot analysis indicated that the level of AFP mRNA in the sham control and summer animals was extremely low and represented less than .01% of the total RNA.

Rate of accumulation of AFP mRNA

The rate of accumulation of AFP mRNA following hypophysectomy was determined using the cytoplasmic dot hybridization method (Fig. 30). The concentration of AFP mRNA was normalized using values from normal winter animals as 100%. In one animal, an increased level of AFP mRNA was detectable the first day following the operation, reached 10% of the winter level 4 days after hypophysectomy and 25% by day 7. This would indicate that approximately 0.125% of total RNA was AFP mRNA in day 7, a 20-fold increase compared to the summer control animals. The accumulation of AFP mRNA in the second animal was slower. Nonetheless, a 8-fold increase over the summer control animals was observed by day 11.

Figure 29. Analysis of flounder cytoplasmic RNA by the dot hybridization technique. Flounder cytoplasmic RNA with different serial dilutions were applied on the nitrocellulose paper and hybridized with ^{32}P-labelled, nick-translated plamid CT5, followed by 24 h autoradiography. A, December ♀, B, September ♀, C, hypex ♀, D, sham-operated ♀, E, December ♂, F, September ♂, G, Hypex ♂, H, sham-operated ♂. Components B, D, F and H showed hybridization only after prolonged exposure and were not detectable after 24 h of autoradiography.

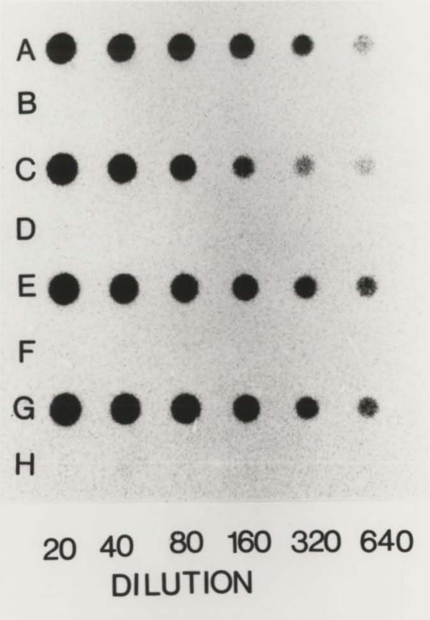
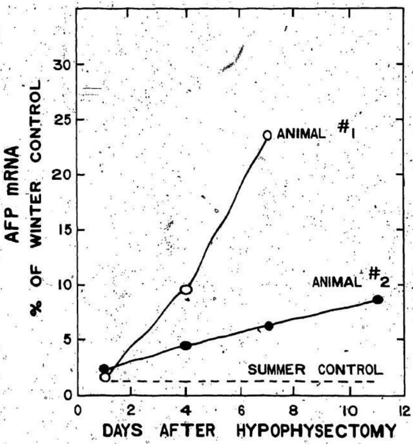

A
B
C
D
E
F
G
H
20
40
80
160
320
640
DILUTION

Figure 30. Rate of accumulation of AFP mRNA after hypophysectomy. Portions of liver were removed from two individual animals at different time intervals. The AFP mRNA concentration was estimated by the cytoplasmic dot hybridization technique and was calculated using the level of AFP mRNA in a winter control animal as 100%.


AFP mRNA
% OF WINTER CONTROL
0
5
10
15
20
25
30
0
2
4
6
8
10
12
DAYS AFTER HYPOPHYSECTOMY
ANIMAL #1
ANIMAL #2
SUMMER CONTROL

Discussion

The induction and maintenance of AFP synthesis in summer by hypophysectomy can be readily demonstrated by the measurement of antifreeze activity (Fletcher et al., 1978), detection of polypeptides by radioimmunoassay (Hew, 1981), in vivo and in vitro protein synthesis (Hew and Fletcher, 1979) and AFP mRNA accumulation reported in the present study. Previous studies have used flounder hypophysectomized in September (Fletcher et al., 1978), at the beginning of the seasonal synthesis of AFP, or in April when an elevated level of AFP is still present in the plasma (Hew and Fletcher, 1979). It could be argued that in these studies the synthesis of AFP was primed for its normal cycle and that hypophysectomy resulted in a continuation of its synthesis. The demonstration of AFP synthesis in this study is significant in that flounder hypophysectomy was performed after all traces of AFP had disappeared from the plasma and before the induction of a new cycle of AFP synthesis had begun. Consequently there is little doubt that the elevated levels of AFP present after hypophysectomy is due to the initiation of synthesis. There is no preferential synthesis of the polypeptides (3 to 9) since the relative concentration of the AFP is similar in both the control (November) and hypex animals. Components 1 and 2, which are not AFP, were absent from the HPLC profile of the hypex plasma sample.

The identity of the AFP mRNA in the hypex animals was confirmed by its electrophoretic migration, product analysis from cell-free translation studies and Northern blot hybridization with a specific AFP cDNA clone. The presence of several AFP and a broad hybridization band detected in the Northern blot study suggests that there is more than one antifreeze mRNA in the winter flounder. The presence of several active AFP and the isolation of DNA sequences from at least six separate genomic antifreeze protein loci in the winter flounder supports this conclusion (Davies et al., 1981; Davies et al., 1984). Due to the similar size of the AFP polypeptides, the mRNA for these polypeptides have probably the same molecular weight making their separation difficult.

The cytoplasmic dot hybridization technique has made it possible to quantify the AFP mRNA level after hypophysectomy as well as determining its rate of accumulation. The AFP mRNA was detectable at day 4 (10%) and reached 25% of normal levels in day 7. After 2-3 weeks its level was as high as that reported for winter animals actively synthesizing AFP. The level of AFP mRNA in the sham control animals was extremely low but consistent with the small but detectable level of AFP mRNA found in summer animals reported by Pickett et al. (1983). These data strongly support the hypothesis that the pituitary gland acts either directly or through some intermediary on the AFP genes resulting in the regulation of the level of translatable AFP mRNA. Unlike many other systems affected by the pituitary (Holmes and Ball, 1974), the present model appears to operate via a negative control mechanism. The rate of accumulation of mRNA depends on the rate of degradation as well as the rate of synthesis, and both these factors have been shown to be involved in controlling the levels of mRNA found in other systems (McKnight and Palmiter, 1979; Innis and Miller, 1979). While the possibility that AFP mRNA accumulation may be due to changes in degradation cannot be eliminated, the extremely low levels of AFP mRNA detected in the control summer flounder suggests that it is a reasonable hypothesis that its accumulation after hypophysectomy depends upon an increase in its transcription. It is interesting to note that a similar observation has been reported for the rat liver histidase. Armstrong and Feigelson (1980) have shown that the de novo synthesis of this enzyme is selectively increased after hypophysectomy. Treatment of hypophysectomized rats with physiological levels of triiodothyronine (T_3) diminishes histidase synthetic rates and catalytic activities to normal levels. Since the thyroid hormone is under pituitary regulation, this hormone may be primary in the hypophyseal suppression of histidase.

Although this study was concerned primarily with the effects of hypophysectomy on AFP mRNA synthesis, other interesting changes were noted. There was a significant increase in liver weight, RNA content and other parameters such as an increase in glycogen content which was observed but not pursued in our study. Johansen (1967) has shown that hypophysectomized goldfish acclimated to summer conditions of temperature and photoperiod, exhibited heat resistance characteristics of cold-acclimated fish. Consistent with this observation, hypex flounder kept under summer conditions exhibit thermal responses that are appropriate only to the winter environment. In contrast to most other vertebrates, where, after hypophysectomy, liver weight decreases (Schapiro et al., 1970), both the hypex goldfish (Walker and Johansen, 1975) and winter flounder have increased liver weight.

The chemical nature of the pituitary factors involved in the regulation of AFP synthesis is not known. A hypothesis consistent with these results is that the pituitary factor(s) responsible for the inhibition of AFP synthesis is normally secreted during the summer months. With the approach of winter, the release of this inhibitor is suppressed and consequently the transcription of AFP mRNA resumes. In accordance with this hypothesis, hypophysectomy mimics the suppression of the inhibitor(s) from the pituitary and consequently genes encoding AFP are reactivated. The observation (Fletcher, 1979) that the administration of pituitary extracts to hypex fish reverses the effect of hypophysectomy and suppresses AFP synthesis lends support to this scheme.

CHAPTER 4

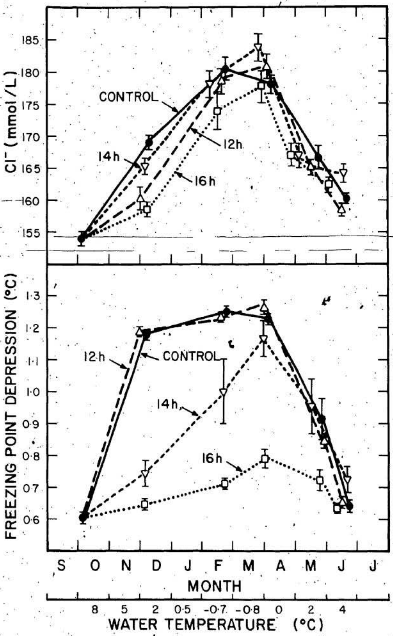
EFFECTS OF PHOTOPERIOD ON ANTIFREEZE mRNA ACCUMULATION

Introduction

The freezing point depression of winter flounder plasma undergoes seasonal changes which correlate with the changes in environmental temperature and photoperiod (Pearcy, 1961; Fletcher, 1977; Petzel et al., 1980; Fletcher and Smith, 1980). Fletcher (1977, 1981) has demonstrated that fish exposed to long day length (≥14 h) in the early fall have significantly decreased concentrations of plasma AFP throughout the winter and also experience a one to two month delay in the appearance and the synthesis of AFP (Fig. 31). It is interesting to note that the flounder require a certain minimal day length to cause this delay since day lengths of 12 h had no obvious effect on the AFP cycle. Although water temperature has little influence on the initiation of synthesis, Fletcher (1981) noted that it is important in the clearance of AFP in the spring. In contrast day length had no effect on the timing of antifreeze disappearance (Fletcher, 1981).

In order to gain a greater understanding of the role of photoperiod in the regulation of the synthesis of AFP in the winter flounder, the previous studies of Fletcher (1977, 1981) have been extended by comparing the concentrations of AFP mRNA in the livers of fish maintained on a 15 h long day with those of fish under normal conditions from September to June. This study has made use of the cytoplasmic dot blot technique (White and Bancroft, 1982; Chapter 3) which provides a rapid, yet sensitive, method of determining the concentration of specific mRNAs in small amounts of tissue.

Figure 31. Effects of day length on the winter increase and spring decrease in plasma Cl^- and freezing point depression. Groups (5-10 fish) of winter flounder were exposed to ambient water temperatures and photoperiods of ambient control, 12 h, 14 h, and 16 h light per day on October 3, 1977. Serial blood samples were taken from the fish on the dates indicated. Values are expressed as means ± one standard error. Water temperatures are mean values for 1974 to 1979 (from Fletcher, 1981).


Cl⁻ (mmol/L)
185
180
175
170
165
160
155
CONTROL
14h
12h
16h
FREEZING POINT DEPRESSION (°C)
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
12h
CONTROL
14h
16h
S O N D J F M A M J J
MONTH
8 5 2 0·5 -0·7 -0·8 0 2 4
WATER TEMPERATURE (°C)

Materials and Methods

Collection of experimental materials

Winter flounder (Pseudopleuronectes americanus) (400-600 g, 30-40 cm long) were collected from Chapel's Cove, Conception Bay, Newfoundland during the first week of September, 1981. At the laboratory they were maintained in 250-L aquaria supplied with flowing seawater (32-33 ⁰/oo). The control flounder were kept at seasonally ambient temperature and photoperiod (Fletcher, 1977). Using fluorescent lamps, the experimental group of flounder was placed under a photoperiod of 15 h of light per day starting on September 15, 1981 and continued to June, 1982. The water temperature remained ambient throughout the experiment.

Blood samples and livers from experimental and control fish were collected and stored as described in Chapter 2, Materials and Methods. Freezing point depression was determined using an Advanced Osmometer (Model 3R, Advanced Instruments Inc., MA., USA), and the plasma Cl^- by chloride titration (Radiometer Copenhagen Model CMT 10) as described by Fletcher (1977).

RNA isolation

Total cellular RNA from 7-12 g of liver from individual fish was isolated by phenol chloroform extraction, followed by a proteinase K digestion as described by Davies and Hew (1980). Antifreze mRNA was purified as described previously (Chapter 3; Materials and Methods). The integrity of the RNA preparations was checked by agarose gel electrophoresis in the presence of methyl mercury hydroxide followed by ethidium bromide staining (Bailey and Davidson, 1976) or by formaldehyde formamide RNA denaturing gel electrophoresis (Lehrach et al., 1977).

Transfer of RNA to nitrocellulose and hybridization to an antifreeze probe

RNA samples from flounder liver were electrophoresed on a 1.5% agarose gel as described above. The gel was prepared for Northern blot transfer and the RNA transferred to nitrocellulose, hybridized and washed as described in Chapter 2, Materials and Methods. The RNA bound to the nitrocellulose was hybridized to a nick-translated genomic clone E_3, which encodes for the most abundant antifreeze protein in the flounder (Davies et al., 1982, 1984). The E_3 clone contains 246 nucleotides coding for an 82-residue antifreeze preproprotein, 5' and 3' flanking untranslated regions and a 0.6 kbp intervening sequence. The E_3 genomic clone was radioactively-labelled to a specific activity of 3.2×10^8 cpm/μg by nick-translation (Maniatis et al., 1975) with [α^{32}P] dCTP using a New England Nuclear nick-translation kit (New England Nuclear, Montreal, Canada). Autoradiography was carried out at -70°C with Kodak X-O Mat, XAR-5 X-ray film with an intensifying screen (Dupont Cronex) as described by Laskey (1980).

Cytoplasmic dot hybridization and RNA quantitation

RNA dot hybridization analyses of cellular cytoplasmic preparations were carried out according to a procedure modified from White and Bancroft (1982). Liver samples (0.2 g) from both control and experimental fish were processed as described previously (Chapter 3; Materials and Methods) to attain a denatured cytoplasmic RNA fraction. Following denaturation, 20 μl of this fraction was diluted to 400 μl and 200 μl was serially diluted with 15 x SSC (0.15 M NaCl/0.015 M Trisodium citrate, pH 7.0) to yield a final volume of 400 μl. An aliquot (150 μl) of each dilution was applied with suction to a 4 mm diameter spot on a nitrocellulose sheet (Ba 85, Schleicher and Schuell) employing a dot blot manifold apparatus (Bethesda Research Labs, Inc., Maryland). The nitrocellulose sheet was then baked at 80°C for 2 h, hybridized to a nick-translated E_3 antifreeze probe and autoradiographed as described above. To ensure linear dependence of radioactive label with the intensifying screen, the X-ray film was preflashed according to the procedure of Laskey and Mills (1975) and Laskey (1980). Scanning of the autoradiograph dot blots was performed on a transmittance/reflectance densitometer (BioRad Model 1650) and the peak areas were integrated over the linear dependence range to derive a relative quantitation of antifreeze mRNA which was expressed as optical density in scanner units.

RESULTS

Plasma Cl and freezing point depression measurements

The seasonal changes in plasma Cl^- concentrations were essentially identical in both the control and 15 h day length experimental fish (Fig. 32). The increase in winter plasma freezing point depression in the fish exposed to 15 h light per day was delayed by approximately two months and the mean values were significantly lower than the control values ($p < .01$) from December to May (Fig. 32). The highest freezing point depression in both groups of fish occurred during February. However, the mean value attained for the long day length fish represented a little more than half the mean freezing point depression value determined in the control fish. Previous studies have shown that the increasing freezing point depression observed in the plasma of the winter flounder during the winter months was due to an increase in the concentration of AFP (Fletcher, 1977; Fletcher et al., 1978). The spring decline in plasma freezing point depression occurs at the same time in both the control and experimental fish (Fig. 32).

Northern blot analysis of AFP mRNA

December fish on a 15 h long day have little or no detectable AFP in their blood compared to control fish. This suggested that the concentration of translatable AFP mRNA would be lower in the experimental fish. In order to test this possibility in December, total RNA was extracted from two control fish and two fish held under 15 h long days, electrophoresed in agarose gels and analysed using the Northern blot technique involving hybridization to a ^{32}P-labelled, nick-translated antifreeze probe E_3 (Fig. 33). Extensive hybridization was detected in the lanes

Figure 32. Effects of day length on the winter increase and spring decrease in plasma Cl^- (A) and freezing point depression (B). Groups (4-12 fish) of winter flounder were exposed to ambient water temperature and photoperiod (normal) and 15 h light per day (LD) on September 15, 1981. The fish were serially blood sampled on the dates indicated. Values are expressed as means ± one standard error. Water temperatures are mean values for 1974-1981.

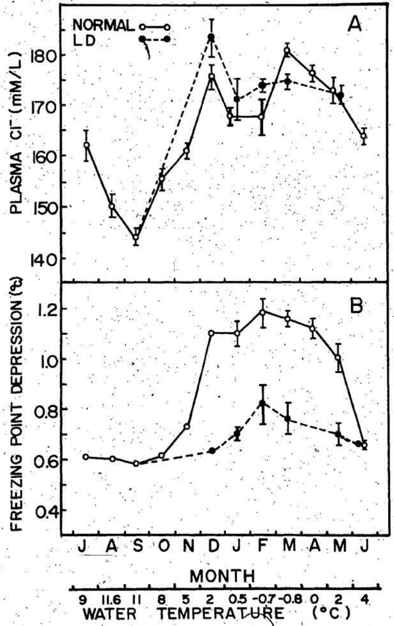
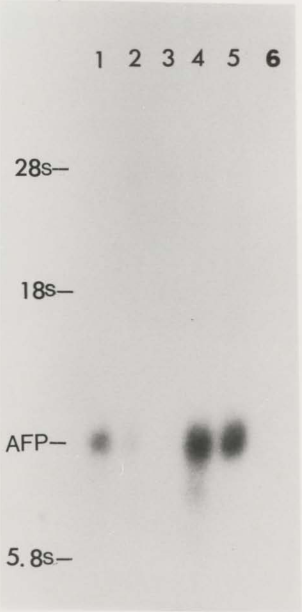

NORMAL
LD
A
PLASMA Cl⁻ (mM/L)
180
170
160
150
140
B
FREEZING POINT DEPRESSION (°C)
1.2
1.0
0.8
0.6
0.4
J A S O N D J F M A M J
MONTH
9 11.6 11 8 5 2 0.5 -0.7 -0.8 0 2 4
WATER TEMPERATURE (°C)

Figure 33. Analysis by Northern blot hybridization of flounder liver RNA extracted from fish maintained under different day length conditions in December. RNA samples were electrophoresed in a 1.5% agarose gel, transferred to nitrocellulose paper and hybridized to a nick-translated E_3 antifreeze genomic clone. Gel lanes contain the following RNA: (1) Pure AFP mRNA 0.5 μg, (2) 20 μg of total RNA sampled from fish (1) on 15 h long day length, (3) 20 μg of total RNA sampled from fish (2) on 15 h long day length, (4) 20 μg of total RNA sampled from control fish (1), (5) 20 μg of total RNA sampled from control fish (2), (6) summer control fish (July), 20 μg total RNA. Flounder 28 S, 18 S and 5.8 S ribosomal RNAs are indicated by the dashes and were determined from a photograph of the ethidium bromide stained gel.


1
2
3
4
5
6
28s–
18s–
AFP–
5. 8s–

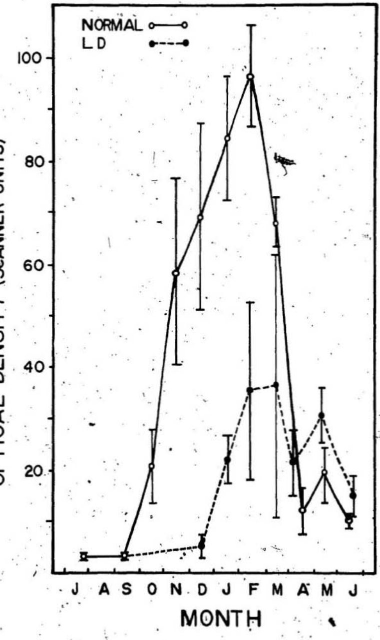
containing purified AFP mRNA and total liver-RNA from control fish kept at ambient temperature and photoperiod. Initially no hybridization was detectable in the total RNA samples taken from fish held under 15 h photoperiod or in the total RNA samples taken from summer control fish. However, longer autoradiographic exposure revealed that weak hybridization occurred in one of the experimental 15 h photoperiod December fish (lane 2) and in the summer control sample. This result provides evidence that long day length retards the accumulation of AFP mRNA in the flounder liver.

Cytoplasmic dot blot estimation of AFP mRNA

Although the Northern blot hybridization study can readily detect the presence or absence of AFP mRNA in the liver, it is difficult to quantitate the levels of AFP mRNA involved. Therefore, a cytoplasmic dot hybridization analysis was carried out (Fig. 34). In the normal seasonal synthesis of AFP mRNA this technique indicated that in late September the concentration of AFP mRNA increased dramatically and reached a maximum value in late January or early February. The concentration of AFP mRNA declined quickly in the spring until late April, then gradually decreased until it reached a minimal but detectable level in late June. This minimal level of AFP mRNA was maintained throughout the summer until the cycle was repeated starting in early fall.

Figure 34 illustrates that the levels of AFP mRNA found in experimental fish on a 15 h photoperiod never reached half the mean value attained by the control fish during the winter months. There was also a significant delay in the accumulation of AFP mRNA found in the liver. Although the experimental fish never accumulated the same amount of AFP

Figure 34. Quantitation of liver AFP-mRNA in flounder held under ambient (Normal O---O) and 15 h long day length conditions (●----●) by cytoplasmic dot hybridization. Cytoplasmic aliquots were prepared from liver and analyzed as described in Materials and Methods (Chapter 3). Autoradiographic spots were scanned and the density integrated according to peak area. Values are expressed as means ± one standard error. N values are as follows: for 15 h photoperiod conditions; D(2), J(2), F(4), M(2), A(2), M(4), J(4) and for control conditions; June(2), S(2), O(2), N(2), D(4), J(2), F(4), M(2), A(2), M(4), July(4). September values of N represent the same fish since the experiment was initiated on September 15, 1981.


NORMAL
LD
OPTICAL DENSITY (SCANNER UNITS)
100
80
60
40
20
J
A
S
O
N
D
J
F
M
A
M
J
MONTH

mRNA as in the controls, they did reach a maximum concentration at approximately the same time as control fish. The decline in the amount of AFP mRNA followed a similar pattern in both control and experimental fish. Although considerable variation was found in the concentrations of AFP mRNA in the two groups of fish, these results demonstrate the dramatic effect of a 15 h photoperiod on the seasonal synthesis of flounder AFP mRNA.

Discussion

The results of the present study demonstrate that long day lengths in fall delay the appearance and suppress the accumulation of AFP in the plasma of winter flounder. These results are in agreement with previous findings (Fletcher, 1981). In addition, the cytoplasmic dot blot hybridization and Northern blot analysis demonstrated that the concentration of flounder AFP mRNA also follows a seasonal cycle and that a long day length photoperiod leads to a significant delay in the time of appearance and a decrease in the accumulation of AFP mRNA in the liver. This was especially evident in the December samples where the suppressed levels of AFP mRNA in the experimental fish were not readily detected as a band in the total RNA preparations following gel electrophoresis. This was not surprising, as it was estimated that the mean value for AFP mRNA in the long day length fish represented less than 0.6% of the level of this RNA normally found in flounder actively synthesizing AFP in December. This suggests that an extended photoperiod suppresses the concentration of AFP mRNA. This study may in fact represent one of the first reports of the effect of photoperiod on the concentration of a specific mRNA.

Although considerable variation exists between the concentration of AFP mRNA found in individual flounder livers, the mean values calculated from the dot blot hybridization are in good agreement with a previous seasonal study of winter flounder AFP mRNA levels (Pickett et al., 1983). Pickett et al. (1983), using a liquid hybridization procedure and a cell free translation analysis, have clearly shown that the concentration of AFP mRNA constitute 0.5% of the total liver RNA in the winter and 0.0007% in the summer. In addition, the timing of the seasonal cycle of the accumulation of AFP mRNA in control fish is similar to that described by Pickett et al. (1983), with the increase and decrease in AFP mRNA concentrations in the fall and spring, respectively preceeding the corresponding increase and subsequent decrease in plasma AFP. Although less evident, the concentration of AFP mRNA in flounder exposed to long day lengths also increases prior to the AFP levels in the plasma. Therefore, it is likely that the same control processes are governing the initiation of AFP synthesis in both normal flounder and flounder exposed to long day lengths.

As mentioned in the Discussion of Chapter 3, the accumulation of AFP mRNA in the liver would depend upon the rate of its degradation as well as its synthesis. Although the possibility that AFP mRNA accumulation may be due to changes in the degradation rate cannot be eliminated in this study, the extremely low levels or absence of AFP mRNA in December fish on a 15 h photoperiod and the lower overall concentrations of AFP mRNA in the fish during the remainder of the winter suggest that photoperiod suppresses the transcription of AFP mRNA. Neither the degradation of AFP mRNA or its protein product is affected by photoperiod as the decrease in the levels of both AFP in the plasma and AFP mRNA in the liver during the spring, occurred at the same time in control and experimental fish. Maintenance of high serum concentrations of AFP during March and April when levels of AFP mRNA are significantly decreased suggests that this is due to a slow clearance of the polypeptides.

The control over the initiation of AFP mRNA synthesis appears to be more complex than simply changing day lengths. Other studies (Fletcher et al., 1978; Hew and Fletcher, 1979; Fletcher, 1979; Chapter 3) have demonstrated that hypophysectomy in the winter flounder leads to elevated levels of plasma AFP and AFP mRNA in the liver regardless of summer photoperiod and temperatures. Therefore, AFP synthesis in the winter flounder may very well be regulated by a pituitary factor that is modulated by photoperiod.

CHAPTER 5

GENERAL DISCUSSION

The seasonal biosynthesis of AFP in the winter flounder is a unique system for studying the environmental regulation of gene expression. A prerequisite for the basic understanding of gene activity and differential gene expression includes characterization of the gene product(s). Therefore, the initial objective of this study was to identify and characterize the AFP found in the plasma of winter flounder. The other objectives of this study were to determine the role that the pituitary plays in controlling AFP gene expression, and to study the effects of photoperiod as an environmental influence in this regulation.

Serum AFP were isolated from winter flounder collected from Nova Scotia, New Brunswick, Long Island (New York) and Newfoundland. Seven AFP components were readily resolved by reverse phase HPLC and SDS PAGE. Two major components comprise the bulk of the antifreeze activity (47%). They share a similar molecular weight (3300) with three other minor components. Two other minor AFP were larger (4500 daltons) and unlike the other five components contained valine. As indicated by amino acid analysis, PAGE and reverse phase HPLC, different populations of winter flounder contain a similar set of AFP. Little variation was noted. However, in addition to the two larger minor components, one of the 3300 dalton major AFP isolated from the New Brunswick population also contained valine. Since the corresponding AFP component from other winter flounder populations did not contain valine, this suggests that some genetic polymorphism exists in flounder.

The polymorphism within winter flounder AFP is in direct contrast to studies involving other vertebrates which demonstrate that most serum proteins are highly polymorphic (Sarich, 1977). The small amount of variation noted in the set of AFP between populations, as well as the close similarity that exists within the AFP components themselves suggest that the structure of all AFP are highly conserved in order to maintain a stringent functional requirement for antifreeze activity. It has been proposed that the principal sequence and secondary structure of AFP are critical to enable specific orientation and binding to the ice crystal lattice, and thus inhibit further ice-crystal growth (Raymond and DeVries, 1977).

The close chemical and physical similarity noted between the seven AFP within an individual raises an interesting question; are these seven components processing products or are they products of separate genes? Neither the presence of additional alanine nor the minor residue variation of valine detected in some AFP is easily explained as post-translational cleavage or processing products. In this and previous studies (Davies and Hew, 1980; Pickett et al., 1983) characterization of primary translation products of AFP demonstrated the existance of a single fluorographed band when analysed by conventional SDS PAGE (Laemmli, 1970). However, preliminary studies suggest that there are a multiple number of AFP precursors present. When cell-free translation studies were carried out using purified flounder AFP mRNA and the products analyzed by two-dimensional gel electrophoresis (O'Farrell, 1975), the improved resolution afforded by this system revealed the presence of five and possibly seven precursor AFP. These AFP were very similar in molecular weight but were resolved by isoelectric focusing in the first dimension. This leaves little doubt that the discrepancy over the number of AFP and their precursors has resulted from technical difficulties involving their resolution and separation. In view of the problem encountered in characterizing the final mature AFP components in the serum, this is not surprising. It is also unlikely that the different AFP represent allelic variations due to genetic polymorphism since the seven components are expressed in individual animals. The most likely hypothesis is that the multiple AFP detected in winter flounder are products of a family of genes coding for AFP.

If the AFP components are in fact products of separate genes, there should be several AFP mRNAs. As noted above, the preliminary analysis of cell-free translation products of total AFP mRNA suggested that this was the case. RNA excess hybridization analysis of winter flounder mRNA also indicated that there is more than one species of AFP mRNA (Lin and Long, 1980). However, only one major mRNA for AFP was detected by denaturing agarose gel electrophoresis or sucrose density gradient contrifugation (Davies and Hew, 1980; Pickett _et al._, 1983). Preliminary studies carried out as a side project to this thesis demonstrated that an improved PAGE system (adapted from that of Lichtler _et al._ (1982)) can resolve a 7.5 S AFP mRNA preparation into two to seven separate components. Specific poly (A)$^+$ digestion of these mRNAs indicated that the differences in their electrophoretic mobilities were due to alterations in the nucleotide sequences of the mRNAs and not a result of variation in polyadenylation. The final piece of evidence that indicates that the AFP in the winter flounder are encoded by separate genes comes from studies by Davies _et al._ (1981, 1984). Analysis of the flounder genome revealed that there are at least six independent nucleotide sequences (gene loci) that cross-hybridize extensively to the AFP cDNA plasmid CT5. Preliminary sequence studies on these genes indicate that the amino acid composition derived from the mature AFP coding region of three of these genes correspond to the amino acid composition of AFP components 3, 6 and 8 as separated by reverse phase HPLC in this study (P. Davies, personal communication). Therefore, there is little doubt that the winter flounder AFP are the products of a group of closely related genes which comprise a gene family.

A gene family can be defined as a set of genes that are descended by duplication and variation from a common ancestral gene. There are many examples of gene families (see Lewin, 1983). The biological significance of a group of genes producing a set of functionally similar antifreezes is as yet unclear. Adaptive evolution may depend on mutations affecting the rate of synthesis of specific gene products (Wilson et al., 1977). In the case of antifreezes, fish that are adequately protected, will survive. Mutations can affect the rate of synthesis in two ways. First, the rate of transcription can be altered. Second, the number of genes coding for a specific product can be altered (Zimmer et al., 1980). The evolutionary importance of the second process may be significant in AFP biosynthesis. In view of the abundance of AFP found in the serum of flounder, Lin and Long (1980) have suggested that a multiple AFP gene system may be necessary to ensure the adequate production of large amounts of AFP in a short period of time. Little is known concerning the time span involved in AFP biosynthesis in cold-water fishes. It is possible that a multiple array of genes simultaneously synthesizing their products is a means of compensating for slower rates of protein synthesis.

A second role for an AFP gene family is the possibility that the multiple AFP represent different gene products produced during the normal chronological development of the flounder. The less abundant components detected in the serum of mature flounder may be the persistent expression of juvenile forms of AFP which were critical for the survival of the developing flounder. This suggestion is not without precedent. The differential expression of the globin gene family represents an example of developmental control, in which different genes are responsible in providing alternate products that fulfill a similar function at different times (Maniatis et al., 1980). Gene families may allow for the coordinate expression of a set of dispersed genes. An intriguing aspect of this biological function is that the synthesis of a set of AFP in a catabolic tissue such as the liver may be coordinately controlled by a common signal. Presumably, AFP will be required by several tisues at the same time to stop the threat of freezing and different genes with slight sequence variations may be required to meet the functional needs of specific tissues. Support for this thought has come from recent studies conducted by O'Grady et al. (1982b) that indicate that in many Antarctic and Arctic fishes only the low molecular weight AFGP are responsible for preventing the intestinal fluids from freezing whereas both high and low molecular weight AFGP are found in the blood. In contrast, the cerebral spinal fluid, bile and egg fluids of both the shorthorn sculpin and winter flounder contain AFP which are very similar, if not identical, to those found in the blood (Fletcher, personal communication). This would argue against the possibility that gene families provide products with slight variations to meet specific tissue requirements. The lack of preferential synthesis of AFP components following hypophysectomy, as noted in this study, strongly suggests that all the AFP are coordinately regulated by a common mechanism. This represents the simplest means of controlling numerous genes.

Little is known concerning the mechanism of action of AFP or AFGP. Most fish contain multiple antifreezes which could indicate that a number of components may be necessary to attain an antifreeze functional activity. It is possible that a collective effect on the freezing point depression of serum occurs with multiple AFP components and that this effect is greater than the sum of activities exerted by the individual components. In this case the gene family would provide different products which share a common biological function. Osuga and co-workers (1978) have reported that there is a cooperative effect between the high molecular weight and low molecular weight AFGP. This interaction "potentiates" or increases the antifreeze activity of the individual components in some Antarctic fish. The existence of this potentiation effect in AFGP has recently being disputed by Schrag and DeVries (1983). These investigators have demonstrated that the sum of the individual freezing point depression activities of each component is equal to the collective activity of all the AFGP in the serum.

The evolution of gene families poses several interesting considerations. Studies of cloned genes and the isolation and characterization of genomic fragments have revealed that duplication events, followed by variation, are of primary importance in the evolution of individual genes. One copy can evolve via mutation while the other retains its original functions. This does not seem to be the case with gene families. A family of repeated genes may undergo concerted evolution (Zimmer et al., 1980), that is to say the repeated genes evolve in unison. Little variation within the gene family is tolerated. The co-evolution of several genes coding for nearly identical products suggests that some mechanism such as gene conversion or cross-over fixation is responsible for the continual regeneration of multiple genes (Zimmer et al., 1980; Lewin, 1983). In addition to globin genes, examples of DNA sequences that exhibit concerted evolution and fluctuation in numbers of copies include satellite DNA (Southern, 1970); genes coding for ribosomal RNA (Brown et al., 1972; Anderson and Roth, 1979; Long and Dawid, 1980); immunoglobins (Hood et al., 1975); and histones (Kedes, 1979). The similar biochemical and physical properties found in the winter flounder AFP strongly suggests that these polypeptides are members of a gene family which undergo concerted evolution.

It has been demonstrated by cDNA sequence analysis that AFP codon usage is biased (Lin and Gross, 1981; Davies et al., 1982). It appears that alanine codons in both pro-sequences and the sequence corresponding to the mature protein favours GCC (68 percent). The alanine codon GCG is not used (Davies et al., 1982). Uneven usage of codons is observed in other eukaryotic mRNAs, such as fibrin (Tsujimoto and Suzuki, 1979), and collagen (Bernard et al., 1983). It has been suggested that the codon pattern in yeast is biased to the most efficient used codons, thus allowing maximum production of key products such as the translation of glycolytic mRNAs (Holland et al., 1981). It is interesting to speculate that the nucleotide sequences of AFP genes may also be geared to achieve maximum translational efficiency.

In summary, the biological significance of an AFP gene family includes several possibilities. It may provide a means of producing an abundance of product in a short period of time. The genes for AFP could be coordinately regulated by a common mechanism or signal. The individual variation encountered within the gene products themselves, may be important to meet a particular set of functional requirements at different developmental times. In addition, the minor variation in size, number and composition of different AFP components may be tailored to the functional requirements of different tissues or be involved in a cooperativity of antifreeze action.

The presence of multiple AFP in the winter flounder is consistent with the recent observations on the large number of AFP seen in sub-Arctic fish (Hew et al., 1980, 1984; Fletcher et al., 1982a) and the multiple AFGP reported for Antarctic notothenids and northern gadoids (Feeney and Yeh, 1978; Hew et al., 1981; DeVries, 1982; Fletcher et al., 1982b). A comparative study of the structure and function of AFP and AFGP would provide a foundation for understanding their structural diversity as well as provide additional insight into how DNA sequences are duplicated and conserved during the course of evolution.

Substantial progress has been made in understanding the control of gene expression in eukaryotic cells. It has been shown that this expression may be regulated at many points from transcription of the gene to the completed gene product (Darnell, 1979, 1982; Breathnach and Chambon, 1981; Nevins, 1983). From current evidence, regulation of the rate of transcription is the most frequent mode of control favoured by bacteria (Gilbert et al., 1973) and eukaryotic cells (Darnell, 1982).

Tissue specific gene expression (Derman et al., 1981) or developmentally regulated gene expression (Groudine et al., 1981) have been shown to be due to transcriptional control. Transcriptional control implies economy and conceptual simplicity. It is well established that many proteins in differentiated cells are produced in increased concentrations in tissues following hormonal stimulation. Furthermore, transcription experiments utilizing isolated nuclei or cellculture have demonstrated that this induction is due mostly to transcriptional control (Tsai et al., 1978; McKnight and Palmiter, 1979; Swaneck et al., 1979; Tata and Smith, 1979; Ucker et al., 1981). In contrast, little is known about how environmental influences regulate the seasonal expression of eukaryotic genes.

Studies on the seasonal concentrations of AFP in the serum attest to its abundance in the winter months and to negligible levels present in the summer. The biosynthesis of the AFP in the liver and its corresponding levels of AFP mRNA has also been investigated. The large buildup of AFP mRNA concentrations in January specimens (0.5% of total RNA) and their decline in the spring to summer concentrations bordering the limits of detectability (0.0007% of total RNA) is considered strong evidence in favour of gene regulation by transcriptional control processes.

Recent evidence indicated that in winter flounder the mechanisms controlling the biosynthesis of AFP are influenced by photoperiod and comes under the control of the pituitary gland (Fletcher et al., 1978; Hew and Fletcher, 1979; Fletcher, 1981). The present study supports this finding and presents evidence to suggest that the role of photoperiod in controlling AFP biosynthesis is mediated by the pituitary through a transcriptional control mechanism. Hypophysectomy in winter flounder leads to elevated levels of plasma AFP and a dramatic increase in its corresponding AFP mRNA apparently regardless of conditions of photoperiod and temperature. The negligible degree of hybridization of an AFP cDNA probe to total liver RNA from control summer fish, demonstrates that there is no large pool of unprocessed message in the cell. This indicates that the absence of AFP synthesis during the summer is due to the lack of synthesis of translatable AFP mRNA. Since there is an absence or a greatly reduced level of AFP mRNA in the liver of control flounder, the most likely hypothesis is that its accumulation following hypophysectomy is dependent upon accelerated transcription. This implies that the pituitary gland regulates AFP mRNA and its corresponding protein synthesis by a negative transcriptional control process.

AFP biosynthesis is influenced by photoperiod presumably by the central nervous system acting on the pituitary gland. Initial findings by Fletcher (1977, 1981) indicated that flounder maintained under conditions of long day length had both a delayed appearance and decreased accumulation of AFP in the serum. These findings were confirmed in this study. In addition, it was also noted that long day length had a similar effect on AFP mRNA accumulation in the plasma. It was estimated that fish sampled in December which had been acclimated previously to 15 h long day photoperiods, had less than 0.6% of the AFP mRNA that was present in control fish. The seasonal fluctuation of AFP mRNA in both the experimental and control fish matched closely but preceeded the rise and fall of plasma AFP levels. In view of the significant decrease in the concentration of stable translatable AFP mRNA following an experimental photoperiod regime, it appears that long day length suppresses the transcription of antifreeze genes.

The mechanisms controlling the expression of the AFP genes must encompass aspects of pituitary regulation and photoperiod influence. Towards this end, I wish to propose a model that explains how AFP biosynthesis in the winter flounder may be regulated by a pituitary factor that is modulated by photoperiod. During the summer months the pituitary regulates AFP synthesis by releasing an inhibitor(s) which acts directly or through a mediator to inhibit transcription of AFP mRNA. With the approach of winter, the release of this inhibitor stops and consequently AFP mRNA transcription resumes. Consistent with this hypothesis, is that hypophysectomy mimics the suppression of inhibitor(s) from the pituitary and the AFP genes are reactivated. In late winter (March), the suppression of transcription resumes but because of the significant levels of AFP present in the blood and its slow rate of clearance, the fish is well protected from freezing.

The seasonal release of AFP inhibitor(s) from the pituitary appears to be finely tuned by photoperiod. During the autumn the flounder perceives a change in photoperiod by photoreceptors, possibly located in the eyes or a pineal body. The lack of long day length (or short nights) once detected is probably encoded as signals which act through the central nervous system to suppress the release of the AFP inhibitor(s) from the pituitary. Recent evidence indicates that the release of AFP inhibitor(s) from the pituitary are in fact controlled by releasing factors from the brain and that the central nervous system normally inhibits the pituitary gland's release of antifreeze inhibitor during the winter (Fletcher *et al.*, 1984).

In the absence of pituitary AFP inhibitor(s), transcription of AFP genes resumes.

Experimental conditions of long day length, delay the accumulation of AFP and AFP mRNA synthesized by the liver, presumably by maintaining the release of AFP pituitary inhibitors. It is highly unlikely that the changes in AFP and AFP mRNA accumulation which occur after hypophysectomy or long day length acclimation studies, are independent events. Preliminary data indicate that the pituitary is required to maintain the suppression of AFP genes in fish maintained under long day length conditions. Hypophysectomy of fish held under long day length conditions results in the immediate synthesis and accumulation of AFP and AFP mRNA. Since hypophysectomy has provided evidence to suggest that the AFP are under a transcriptional control mechanism, the above information suggests that photoperiod may ultimately control the onset of synthesis of AFP mRNA by acting as a trigger which signals the cessation of the release of a pituitary AFP inhibitor(s).

Our knowledge of how the winter flounder regulates the biosynthesis of AFP is rapidly growing. Many important aspects of this regulation have yet to be investigated. Among these are: the chemical nature of the pituitary inhibitor(s), the presumptive photoreceptors that are involved in the conveyance of photoperiod signals, the processes regulating degradation and clearance of AFP and AFP mRNA, and the factors controlling the termination of AFP biosynthesis. In addition, there is some evidence to suggest that an endogenous antifreeze cycle exists and that some other levels of control may be involved in the fine tuning of AFP regulation (Fletcher and Smith, 1980; Lin, 1979; Pickett et al., 1983). The remainder of this chapter will discuss the scope of our knowledge on some of these aspects and their major implications on AFP biosynthesis. Emphasis will also be placed on possible future experimentation.

The chemical nature of the pituitary inhibitor(s) involved in the regulation of AFP synthesis in winter flounder is not known. It is known that the pituitary of fish contain at least six cell types capable of releasing seven different hormones (Campbell and Idler, 1976; Ng and Idler, 1979; Ng, 1980; Burton et al., 1981; Eales and Fletcher, 1982; Ng et al., 1982; Gorbman et al., 1983). In addition, many of these pituitary hormones have been implicated in causing both androgenic stimulation and thyrotropic stimulation (Ng and Idler, 1980; Ng et al., 1982). Therefore, a large number of pituitary or pituitary influenced hormones are potential candidates for inhibition of AFP transcription. However, it is difficult to assess the most likely candidate on the basis of our present knowledge of the seasonal changes in these hormones in fish. For example, DeVries (1982) notes that the appearance of antifreeze production in the autumn appears to be correlated with gonadal production and suggests that the initiation or termination of the antifreeze cycle may be controlled by levels of steroid hormones produced by the testes and ovaries. However, there are several problems associated with this interpretation. The most obvious of these is the fact that sexually immature fish contain AFP during the winter. It is also difficult to correlate gonadotropins with AFP biosynthesis since there is no reliable means for measuring these hormones in this species. There are two gonadotropins in the winter flounder (Ng and Idler, 1979; Ng, 1980) but the presence of both these gonadotropins in the juvenile fish appears unlikely thus preventing further speculation on their involvement in AFP biosynthesis.

Recently a thyroid stimulating hormone (TSH) separate from the gonadotropins has been isolated from flounder pituitary extracts (Ng, 1980; Ng et al., 1982). It is possible that this hormone may play a role in setting up a chain of commands such that the thyroid becomes involved in the inhibition of AFP synthesis. Eales and Fletcher (1982) have shown that seasonal changes in plasma concentrations of thyroxine (T_4) were highest from April to June and lowest during the fall. Since hypophysectomy results in the cessation of T_4 secretion it is possible that this hormone is involved in AFP synthesis. Plasma levels of triiodothyroine (T_3) were highest when (T_4) levels were lowest and consequently it is unlikely that AFP synthesis is inhibited by (T_3).

The pituitary also contains growth hormone (GH) and prolactin (PRL) which could be possible inhibitors of AFP mRNA transcription. Prolactin is involved in the osmoregulation of freshwater fish but its exact role in marine teleosts is in question (Bern, 1983). Similarly, the function of GH in teleosts is attributed to general growth and differentiation but its specific nature or mode of activity in different tissues is unknown (Bern, 1983; Gorbman, 1983). No correlation between the seasonal variation of either of these two hormones and the AFP seasonal cycle can be made since a reliable means for quantitating the concentrations of these two hormones (ie. radioimmunoassay) is still lacking. In the absence of adequate experimental data and more comprehensive information it is difficult to assess the hormones mentioned above and other pituitary hormones such as melanocyte stimulating hormone and adrenal corticotropin hormone as possible AFP pituitary inhibitors.

The fact that AFP synthesis is under the negative control of the pituitary should allow individual hormones to be tested for their actions on hypophysectomized fish. Fletcher (1979) has demonstrated that pituitary extracts administered to hypex fish suppress AFP synthesis. However, efforts to identify these extracts to specific hormones or specific pituitary cell types have resulted in inconclusive information (Fletcher, personal communication).

An initial question concerning the control of flounder AFP biosynthesis is how photosensory information is received and relayed. The pineal body in many vertebrates has been implicated as the major structure associated with the integration of photic information (Ralph, 1983). There is good evidence to suggest that the pineal complexes of fishes participate in mediating photoperiod effects on seasonal reproductive processes (deVlaming and Olcese, 1981). Consequently, it is conceivable that a pineal body may play a principal role in timing the annual AFP cycle. The presence of a pineal body or specific photoreceptors indicative of this structure has not yet been found in winter flounder (Crim, personal communication).

Several investigators have shown that some annual cycles are regulated by light (Goss and Rosen, 1973; Gwinner, 1973; Goss, 1976, 1977; Ralph, 1976, 1983; Ralph et al., 1983). Photoperiod has been implicated as the major cue signalling tissue specialization and protein synthesis in a number of systems. For example, the synthesis of vitellogenins and diapause proteins in the Colorado potato beetle, Leptinotarsa decemlineata is influenced by the duration of day length (Dortland, 1978; Dortland and DeKort, 1978; Dortland and Esch, 1979). Heinze and co-workers (1980) have demonstrated that light induces greening in barley plants by initiating genes or influencing post-transcriptional processing to produce a group of poly(A)-rich mRNAs. Although the characterization of these mRNAs was not carried out, it is believed that they will encode for proteins responsible for the greening of etiolated plants. Therefore, a photoperiod influence on AFP biosynthesis in winter flounder is not without precedent.

Fletcher (1981) has suggested that the control over the antifreeze cycle may be endogenous, with photoperiod acting as zeitgeber (prominent signal) for entraining the precise time at which the cycle is initiated. In many teleosts endogenous endocrine rhythms in the hypothalamo-pituitary-gonad axis have been observed (Crim, 1982). These annual hormonal cycles, such as in the case of the gonadotropins utilize light and temperature as seasonal cues in gonadal development. Many insect larvae which over-winter appear to synthesize antifreeze proteins in response to temperature and photoperiod influences (Duman, 1977c, 1980; Patterson and Duman, 1978; Horwath and Duman, 1983a; Duman and Horwath, 1983). Recently it has been demonstrated that insect antifreeze biosynthesis is subject to hormonal regulation with a biological timing of circadian nature playing an important aspect in this control (Horwath and Duman, 1982, 1983b). The regulation of winter flounder AFP biosynthesis appears to be mainly under transcriptional control and there is some evidence to suggest that an endogenous cycle is involved in the timing of this synthesis (Fletcher and Smith, 1980). It is possible that an endogenous rhythm responsive to hormonal influences is involved in AFP biosynthesis but at best these investigations are preliminary.

The advantage of photoperiod being the zeitgeber rather than water temperature is due to its reliability as an indicator of the approach of winter. It is evident from the seasonal studies to date that the strategy of the winter flounder is to synthesize antifreeze proteins well before they are required to prevent cryoinjury. The initiation of AFP synthesis occurs at temperatures of 4-6°C (Fletcher, 1977; Hew et al., 1978). The present study indicates that AFP mRNA synthesis occurs in October when the water temperatures are 8 to 10°C. The average water temperature in Newfoundland during the summer is 12°C, however, wind or wave action can destroy the thermocline rapidly to produce water temperatures as low as 6-8°C at any time during the summer, and particularly during June and July. Clearly temperature would be a poor indicator of the approach of winter to fish such as winter flounder which inhabit shallow inshore water. Temperature would be a more reliable cue for the initiation of degradation and clearance of AFP in the spring when the threat of freezing is long past.

The accumulation of a specific mRNA can be regulated by either an increase in its rate of synthesis or a decrease in its rate of degradation. The dramatic rise in concentrations of AFP mRNA and their corresponding AFP suggests that the regulation of AFP biosynthesis is primarily at the level of transcription. However, the demonstration that the concentration of specific mRNAs increases is not absolute proof for transcriptional control (although it has proven to be true in most cases). Measurement of transcriptional rates must be conducted in order to establish transcriptional control. To date, actual transcriptional rate studies have not been done in many systems whose gene regulation have been investigated. In order to demonstrate specific initiation of AFP transcription apart from accumulation of gene products, new experiments are required. These studies should involve the investigation of transcriptional control by using isolated nuclei or cell-culture and following AFP mRNA induction and synthesis through pulse-labelling RNA incorporation. Only from this type of experiment can mRNA induction and transcription rates be determined (Cox, 1976; McKnight and Palmiter, 1979; Nevins and Darnell, 1978; Darnell, 1982). At a more molecular level, a flounder liver cell-culture responsive to pituitary influences would be useful for deciphering the molecular events leading to the suppression and induction of the AFP genes.

At the present time little is known about what determines the termination of AFP mRNA and AFP synthesis and the clearance of these gene products. Studies of AFP levels in fish acclimated to water temperatures of 6 to 8°C suggest that AFP synthesis stops during February and March. In vitro investigation of the livers capacity to synthesize AFP indicates that synthesis stops during March (Sclater and Hew, unpublished results). The results of the present study and those of Pickett et al. (1983) demonstrate that AFP mRNA levels decline during March. Photoperiod appears to have no influence on the loss of AFP from the plasma (Fletcher, 1981) or the time at which AFP mRNA concentrations decline in the liver (present study). Temperature does affect the rate at which AFP disappears from the plasma; however, low water temperatures do not appear to extend the time the liver is capable of synthesizing AFP (Fletcher, 1981). Even less is known about the controls involved in the degradation and clearance of AFP mRNA or its products.

It is important to note that the presence of transcriptional control for a particular gene does not preclude other levels of control. In addition to transcriptional regulation, there might be regulation of RNA processing in the nucleus, mRNA transport from the nucleus, mRNA stability and frequency of transport in the cytoplasm (Darnell, 1982; Nevins, 1983). There is some evidence to suggest that processing of nuclear RNA does not play an active role in AFP regulation since hybridization studies have failed to detect a larger unprocessed nuclear RNA (Fourney, preliminary results). This strongly suggests that there is no large pool of unprocessed nuclear AFP message in the cell and if a large AFP precursor is involved it is subject to rapid processing and transport to the cytoplasm. There is, however, some indication that translational control may play a minor role in the "fine tuning" regulation of AFP biosynthesis. As noted in this study and another investigation (Pickett et al., 1983), the seasonal appearance of AFP mRNA slightly preceeds the rise in AFP accumulation in the serum by approximately one month. This means that there is a significant concentration of AFP mRNA present when levels of AFP are low. This may imply that translation requires some unknown factor for initiation or the availability of a predetermined minimum concentration of message. AFP regulation may also be subject to variation of transcription rate. The presence of a minimal but detectable amount in AFP mRNA in the liver during the summer (Pickett et al., 1983) may indicate that regulation of the concentrations of AFP in the serum is not achieved by simply induction or cessation of transcription. These authors have suggested that the rate of transcription may be modulated possibly by one of several AFP genes being transcribed at a low level during the summer.

The study of biological antifreezes is a relatively new scientific endeavour. As is the case in most investigations, many aspects of this study have become the source of new questions. Important information concerning the mechanism of antifreeze activity, or the biological significance and evolutionary ramifications of a multigene AFP family is lacking. Some questions will involve simple solutions such as the presence or absence of AFP in body tissues other than blood or the nature of intracellular antifreezes such as that found in the eggs of winter flounder or shorthorn sculpin. In contrast, the study of the genomic organization and structure of the AFP genes may prove more difficult. Unlike many systems affected by the pituitary (Holmes and Ball, 1974), the present model appears to operate via a negative control mechanism. This is in direct contrast to hormone induced synthesis of many proteins found in other animals (For Review: Tata and Smith, 1979; Darnell, 1982).

One of the more challenging aspects of studying AFP regulation will be deciphering some of the clues concerning the diversity of their regulation. Arctic and Antarctic fish synthesize antifreeze proteins throughout the year which suggests that these species have either lost their ability to regulate AFP synthesis or the regulation of this synthesis has never evolved. In contrast, the winter flounder and many other sub-Arctic species, synthesize AFP according to their seasonal biological needs. In addition, the cycles of AFP synthesis are closely correlated to specific local environmental needs such that more northern populations of fish have longer cycles with initiation of synthesis occurring earlier and termination later than southern populations (Fletcher <u>et al.</u>, 1982a). Perhaps equally as interesting is the observation made by Duman and DeVries

(1975) that the same species of fish which synthesize antifreezes in northern populations may lack these proteins in southern populations. There is also an indication that some sub-Arctic fish synthesize AFP year-round regardless of the fact that summer water temperatures do not necesitate their presence for survival (Fletcher and Hew, personal communication). This could imply that full control of AFP synthesis is lacking or not fully evolved in these species. Collectively there is certainly enough information and tantalizing data to warrant an investigation of the diversity of AFP regulation in fish.

The study of the detailed interplay between the pituitary gland, environmental signals and the subsequent transcription of AFP mRNA is an exciting model to study gene expression.

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