

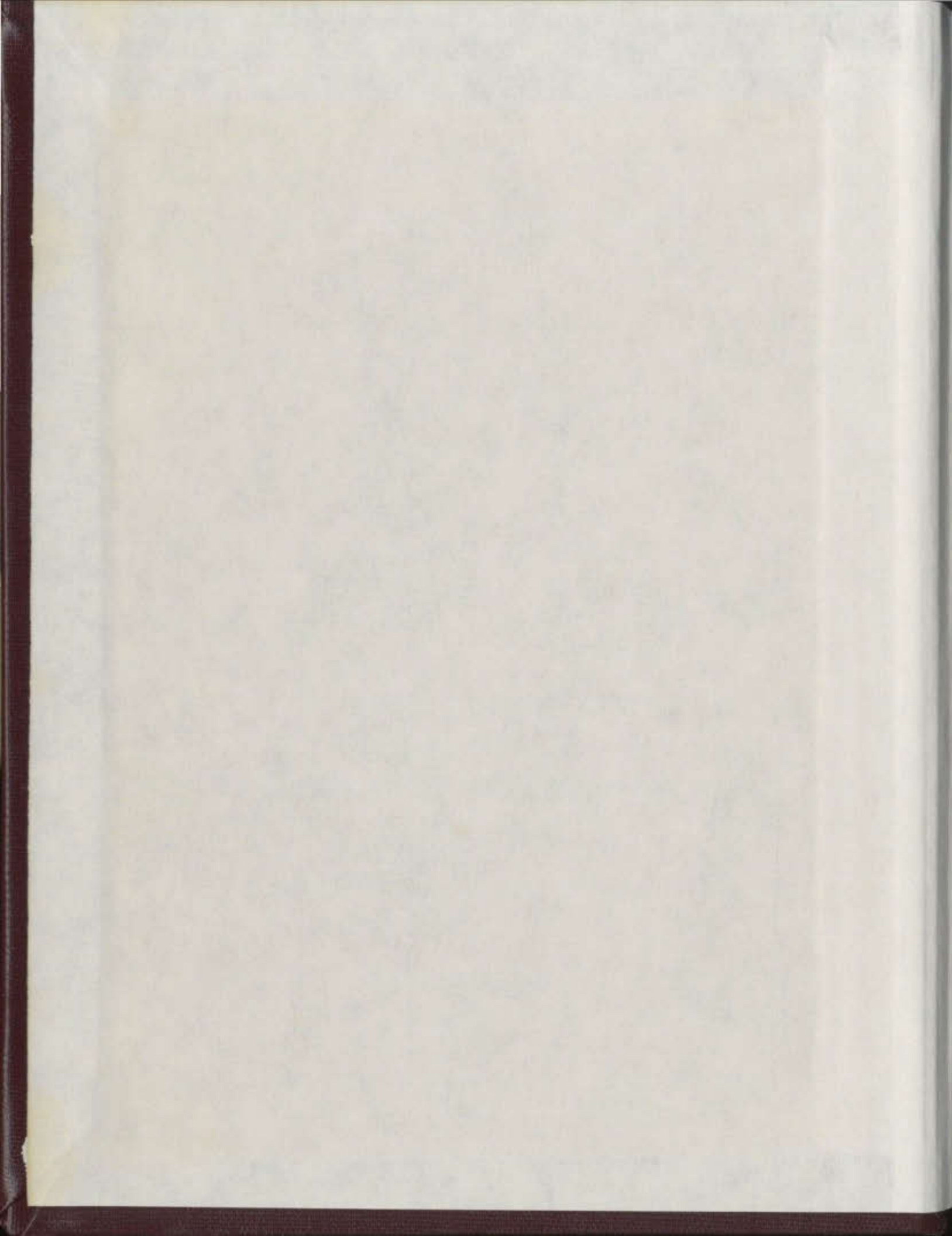
BIOSYNTHESIS OF THE
ANTIFREEZE PROTEIN IN THE
WINTER FLOUNDER,
PSEUDOPLEURONECTUS
AMERICANUS: IN VITRO STUDIES

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BIOSYNTHESIS OF THE ANTIFREEZE
PROTEIN IN THE WINTER FLOUNDER,
PSEUDOPLEURONECTUS AMERICANUS:
IN VITRO STUDIES

by

Anne Louise Sclater, B.Sc.



A Thesis submitted in partial fulfillment
of the requirements for the degree of

Master of Science

Department of Biochemistry
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March 1979

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ABSTRACT

The serum of the winter flounder Pseudopleuronectes americanus contains one principal antifreeze protein of 10,000 daltons in the winter. In vitro incorporation with radioactive alanine in the flounder's liver showed the occurrence of one major radioactive component of 16,000 daltons on Sephadex G-75 columns in 0.05 M NH_4HCO_3 buffer, pH 8.0, as well as in 8 M urea, 0.1 M tris-glycine buffer, pH 8.6, containing 50 mM β -mercaptoethanol. One major radioactive component was observed on SDS and urea tris-glycine disc gel electrophoresis. Upon mild tryptic digestion, this component yielded fragments having the same elution position on the gel filtration column as compared to authentic antifreeze protein. The synthesis of this 16,000 component in the fall (October-November) corresponded closely with the serum antifreeze level and was absent from fish caught in the summer. However, the radioactivity in this in vitro biosynthetic product could not be converted to antifreeze protein using pulse chase experiments with puromycin, nor with cold alanine.

An attempt was made to purify this antifreeze-like polypeptide from bulk materials. Amino acid analysis and enzymic peptide finger prints showed close structural similarity between this material and authentic antifreeze protein.

It is concluded that this liver 16,000 dalton component represents the precursor protein for the antifreeze protein.

2

Dedicated to my husband, Keith.

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LIST OF ABBREVIATIONS

AFP	antifreeze protein
AFGP	antifreeze glycoprotein
ANS	anilinonaphthalene sulfonate
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid

INTRODUCTION

Strategies for Survival in Sub-Zero Environments

Many organisms live and survive in sub-zero temperature environments. This freezing habitat poses serious difficulties for the survival of the organism because these cold temperatures are often lethal due to the formation of ice in the tissues. The crystallization of water to ice can result in histological injuries, both by mechanical damage and dehydration as water is being removed to form ice. Dehydration can result in changes in pH, concentration of certain solutes to toxic levels, and increased electrolyte concentration.

Consequently, various strategies have been evolved in order for the organisms to survive in these environments.

(i) Some species, such as cod, Gadus morhua, avoid freezing by migrating to a warmer environment. (ii) Other species, as varied as the Alaskan beetle, Pterostichus brevicornis

(1) and the deep water fish Icelus spatula from Hebron Fjord, Labrador (2), survive by supercooling. This cooling of body fluids below the freezing point in a liquid state is accomplished by the accumulation of small molecular weight compounds such as salts, polyols, and sugars.

However, the resultant freezing point depression of the body fluids is an unstable condition. For example, under laboratory conditions when the water surrounding a supercooled

fish was seeded with ice (2), the fish was immediately frozen to death. (iii) Various intertidal mussels and invertebrates can tolerate freezing. Incapable of migration, these species endure freezing and thawing twice daily for many months as a result of exposure to the atmosphere during the rise and fall of tides (3,4). (iv) Many species manage to survive in the freezing environments by synthesizing serum macromolecules. These organic anti-freezes have been observed in species as varied as the Antarctic fishes Trematomus borchgrevinki and Dissostichus mawsoni (5,6,7), the Arctic polar cod Boreogadus saida (8), the winter flounder Pseudopleuronectes americanus (9), and possibly the intertidal mussel Mytilus edulis (10) and the overwintering larvae of the darkling beetle, Meracantha contracta (11).

Background to the Study

A. Blood Serum Macromolecules and Freezing Resistance

The first suggestion that differences in resistance to cold might depend on differences in the physical and chemical properties of the blood of animals was made by De Réaumur in 1736 (12). He observed that the blood of caterpillars of different species froze at different temperatures. He likened their blood to brandies of different strengths based on the observation that a weak brandy freezes at a temperature at which stronger liquor remains fluid. De Réaumur stated that although the blood of birds and mammals freezes at temperatures much higher than that of insects, these animals are protected against freezing by their "innate heat".

Much later, Arctic explorers became intrigued with the manner in which fish survived freezing. Many tales were recorded such as that of Turner in 1886 (13). He described how frozen Alaskan blackfish, Dallia pectoralis, were chopped out of ice blocks in which they had been stored for weeks and fed to sled dogs. Much to the bewilderment of the dogs, and the explorers, the dogs regurgitated the fish which had been thawed and came back to life in the warmth of their stomachs. This astounding resistance of polar fish to freezing was reinvestigated by Scholander and his colleagues (13). Their studies (2,14,15) indicated

that the serum of many Arctic fishes had a lower freezing point than that of fishes from more temperate zones. Their data indicated that in the serum, there were trichloroacetic acid-soluble, non-salt compounds which aided in the lowering of the serum freezing temperature.

The freezing points of blood serum from human and most fish are within the range of -0.5 to -0.8°C (16). However, the serum from certain fish in Antarctica, where water temperatures can be as low as -2°C , has a much lower freezing point: -2.07°C for T. borchgrevinki, and -1.99°C for D. mawsoni. When such serum was subjected to extensive dialysis, it was observed that 70% of the freezing point depression was due to the presence of dialyzable salts, while 30% could be attributed to nondialyzable high molecular weight glycoproteins (16). Similarly, 40% of the low blood serum freezing temperature of the winter flounder can be attributed to the presence of a serum antifreeze protein (9).

B. Occurrence of Antifreeze Proteins

Scholander's initial observation that antifreeze macromolecules occurred in Northern polar fish was further substantiated by Scholander and Maggert (17), Hargens (18), and Raymond et al. (19) who noted its presence in Eleginus gracilis, the saffron cod. An antifreeze substance was also found in the winter flounder Pseudopleuronectes

americanus (9,20), a species indigenous to the Northern Atlantic coastal waters of Canada and the United States, and in the polar cod Boreogadus saida from the Barents Sea north of Russia (21).

At present, there are two distinct types of antifreeze proteins found in fishes - the protein antifreeze (AFP), and the glycoprotein antifreeze (AFGP). The AFP has no sugar groups associated with it and is found in cold water fish such as the winter flounder (9,20) and the short-horn sculpin Myoxocephalus verrucosus (19). To date, antifreeze proteins have been found only in fish from cold, but not polar waters (22). DeVries and Wohlschlag (5) were the first to report on the existence of an antifreeze glycoprotein in Antarctic fish. Antifreeze glycoproteins are found in Antarctic fishes such as Trematomus borchgrevinki, Trematomus bernacchii, Dissostichus mawsoni (16), and in Arctic fishes such as the Saffron cod E. gracilis (9,18,19) and the polar cod B. saida (21).

C. Characterization of Antifreeze Proteins

The antifreeze glycoproteins from the Antarctic fishes (6,23,24,25) were found to consist of a family of 8 proteins and were designated AFGP 1-8 based on their relative migration on gel electrophoresis (16). The molecular weight range of AFGP 1 to 5 is 30,000 to 10,500 daltons. The basic structure of AFGP 1 to 5 (as shown in Figure 1)

is that of a repeating glycopeptide unit composed of, alanyl-alanyl-threonine, with a disaccharide glycosidically linked to the threonine. The disaccharide is galactosyl-N-acetyl galactosamine with a β 1 \rightarrow 3 internal linkage. This basic unit is repeated 17 to 50 times in these active AFGP molecules (16). The carboxyl terminal of each polymer ends with one or two alanyl residues. The AFGP 6 to 8 are smaller components (16,22) which contain prolines following some of the threonines in the peptide chains (26). Unlike glycopeptides 1 to 5, AFGP 6 to 8 have been considered to have weak activity (16), or no activity when tested alone (22). However, when these smaller antifreeze glycoproteins, AFGP-7 and AFGP-8, were tested as mixtures with the larger AFGP 1 to 5, a 2 to 8-fold potentiation of antifreeze activity was observed (27). Recently, Osuga and Feeney (28) have conducted a detailed comparison between the glycoproteins of the Arctic B. saida and the Antarctic T. borchgrevinkii. Despite differences in the number of multiple molecular forms, the sequence and composition of the active fractions appeared to be identical in both species (28).

In contrast to those species with AFGP, the serum of the winter flounder Pseudopleuronectes americanus contains one major antifreeze protein of molecular weight 10,000 daltons (20,29). This estimation of molecular weight was based on gel filtration chromatography, SDS disc gel

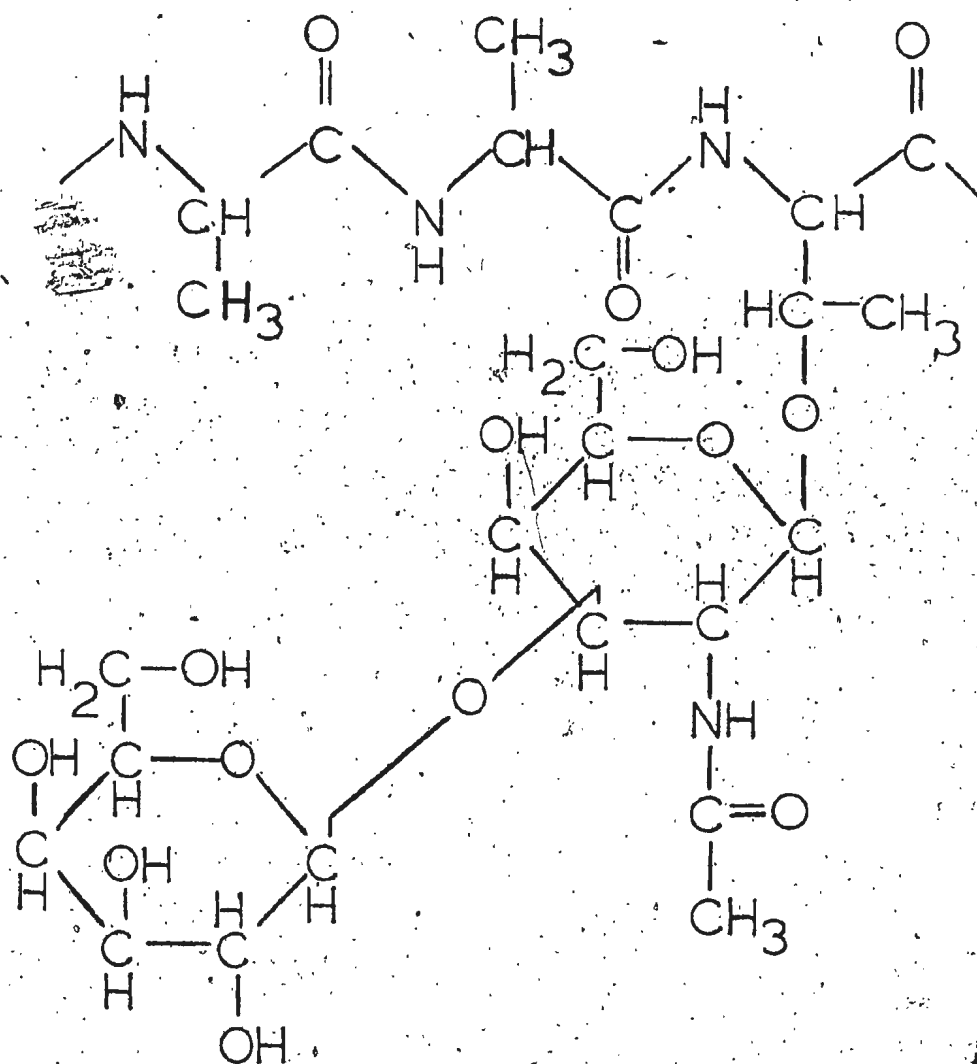


Figure 1 Structure of antifreeze glycoprotein
(reference 14)

electrophoresis, and amino acid analysis. Other researchers (30) have reported the presence of three peptides of molecular weight 12,000, 8,000, and 6,000 daltons in flounder serum using ion exchange chromatography. Recently the molecular weight of the third peptide was corrected to a value of 3,300 differing from that of the previously reported value of 6,000 (31). The amino acid compositions of these peptides (30) are in close agreement with that of the 10,000 MW antifreeze protein shown in Table 1. It is likely that flounder serum contains proteases which degrade the 10,000 MW component to peptides of varying smaller molecular weights in the spring during its normal clearance.

Amino acid analysis of AFP (Table 1) reveals that alanine accounts for approximately 60% of the residues (32). The first 1-28 amino acids have been determined by protein sequencing as well as by the isolation of enzymically digested peptides (32). As seen in Figure 2 there are no obvious repeating units in the structure. However, the clustering of alanyl residues is apparent. Similar antifreeze proteins have been reported in the sculpin Myoxocephalus verrucosus (19).

AFGP has been the subject of extensive chemical modification studies. One strategy employed in the study of structure-function relationships in AFGP is the degradation

TABLE 1
AMINO ACID COMPOSITION
OF THE WINTER FLOUNDER
ANTIFREEZE PROTEIN

Amino Acids	Yield in μ mole	Number in residues
Aspartic acid	0.256	14
Threonine	0.215	12
Serine	0.059	4
Glutamic Acid	0.034	2
Alanine	1.302	76
Leucine	0.131	7
Lysine	0.059	4
Arginine	0.041	2
Total residues		- 121
M. wt.		10457

(Reference 28).

Asp-Thr-Ala-Ser-Asp-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asn-Asx-Lys-Ala-Ala-Glu-Leu-Thr-Ala-Asp-Asn-Lys-

 20 28

Figure 2 N-terminal amino acid sequences of the anti-freeze protein (reference 28)

of the polypeptide chains with subtilisin or with fungal proteases (23). This treatment resulted in a loss of antifreeze activity. Of particular interest was the observation that hydrolysis of fewer than two peptide bonds in glycoprotein 5 (MW 10,500 g/M) resulted in a loss of antifreeze activity (16). Furthermore, when smaller glycopeptides were isolated in pure form from enzymic hydrolysis of AFGP 5, they had no significant antifreeze activity.

A second approach in the study of structure-function relationships of the AFGP involves the chemical modifications of the carbohydrate side chains. Oxidation of the terminal hydroxyl groups (C-6 hydroxyls) of the sugar of AFGP to an aldehyde by D-galactose oxidase does not destroy antifreeze activity (25). However, all other chemical modifications of the disaccharide side chains result in the destruction of the antifreeze activity (6,7,24,25,33,34,35). For example, the further oxidation of these aldehydes to acids with iodine, or formation of a complex with bisulfite results in a loss of antifreeze activity (25). Treatment of the bisulfite adduct with acidic solution, which removes the sulfite, restores biological activity. Periodate oxidation of 80% of the galactose residues, elimination of carbohydrate with 0.5 N NaOH, or acetylation

of 35% of the carbohydrate hydroxyls with acetic anhydride, also destroyed the activity (23). Complete restoration of the antifreeze activity was affected by deacylation of the inactive acetyl glycoproteins with hydroxylamine (23). Complexing of borate (6) with the carbohydrate moiety also caused a loss in antifreeze activity (7,34). Thus, studies in structural requirements for antifreeze activity imply that both the carbohydrate side chains and the polypeptide chain are important (16).

Structural studies have indicated that there are fundamental differences in secondary and tertiary structure between the AFGP and AFP. Based on intrinsic viscosity measurements of AFGP at 0.5° and 11°C, Antarctic fish glycoproteins are believed to have no ordered structure and to exist in the random coil configuration (6). In contrast, circular dichroism studies (36) indicate that flounder AFP exists in an approximately 85% α helix conformation in aqueous solution at -1°C. In addition, viscosity measurements on dilute solutions indicate that at -1°C, AFP assumes an asymmetric or rod-like shape, whereas at 20°C it exists in a more extended structure. These differences in the secondary and tertiary structure between AFP and AFGP have been attributed to the presence of disaccharide moieties in the Antarctic AFGP (36).

Circular dichroism studies of the AFP of M. verrucosus (37) showed, like those reported earlier for P. americanus (36), an approximate 70 to 80 percent right-handed helix content. It has been suggested that because AFP and AFGP have such dissimilar conformations in solution the biological activity of the antifreeze does not require their having a particular secondary structure (37).

D. Properties of Antifreeze Proteins Related to the Behaviour of Ice in Serum

AFGP and AFP have a number of properties in common regarding the freezing behaviour of ice in serum. Normally, "freezing point" is the temperature at which a solid is in equilibrium with its liquid form, and thus freezing point and melting point are considered to be the same. However, AFGP and AFP lower the temperature at which ice will form in the serum but do not lower the melting point of ice. Thus the proteins are regarded as having "anti-freeze" properties, the emphasis being that only the freezing behaviour and not the melting behaviour of the serum is affected. This freezing point and melting point difference has been referred to as a thermal hysteresis in the freezing-melting behaviour (38). It has been noted that fishes living in the coldest ice-laden water have the largest freezing and melting point difference. Thus the Antarctic T. borchgrevinki inhabiting -1.9°C water has a

serum freezing point of -2.34°C , a serum melting point of -1.07°C , and a freezing point-melting point difference of 1.27°C (39). By way of comparison, the North-temperate P. americanus inhabits -1.2°C water in winter and has a serum freezing point of -1.47°C , a serum melting point of -0.71°C , and a thermal hysteresis of 0.76°C (39). This hysteresis in the freezing-melting behaviour provides additional support for the fact that antifreeze proteins act in a non-colligative manner (39). If the low serum freezing temperature were attained solely by colligative means, the concentration of colligatively acting substances such as sodium chloride would exert osmotic pressures far in excess of what most tissues can tolerate (16).

It has been reported (6) that the active glycoproteins lower the freezing point of water 200 times more than do other comparably sized proteins such as chicken lysozyme (Figure 3). As can be seen in Figure 3 the AFGP is more active than a comparable weight of NaCl. These freezing point-depression curves indicate that there is a saturation phenomenon at higher concentrations which is not found with other agents which lower the freezing point. In addition, there is an additive effect when a mixture of AFGP and sodium chloride were tested together.

Various mechanisms have been proposed for the action of the antifreeze proteins. Experimental evidence suggests

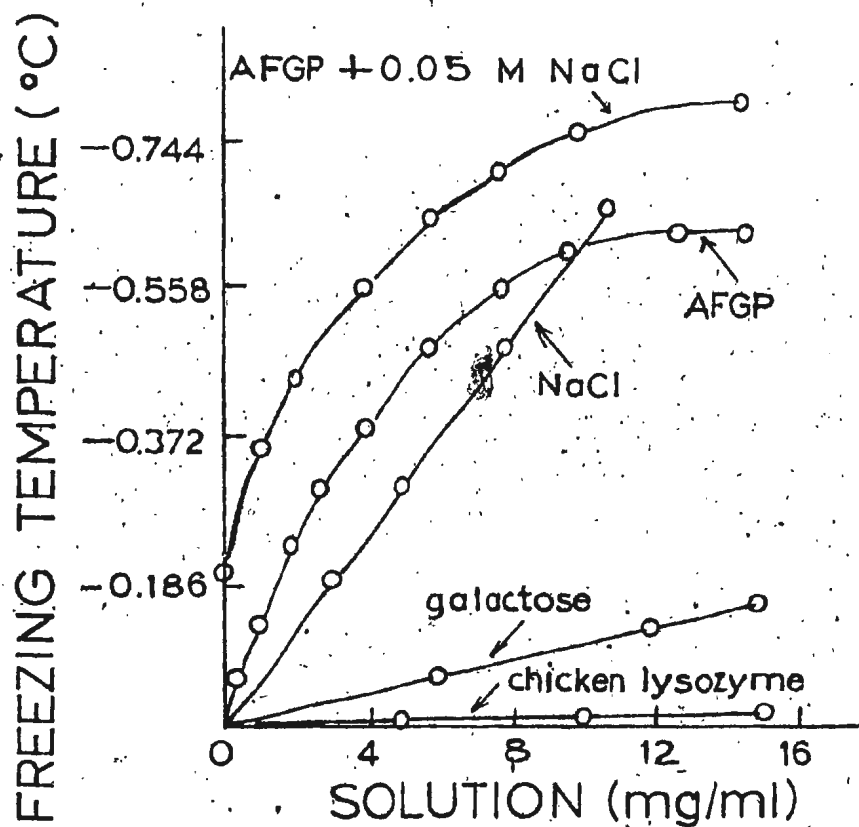


Figure 3 Freezing points of solutions of sodium chloride, galactose, lysozyme, and AFGP 3, 4, and 5. (a 1 milliosmol solution has a freezing point of -0.00186°C) (reference 6)

that the site for the functioning of antifreeze molecules is at the ice-water interface (25,40), a so-called interfacial surface interaction (21), where the proteins adsorb to the ice surface and inhibit further crystal growth by an adsorption inhibition mechanism. One of the important aspects of this model is that the AFGP and AFP recognize the ice, bind to it, and inhibit further crystal growth by presenting a barrier to the advancing front of water molecules which are attempting to link with the ice lattice.

There is experimental evidence (41) that specific binding does occur because during freezing the glycoproteins are equally partitioned between the liquid and solid phases. Antifreeze glycoproteins, unlike solutes such as sodium chloride, galactose, and large molecular weight dextrans (MW 20,000-40,000 daltons), are not excluded from the solid phase during freezing. Galactose and dextran tend to concentrate in the liquid phase when frozen in solution. If tubes containing solutions of the glycoproteins and NaCl are frozen in the lower half of the tubes, the unfrozen upper portion of the solutions does not show an increase in NaCl concentration. This is contrary to the behaviour exhibited by NaCl alone in solution (40,42). In this case the glycoproteins do not

prevent the concentration of the NaCl, but rather affect mobility of the NaCl by compartmentalization of the brine in spaces between ice spicules. This compartmentalization has been attributed to the unusual needle-like structure imposed on ice when adsorbed to the glycoproteins.

The detailed mechanism by which the antifreeze molecules bind to the ice lattice remains to be elucidated. One feature in common between the Antarctic fish glycoprotein and the flounder antifreeze protein is that their molecules are composed primarily of alanine. The methyl groups of these alanine residues may play a critical role in preventing the formation of ice crystals (25). The interaction of the hydroxyl group in the disaccharide of AFGP with the ice nuclei could expose the hydrophobic methyl groups to the so-called water side of the ice-water interface. It has been suggested that these hydroxyl groups of AFGP can be replaced by the carboxyl groups of the aspartic and glutamic acid of AFP (30). A synthetic polypeptide with structural similarities to the flounder AFP has been synthesized (43). This polypeptide is a random copolymer of alanine and aspartic acid (Ala:Asp = 2:1 in molar ratio) with approximately one third of the antifreeze activity of the authentic antifreeze protein of winter flounder. Systematic synthesis of various analogues should elucidate more precisely the role of hydrophilic

and hydrophobic groups in AFP and AFGP (21).

E. Determination of Freezing Resistance in Fish

When one mole of non ionic solute is added to one kilogram of water, the colligative properties of the resulting solution are usually changed; freezing point is lowered 1.86°C , vapour pressure is lowered 0.3 mm Hg (vapour pressure of pure water = 17 mm Hg), boiling point increases 0.52°C , and osmotic pressure increases 17,000 mm Hg (17,000 mm Hg = 22.4 atmospheres).

Osmolality was first defined in 1934 (44) as the osmotic concentration of osmotically active particles. This is a widely accepted measure of biological concentration, and the common unit of expression is milliOsmol/kg H_2O , or simply milliOsmols. Methods of molality measurement provide an opportunity to define osmolality because

$$\text{osmolality} = \phi n \text{ molality}$$

where ϕ = osmotic coefficient or percent deviation from "ideal" behaviour (complete dissociation, no water binding, etc.)

n = number of particles into which molecule can be broken (1 for sucrose, 2 for NaCl, 3 for K_2SO_4 , etc.)

Any of the colligative properties can be used to indicate osmolality. Each method has its advantages and disadvantages. However, freezing point determination, with the available commercial instruments is the most sensitive of all the colligative techniques. Furthermore, it provides the only technique in which sample can be recovered.

At the present time, the problem of describing freezing resistance in fishes is greatly simplified by equating the freezing point to the temperature of ice formation in the serum (39). Thus, by common usage, osmolality is directly proportional to the freezing temperature. The technique of measuring freezing point is cryoscopy. Usually there is a lowering of the freezing point below 0°C due to the presence of salts in water. The greater the concentration of salt per unit of solvent, the lower the freezing point. Thus there is a nearly linear relationship between osmolality and freezing point such that:

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

Advanced osmometers are instruments designed to measure freezing points of liquids. Briefly, the method used in a freezing point osmometer involves placing the sample to be measured in a glass sample cup. When the head of the machine is lowered the sample is immersed in a cold bath and supercooled several degrees below its

freezing point. The curve of freezing (Figure 4) is traced out by a galvanometer spotlight and after the solution is supercooled, the sample is vibrated violently for one second via a stir wire. As crystallization occurs, heat of fusion is liberated and the sample temperature raises. The sample will stay at equilibrium between freezing and thawing because the ice crystals form a "blanket" of slush which insulates the centre of the sample from the cold bath. Thus the centre of the sample becomes independent of the environment or "adiabatic". Any changes that occur are due only to internal causes, that is the heat of fusion is equal to the heat released when the supercooled liquid crystallizes. During this period of isolation, the centre of the sample alternates between thawing and freezing and the heat of fusion released when one crystal is formed melts an adjacent one. The heat absorbed during this melting causes the first crystal to freeze again - and so the process continues. This temperature plateau makes the measurement of the freezing point of the solution relatively easy. The slush blanket will finally break down and allow the heat to leak out to the bath. The sample turns solid and cools to bath temperature.

Measurements of the osmolality of the winter flounder's serum at various times of the year with an Advanced

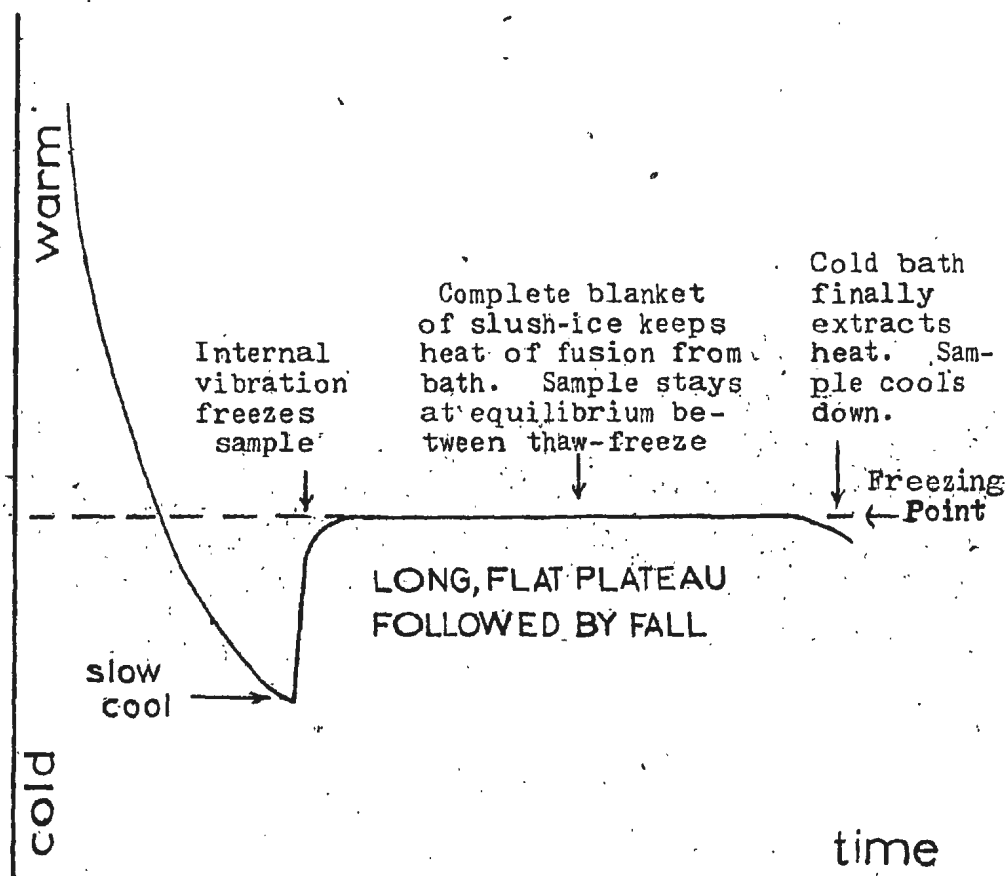


Figure 4 The curve of freezing in an Advanced osmometer

osmometer have shown that the osmolality of the flounder's serum is inversely proportional to the ambient sea water temperature (45). As can be seen in Figure 5, the osmolality of the flounder serum increases from 330 mOsm in October to more than 600 mOsm in January and February when the monthly average temperature of the seawater is at its lowest of -1°C . As the seawater warms up the osmolality starts to decline and reaches a minimum value of 320-330 mOsm in June and July when seawater temperatures are $12-13^{\circ}\text{C}$. These measurements are the monthly average of 4 years results (June, 1972-May, 1976) with approximately 10-30 fish per point. Almost 40% of this increase in osmolality in the flounder serum has been shown to be due to the presence of antifreeze protein (9). Thus, when the winter seawater temperature is as low as -1.2°C inorganic salts present in the serum of the winter flounder lower the serum freezing point to -0.7°C . The presence of the serum antifreeze protein lowers the blood serum freezing point the additional 0.5°C necessary to protect the flounder from freezing. It has been established that antifreeze protein is not found in flounder serum during the summer (32). This seasonal appearance of antifreeze protein is believed to be regulated by environmental factors such as temperature, photoperiod (46), and the endocrines (47).

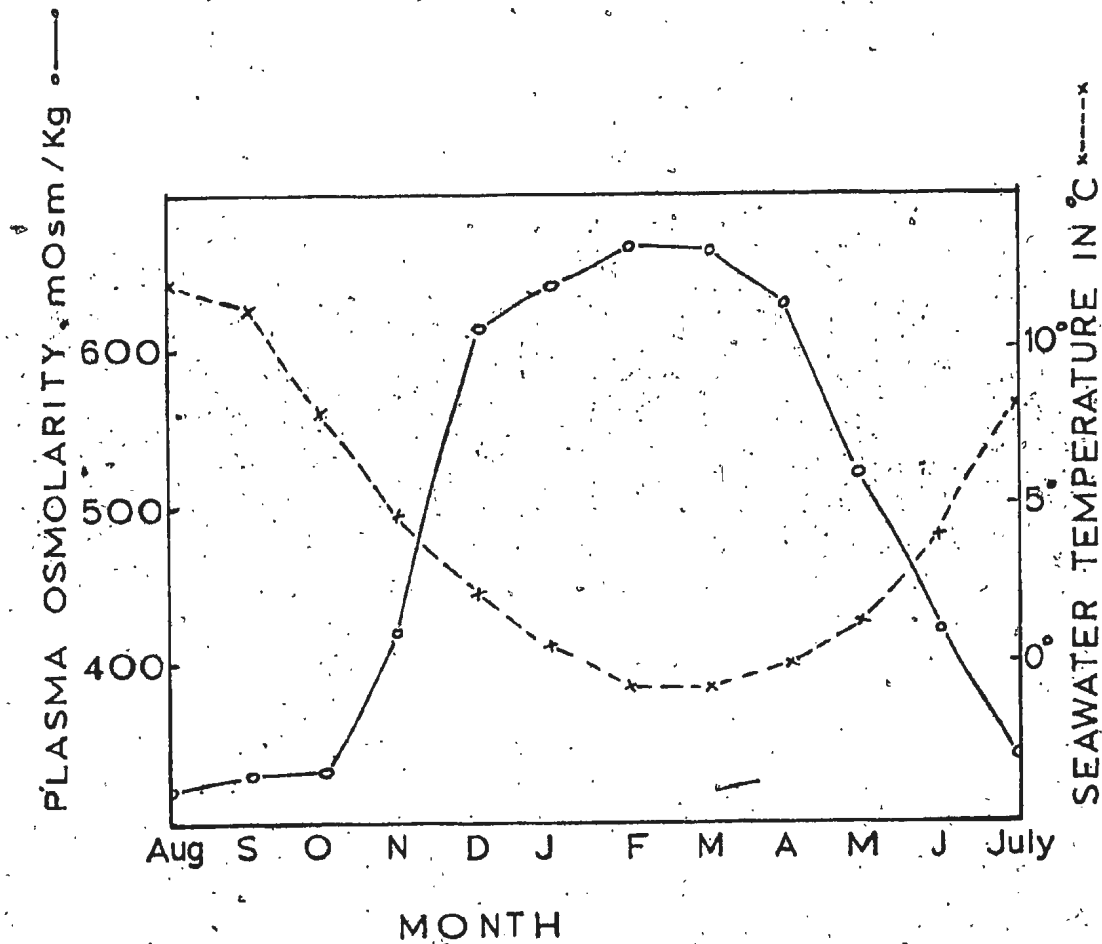


Figure 5 The seasonal variation in the plasma osmolality of the winter flounder (reference 45)

Relevance and Purpose of the Study

Many facets of the phenomenon of temperature acclimation are not fully understood. Studies on the temperature acclimation of fish could provide information about the adaptability of these organisms to environmental temperature variation, and the physiological mechanism underlying these adaptations. A thorough understanding of the adaptive processes of fish relative to environmental parameters may indicate the extent to which environmental manipulation is possible. Furthermore, it could provide the criteria whereby genetic selection could be controlled to alleviate problems arising from environmental changes, and even improve on the existing stocks of aquatic food resources. Ultimately, such studies could provide insight into the adaptive processes in humans, and possible mechanisms for the regulatory control of metabolism in general.

Because of the seasonal appearance of antifreeze protein in the winter flounder (32,45), and its probable regulation by environmental factors such as temperature, photoperiod (46), and the endocrines (47), the flounder offers an interesting system for studies of gene expression. It has been shown that winter flounder antifreeze protein is synthesized ribosomally (20). Thus, 6-10 S poly A-rich

polysomal RNA, isolated from flounder liver, was translated to a product identifiable as flounder antifreeze protein when injected into Xenopus laevis oocytes (20). In vivo biosynthetic studies have demonstrated the occurrence of a "large" antifreeze protein (molecular weight 15,500 daltons) in the serum (45). In addition, pulse chase experiments suggest that the 15,500 MW "large" antifreeze protein is converted to the 10,000 MW antifreeze protein in the serum (45).

As a first step to better understanding the synthesis and regulation of antifreeze protein in the winter flounder, an investigation was planned with these objectives:

1. The demonstration of a "large" antifreeze protein in flounder liver using an in vitro incubation system.
2. The isolation of the "large" in vitro antifreeze protein.
3. The characterization of the "large" in vitro antifreeze protein.
4. The demonstration of a structural relationship between the "large" antifreeze protein synthesized in the liver and the 10,000 MW antifreeze protein found in the serum.
5. The production of specific antibody against flounder antifreeze protein as a tool for biochemical studies.

MATERIALS

A. Collection of Experimental Materials

Winter flounder were collected from Chapel's Cove, Conception Bay, Newfoundland, and were kept in 250 to 2500 *l* aquaria supplied with flowing seawater. Seasonally normal photoperiod and water temperatures were maintained. The seawater temperature fluctuated from 12 to 13°C in August to -1 to -2°C in February and March.

B. Chemicals

Trypsin, phenylmethylsulfonyl fluoride, and thermolysin (Type x), were supplied by Sigma Chemical Company, St. Louis, U.S.A. Trasylol was supplied by FBA Pharmaceuticals, Montreal, Canada. The magnesium salt of 1-anilino-8-naphthalene sulfonate was obtained from Eastman Organic Chemicals, Rochester, New York. Freund's adjuvant was obtained from Difco Laboratories, Detroit, Michigan. Sephadex was supplied by Pharmacia Fine Chemicals, Montreal, Canada. Methanol and diethyl ether were reagent grade and were distilled under reduced pressure from 1,5 - difluoro-2,4 -dinitrobenzene (DFDNB) in the ratio 1 g reagent/1 litre solvent. All other chemicals were reagent grade.

METHODS

A. Isolation of the *In Vitro* Biosynthetic Product1. *In vitro* liver slice incubation.

Only male winter flounder were used to avoid the possible

complication and contamination arising from vitellogenesis in the female. Vitellogenesis in the flounder, which occurs during August to February (48), overlaps with the synthesis of antifreeze protein.

Flounder were stunned by a blow to the head. After a blood sample was obtained, the liver was immediately extirpated, weighed, and washed in ice-cold Krebs-Ringer bicarbonate solution, pH 7.4, containing 0.14 M Na^+ (49). Before use, the buffer was equilibrated by gassing it with $\text{O}_2:\text{CO}_2$ (95:5 v/v) for at least 10 minutes. Liver slices (0.5 mm in thickness) were prepared using a mechanical tissue chopper (the McIlwain mechanical tissue chopper, Mickle Laboratory Engineering Co., Gomshall, Surrey, Great Britain). The slices (1 gm each) were placed in a 25 ml erlenmeyer flask containing 2 ml of the bicarbonate buffer and were preincubated for 10 minutes at 15°C with constant shaking (90 cycles/min). After preincubation, the tissue was then transferred to another flask containing 2 ml of buffer, 10 μCi of C^{14} alanine (L-alanine- ^{14}C , uniformly labelled, specific activity 120mCi/mmole, New England Nuclear) and 100 μl of the protease inhibitor, Trasylol (50). The flask was gassed with $\text{O}_2:\text{CO}_2$ (95:5 v/v), stoppered, and incubated in a water bath at 15°C for 4 hours with constant shaking. The flask was gassed at 30 minute intervals. The tissue was then washed

two times with 5 ml of buffer containing 1 mg/ml alanine. The tissue was resuspended in 3 ml of buffer and homogenized in a Potter-Elvehjem homogenizer with Teflon pestle. The homogenized tissue was then treated with an equal volume of ice-cold 20% (w/v) trichloroacetic acid. The acid was added in a drop-wise fashion as the sample was being vortexed. The acid-treated homogenate was equilibrated at room temperature for 10 minutes and then centrifuged for 5 minutes at 10,000 rpm. Flounder antifreeze protein has been reported to be soluble in 10% trichloroacetic acid, and this observation was utilized in the purification procedure. The acid-soluble materials were desalted on a Sephadex G-25 column (1.5 x 40 cm) in 0.05 M NaCl. Forty fractions (2.2 ml per fraction) were collected on a LKB fraction collector at room temperature. Aliquots of 100 *ul* were taken for radioactivity counting using 10 ml of Aquasol 2 (New England Nuclear, Boston, Mass., U.S.A.) in a Packard Liquid Scintillation Spectrometer, Model 3375. Protein fractions coming off in the void volume were pooled and lyophilized. The lyophilized material was dissolved in 2 ml of 0.05M ammonium bicarbonate, and chromatographed on a Sephadex G-75 column (1.6 x 86 cm) equilibrated with 0.05 M ammonium bicarbonate at 4⁰C, and one hundred fractions (2.2 ml per fraction) were collected. Aliquots of 500 *ul* were taken for radioactivity counting. The radioactive

fractions in the elution profile were pooled, lyophilized, and subjected to amino acid analysis. The lyophilized material identified as liver "large" antifreeze protein was stored in the freezer at -60°C . The 10% trichloroacetic acid precipitate was dissolved in 0.5 N sodium hydroxide and aliquots were taken for radioactivity counting and for protein determination using a modification of the Lowry procedure (51).

2. Effect of incubation time and incubation temperature on the *in vitro* system

In order to choose the incubation conditions that would maximize the incorporation of radioactivity into the acid-soluble fraction, experiments were conducted on the *in vitro* system to determine the effect of varying incubation times and incubation temperatures. Liver slices (1 gm each) obtained from one animal were incubated for 1, 2, 3, 4, and 5 hours respectively, at 15°C . Similarly, liver slices obtained from one animal were incubated at various temperatures: 0° , 5° , 12° , and 15°C respectively for 4 hours. The slices were processed as previously described at the end of the incubation period.

3. Pulse chase experiments in the presence of cold alanine or puromycin.

After 1 hour of incubation, excess cold alanine (2 mg/ml) was added to the *in vitro* system, and the liver slices were incubated for 3 hours longer. A second control flask

was incubated for a total of 4 hours.

Similarly, after 1 hour of incubation, puromycin (30 μ g/ml) was added to the incubation mixture. Liver slices were incubated for a total of 4 hours, and the liver slices were processed as usual.

B. Determination of Plasma Osmolality and Sea Water Temperature

Flounder blood samples were obtained from a caudal blood vessel using 23-gauge syringe needles, and placed in heparinized centrifuge tubes on ice. Red cells were separated from the plasma within 15 minutes of sampling by low speed centrifugation. The plasma osmolality was determined with an Advanced osmometer (model 3D, Advanced Instruments, Needham Heights, Mass., U.S.A.). Ambient sea water temperatures were those recorded to the nearest 0.1°C at the Marine Sciences Research Laboratory.

C. Determination of Protein

The protein content was determined using the Lowry procedure as described by Hartree (51). Bovine serum albumin was used as the protein standard.

D. Recovery of Antifreeze Protein by the 10% Trichloroacetic Acid Procedure

Liver slices (2 gm) were equilibrated in 4ml of Krebs-Ringer bicarbonate buffer containing 10 μ l of 3 H antifreeze protein (4.56×10^6 cpm, prepared by tritium gas exposure labelling) at 4°C. The tissue was homogenized and an

aliquot taken for radioactivity counting. The homogenate was treated with an equal volume of ice-cold 20% trichloroacetic acid (w/v), vortexed, and centrifuged. Aliquots were taken from the trichloroacetic acid-soluble and acid-insoluble fractions for liquid scintillation counting.

E. Isolation of Liver Antifreeze Protein

Male flounder livers from 5 to 10 specimens were homogenized in 50 ml of ice-cold buffer NS (20 mM Tris, 10 mM KCl, 40 mM NaCl, 5 mM $MgCl_2$, 6 mM β -mercaptoethanol, 0.25 M sucrose, pH 7.6). The homogenate was centrifuged at 6,000xg for 10 minutes (SS-34 rotor). The supernatant obtained was centrifuged at 45,000 rpm for 1 hour in a Spinco SW 50.1 rotor. The pellet obtained from high speed centrifugation was suspended in buffer N (buffer NS with no sucrose added) and further processed for the isolation of flounder liver polysomes (p 46). The supernatant was treated with an equal volume of 20% ice-cold trichloroacetic acid and allowed to sit for 10 minutes. After centrifuging at 10,000 rpm for 5 minutes, the acid-soluble materials were dialysed against 0.05 M NH_4HCO_3 overnight (dialysis membrane m. wt. cut off 3,500), and then lyophilized. The lyophilized material was chromatographed on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M NH_4HCO_3 . The absorbance of each fraction was measured at 230 nanometers. The materials from each recorded peak were pooled, lyophilized, and

subjected to amino acid analysis. The eluted peak corresponding to the "large" liver antifreeze protein was re-chromatographed on the Sephadex G-75 column, lyophilized, and stored at -60°C .

F. Characterization of the Liver "Large" Antifreeze Protein

1. Electrophoresis

(a) Polyacrylamide gel electrophoresis (PAGE) in 4 M urea, 0.1 M tris buffer, pH 9.2

Stock solution A was prepared by dissolving 36.6 gm of Tris and 360 gm of urea in 500 ml of distilled water. The pH of the solution was adjusted to 9.2 with 2 N HCl and the final volume was brought to 1 liter with distilled water. Stock solution C was prepared by dissolving 60 gm of acrylamide and 4 gm of Bis (N,N' - methylene-bis(acrylamide)) in a final volume of 100 ml of water. Stock solution D was prepared by dissolving 10 gm acrylamide, 2.5 gm Bis, and 36 gm of urea in a final volume of 100 ml of water. Stock solution E was prepared by dissolving 4 mgm riboflavin and 36 gm of urea in a final volume of 100 ml of water. All solutions were filtered before use. The ammonium persulfate solution was freshly prepared by dissolving 0.14 gm ammonium persulfate in 100 ml of water. The tracing dye solution was 0.001% bromophenol blue in water.

A 10% small pore solution was prepared by combining

2.0 ml stock solution A, 2.66 ml stock solution C, 3.34 ml distilled water and 0.02 ml TEMED. An equal volume of the ammonium persulfate solution was added to this and mixed by inversion to start the polymerization.

The large pore solution was prepared by mixing 1.0 ml stock solution A, 2.0 ml stock solution D, 1.0 ml stock solution E, and 4.0 ml distilled water.

The stock buffer was prepared by dissolving 6 gm of Tris and 28.8 gm of glycine in 500 ml of water. The pH was adjusted to 9.2 with 2 N NaOH and water was added to a final volume of 1 L. The electrophoresis buffer was prepared by diluting 125 ml of stock buffer to 1000 ml with water.

Using a disposable pasteur pipette, the small pore mixture was delivered immediately after mixing into 10 cm electrophoresis glass tubing which was stoppered at the bottom with parafilm. The tubes were filled to approximately 2.5 cm from the top end. A small amount of water was carefully layered on top of the gel mixture before it began to polymerize. Polymerization was usually completed within 20 minutes. The top of the gel was rinsed with water. Approximately 2 cm of the large pore solution was pipetted on top of the small pore gel and water was again layered on top of the gel. The large pore gel was polymerized by exposing the tubes to fluorescent light.

After polymerization was complete, a small amount of Sephadex G-200 was layered on top of the large pore gel with a small spatula. The parafilm was removed from the bottom of the gel tubes and the gel tubes were set up accordingly in the electrophoresis unit. The sample, dissolved in a maximum volume of 100 *ul* of electrophoresis buffer, was applied to the Sephadex on top of the gel followed by 2 drops of tracing dye solution. The protein and tracing dye solution were absorbed by the Sephadex and provided better resolution of protein bands on the gels. The gel tube was topped carefully with buffer and the upper tank was filled with sufficient buffer to cover the top of the gel tubes. An initial current of 2mA per tube (positive pole on bottom of gel) was applied until the marker dye had entered the small pore gel. The current was then increased to 5 mA per tube. The electrophoresis was terminated when the dye front reached the bottom of the tube. Gels were removed from the gel tubes by inserting a fine wire between the gel and the gel tube. The tubes were immersed in a water tank and the gels forced out by applying gentle pressure with a pasteur pipette bulb.

(b) Sodium dodecyl sulfate (SDS) gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted as described by Weber and

Osborn (52).

(c) Fluorescent staining with anilinonaphthalene sulfonate (ANS)

Earlier observations in our laboratory have indicated that AFP stains poorly with coomassie brilliant blue. Thus, anilinonaphthalene sulfonate, a fluorescent reagent, was tried instead.

Anilinonaphthalene sulfonate (ANS) does not fluoresce in water, but fluoresces brightly in the visible range when dissolved in organic solvents or in aqueous solutions of various proteins (53). This enhanced fluorescence has been attributed to binding of the fluorophore to hydrophobic sites on the protein surface (54). In addition, most proteins can be made to enhance ANS fluorescence following thermal or chemical denaturation (55).

An aqueous stock solution (1 mg/ml) of the magnesium salt of 1-anilino-8-naphthalene sulfonate was prepared. This solution has been reported to be stable at 4°C for at least 6 months (56). The staining solution was prepared immediately before use by dilution of the stock solution with 0.1 M potassium phosphate buffer (pH 6.8) to produce a final concentration of 0.003% ANS. Freshly run gels were removed from their tubes and immersed for 1 minute in the staining solution. The gels were then immersed in 1 N HCl for a few seconds to enhance surface denaturation of the protein, rinsed in water, and restained

for 1 minute in the staining solution. The fluorescent gels were then viewed under a mineral light ultraviolet source.

(d) Detection of radioactive macromolecules in acrylamide gels

At the end of electrophoresis, radioactive macromolecules were detected by cutting the acrylamide gel into 2 mm slices with a scalpel. Each slice was transferred to a glass scintillation vial and crushed against the side of the vessel. The gel slices were dried overnight at 60°C. Before determination of radioactivity it was necessary to dissolve the gel. An ammoniacal hydrogen peroxide (H_2O_2) solution was prepared by combining 9.9 ml of 30% H_2O_2 and 0.1 ml of concentrated ammonium hydroxide. Three hundred microliters of this solution was added to each vial, and the gels were soaked in the solution. The vials were capped tightly and put in an oven at 60°C for 2-4 hours. The tray containing the vials was placed at a 45° angle so as to keep the gel immersed in the hydrogen peroxide solution. After the gel had been completely dissolved, the vials were cooled, 10 ml of scintillation fluid was added to each vial, and the radioactivity was determined with the scintillation spectrometer.

2. Molecular weight determinations by gel filtration chromatography

(a) Sephadex G-75 chromatography of the in vitro biosynthetic product and ^3H antifreeze protein in 8 M urea, tris-glycine buffer

The protein samples were dissolved in a minimal volume of 8 M urea, 0.1 M Tris, 0.5 M glycine, 50 mM β -mercaptoethanol buffer, and equilibrated at 37°C for 1 hour. After cooling, the samples were applied to a Sephadex G-75 column (1.6 x 86 cm) equilibrated with the urea, tris-glycine buffer, and 100 fractions (2.2 ml per tube) were collected. The column was calibrated with standard protein markers. The K_{av} value for each protein standard was calculated and was used for the molecular weight estimation.

Approximately .3 mg of the in vitro biosynthetic product was dissolved in 500 μl of distilled water. Approximately 100,000 cpm (40 μl) of the in vitro biosynthetic product was equilibrated in .5 ml of the urea, tris-glycine buffer at 37°C for 1 hour and then applied to the Sephadex G-75 column. Aliquots of 500 μl were taken from the eluted fractions for counting with 10 ml of scintillation fluid. The K_{av} value for the in vitro biosynthetic product was calculated. Similarly, the K_{av} of ^3H antifreeze protein was determined.

(b) Succinylation of flounder liver antifreeze protein

Purified flounder liver antifreeze protein (4.5 mg)

in 0.2 ml of phosphate buffer, 0.05 M pH 7.5, was added to a scintillation vial containing 50 μ Ci of succinic anhydride 1,4- 14 C (specific activity 2-10 mCi/mmol, New England Nuclear, Boston, U.S.A.) for 15 minutes at 4°C with stirring. The mixture was then diluted with 2 ml of cold phosphate buffer. Solid succinic anhydride (25 mg) in aliquots was added and the pH of the solution maintained between 7-9 with 2 N NaOH. After 1 hour, the reaction mixture was treated with hydroxylamine (0.8 M, pH 9.5) for 15 minutes, desalted on a Sephadex G-25 column (1.5 x 40 cm) in 0.05 M NaCl and lyophilized. The molecular weight of the succinylated liver antifreeze protein was determined on the urea, tris-glycine Sephadex G-75 column as described earlier.

3. Selective enzymic cleavage of the *in vitro* biosynthetic product

(a) Tryptic digestion of the *in vitro* biosynthetic product

The sample, containing approximately 100,000 cpm of the *in vitro* biosynthetic product and .5 mg of the unlabelled liver antifreeze protein, was dissolved in 0.5 ml of 0.2 M ammonium bicarbonate, pH 8.0. The sample was digested with 10 μ g of trypsin at 37°C for 1 hour. The resultant enzymic digest was treated with 10 μ l of phenylmethylsulfonyl fluoride (1 mg in 1 ml of ethanol). The sample was then chromatographed on a Sephadex G-75 column (1.6 x 86 cm)

previously calibrated and equilibrated with 6 M urea, 0.1 M tris-HCl, pH 8.6. Aliquots were taken for radioactive counting from the eluted fractions.

(b) Thermolysin peptide mapping of the "large" liver antifreeze protein and serum antifreeze protein

The "large" liver antifreeze protein and the serum antifreeze protein were digested separately with thermolysin in 0.2 M ammonium formate pH 8.0, containing 0.002 M CaCl_2 (enzyme to substrate ration 1/50) for 1 hour at 37°C . The digest was then analysed on a Beckman 121 amino acid analyzer using a single-column physiological fluid analysis with W-2 resin (57). The column was initially equilibrated with 0.2 N lithium citrate buffer at pH 2.83. A buffer gradient was used to increase the pH to 3.75 and the lithium concentration to 1.0 N with a temperature gradient of 40°C to 66°C .

4. Separation on DEAE ion exchange

A DEAE cellulose column (0.9 x 12 cm) was washed with 0.5 M NH_4HCO_3 , and then equilibrated with 0.01 M NH_4HCO_3 at room temperature. The in vitro biosynthetic product (1.6×10^5 cpm) was dissolved in 1 ml of 0.01 M NH_4HCO_3 , and then applied to the DEAE column. Ten fractions (2.6 ml per fraction) were collected with the 0.01 M NH_4HCO_3 buffer. The column was then eluted with a linear gradient, 0.01 M to 0.5 M, in NH_4HCO_3 . Aliquots of 100 μl were taken

from the eluted fractions for counting with 10 ml of scintillation fluid. The conductivity of each sample was measured with a conductivity meter.

5. Amino acid analysis

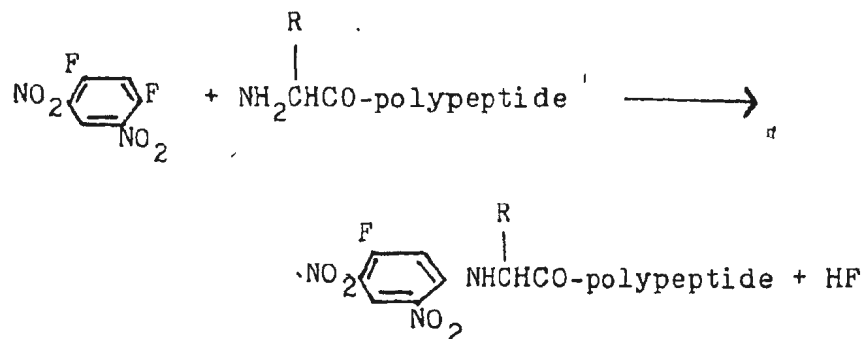
Samples for amino acid analysis was hydrolyzed in constant boiling HCl at 110°C for 18 hours. Analyses were performed on a Beckman 121 amino acid analyzer.

G. Preparation of Antibodies Against Antifreeze Protein

1. Preparation of antigens

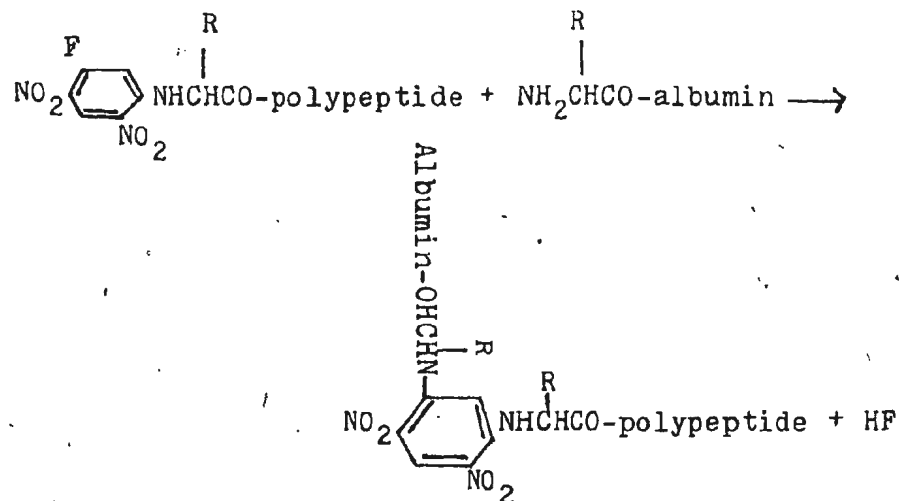
(a) Coupling of antifreeze protein to albumin with difluorodinitrobenzene

A generally useful procedure for the coupling of peptides to albumin has been developed (58) which allows an easily manipulated coupling ratio and a high yield of conjugation, thus eliminating losses of scarce material. The process is a two stage reaction scheme. Initially, the peptide is monofunctionally derivatized with the bi-functional reagent 1,5-difluoro-2,4-dinitrobenzene (DFDNB), which reacts with primary amine groups (and also side-chain phenolic, thiol, and imidazole groups). The peptide is converted to the fluorodinitrophenyl (FDNP) derivative in the presence of excess reagent in aqueous-organic solvent.



fluorodinitrophenyl-peptide (FDNP-peptide).

After the unreacted acylating agent is removed by ether extraction, the activated peptide is conjugated to albumin in aqueous solution.



This two stage reaction has several advantages. Reaction kinetics are simplified by decreasing the number of reacting species in the mixture. The peptide can be derivatized in the presence of a very large excess of reagent thus providing a high degree of reaction and minimizing polymerization of the material. The terminal coupling can be accomplished after removal of the reagent, thus minimizing polymerization of the albumin. The reported efficiency of coupling approaches 90% over a wide range of peptide and albumin concentrations.

i) Activation of peptide

Purified antifreeze protein was dissolved in 7 M guanidine hydrochloride, 0.1 M potassium phosphate, pH 7.2, at a concentration of 1 mg/ml. A solution of DFDNB (30 mg/ml in purified methanol) was added to the peptide in the ratio of 5 volumes of reagent to 1 volume of protein solution. The resulting solution was mixed well and allowed to stand at room temperature for 15 minutes. The reaction mixture was cooled on ice and 4 volumes (based on the final volume of the mixture) of ice-cold, purified ether were added. After vigorous agitation, the resulting mixture was briefly centrifuged in the cold to separate the two phases of solution. The upper ether phase was carefully removed by aspiration and discarded. Another 3 volumes of cold ether were added to the lower phase, and the test tube

was agitated again to deposit in a thin layer a precipitate of the guanidine hydrochloride and other salts in the original reaction mixture, as well as the FDNP-antifreeze protein. The ether phase was removed by decantation and 10 volumes of cold ether were added. After the tubes had stood on ice for about 1 minute, the ether was again decanted and the tubes drained.

ii) Coupling of peptide

Albumin was dissolved in 0.4 M sodium borate buffer, pH 10 at a concentration of 10 mg/ml. A 5 ml volume of the albumin solution was added to the tube containing the precipitated peptide, guanidine hydrochloride, and other salts. The tube was allowed to stand at room temperature uncovered for a short time to allow the evaporation of residual ether. The flask was then covered and stored at room temperature for 24 hours to allow for the completion of the reaction and the formation of the antifreeze-dinitrophenyl-albumin. Although the final product is reported to be light-stable, the activation and coupling procedures were performed in indirect light, and the reaction mixtures were stored in the dark.

iii) Analysis of product

The antifreeze-dinitrophenyl-albumin solution was dialysed versus 4 litres of 0.05 M NH_4HCO_3 for 4 hours with one change of buffer. The dialysed solution was

centrifuged and the supernatant was decanted and lyophilized. The remaining precipitate was also lyophilized. The lyophilized supernatant was dissolved in a minimal volume of 0.05 M NH_4HCO_3 and chromatographed on a Sephadex G-100 column (1.6 x 83 cm) previously equilibrated with 0.05 M NH_4HCO_3 . One hundred fractions (2.2 ml/tube) were collected, and the absorbancies of each fraction were measured at 280 nm. The appropriate fractions were pooled and lyophilized. Similarly, the lyophilized precipitate was chromatographed on a Sephadex G-100 column in the presence of 0.05 M NH_4HCO_3 , and the absorbancies of the fractions were measured at 280 nm. A sample of bovine serum albumin and of the antifreeze-albumin conjugate were subjected to amino acid analysis.

2. Immunological techniques

The following were used as antigens: the serum "large" antifreeze protein (M.Wt. 16,000), the antifreeze protein (M. Wt. 10,000), and the antifreeze-albumin conjugate. Each was dissolved in 0.9% NaCl, 0.15 M sodium phosphate buffer pH 7.4. The protein concentrations were approximately 1 mg/ml. Equal volumes of protein solution and complete Freund's adjuvant were emulsified using glass syringes. Preimmunization sera were collected from each animal prior to the initial immunization. The blood was collected in a centrifuge tube, and the blood

was allowed to clot for 1 hour at room temperature. After centrifugation (in a SS-34 rotor) the serum was carefully decanted and stored at -40°C until needed.

Individual New Zealand white rabbits were immunized with one of the prepared antigens by injecting 1.0 ml of the emulsion intramuscularly into the large hind thighs of the rabbit. Immunizations were repeated at 7 day intervals with freshly prepared emulsions using incomplete Freund's adjuvant. A similar immunization program was followed for each antigen using guinea pigs. Test bleedings were performed 21 days and 3 months after initial immunization.

3. Characterization of blood sera for antifreeze protein antibodies

a) Ouchterlony test

The buffer solution used in the preparation of the ouchterlony plates was prepared by mixing 67 ml of solution I ($\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 26.7 g/L) and 33 ml of solution II (KH_2PO_4 , 20.4 g/L). One hundred ml of the prepared buffer was combined with 300 ml of water. To this was added 4 g of agar and .1 g of sodium azide. The mixture was heated, and when the agar was completely dissolved, the gel solution was poured into disposable petri dishes. Once the gel had set, a central well, surrounded by six peripheral wells, was cut into the gel. The bottom of each hole was layered with agar. The central well of the plate was filled

with the appropriate immunized serum. Peripheral wells were filled with varying concentrations of the antigen. The plates, covered with a plastic lid lined with moist filter paper, were incubated at 37°C for 2-4 days. The plates were inspected every 12 to 14 hours.

b) Immunoabsorption utilizing ^3H antifreeze protein

Control and immunized sera were equilibrated with ^3H antifreeze protein for 1 hour at room temperature. Each sample was chromatographed on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M NH_4HCO_3 . The absorbancies of the eluted fractions were monitored at 280 nm. Aliquots (500 μl) were taken for radioactivity counting.

H. In vitro biosynthesis of antifreeze protein on polyribosomes in flounder liver

a) Isolation of total polyribosomes

The in vitro biosynthesis was conducted as previously mentioned (p 26). After 2 hours of incubation at 15°C, the combined tissue (2 g) was washed with 5 ml of the Krebs buffer containing 1 mg/ml alanine. The tissue was then resuspended in 4.5 ml of buffer NS (20 mM Tris-HCl, pH 7.6, 10 mM KCl, 40 mM NaCl, 5 mM MgCl_2 , 6 mM β -mercaptoethanol, and 0.25 M sucrose) containing 1 mg/ml heparin and 5 mg/ml cyclohexamide, and homogenized in a Potter-Elvehjem homogenizer with Teflon pestle. The homogenate was centrifuged at 10,000xg for 10 minutes in a SS-34 rotor.

The pellet, containing nuclei, mitochondria, and cell debris was discarded. The supernatant was made 1% in Triton X-100. The polysomes were pelleted through 1M sucrose in buffer N at 49 k for 90 minutes in a Ti 50 rotor. The pellet containing the total polyribosomes was rinsed twice with buffer N, drained, and resuspended in buffer N (.5 ml total volume) by gentle homogenization in a disposable syringe. Aliquots were taken from the pellet for absorbancy measurements at 260 nm, and for radioactivity counting.

b) Isolation of free and membrane-bound polyribosomes

After 2 hours of incubation at 15°C, the combined tissue (2 g) was washed with 5 ml of the Krebs buffer containing 1 mg/ml alanine. The tissue was then resuspended in 4.5 ml of buffer NS (20 mM Tris-HCl, pH 7.6, 10 mM KCl 40 mM NaCl, 5mM MgCl₂, 6 mM β -mercaptoethanol, and 0.25 M sucrose) containing 1 mg/ml heparin and 5 μ g/ml cyclohexamide, and homogenized in a Potter-Elvehjem homogenizer with Teflon pestle. The homogenate was centrifuged at 6000 xg for 10 minutes in a SS-34 rotor. The pellet, containing nuclei, mitochondria, and cell debris was discarded. The supernatant was centrifuged at 45,000 rpm for 1 hour in a Spinco SW50.1 rotor. The decanted supernatant was set aside for purification of the liver antifreeze protein.

Each pellet was re-suspended in .4 ml of buffer N, pooled, and homogenized gently. The homogenized material (.2ml) was layered on top of a discontinuous sucrose concentration gradient of 2.4 ml each of 1.25 M - and 2M - sucrose in buffer N. Centrifugation in the sucrose gradient was conducted at 40,000 rpm for 16 hours in a SW50.1 rotor. Under these conditions the free polysomes were pelleted at the bottom of the gradient, the rough endoplasmic reticulum was banded at the interface, and the top of the gradient contained the smooth endoplasmic reticulum. The supernatant was decanted with a water aspirator and discarded. The membrane-bound polysomes were collected from the 1.25 M/2.0 M sucrose interphase. They were diluted with an appropriate volume of buffer N to bring the sucrose concentration from 1.25 M to 0.25 M, and then made 1% in Triton X-100 by treatment with a 10% solution of Triton X-100 in buffer N. The polysomes released from the membrane by the detergent treatment were pelleted through 1 M sucrose in buffer N at 49 k for 90 minutes in a Ti 50 rotor. The pellets containing the free and membrane-bound polyribosomes were rinsed twice with buffer N, drained, and resuspended in buffer N (.3 ml total volume). Aliquots were taken from the pellets (free and membrane-bound) for absorbancy measurements at 260 nm, and for radioactivity counting.

c) Analysis of Polyribosome Suspensions

Polyribosome suspensions were layered in an amount of 100 μ l (approximately 1 OD₂₆₀ unit) over a sucrose concentration gradient (15-30%) prepared in buffer N. Centrifugation was conducted at 49,000 rpm for 30 minutes in a Spinco SW50.1 rotor. The gradient was displaced with 50% (w/v) sucrose and monitored at 260 nm with an ISCO absorbance monitor. Fractions of the gradient (300 μ l each) were collected on an Isco density fractionator, and were used to determine radioactivity by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

SECTION I The Identification and Characterization of
the In Vitro Biosynthetic ProductIntroduction

Hew et al. (45) have recently demonstrated the presence of a "larger" antifreeze protein of 15-16,000 daltons in the serum. This raises the possibility that flounder AFP (M. Wt. 10,000) may be synthesized via a larger precursor protein. In order to better understand the synthesis and regulation of AFP, an investigation was undertaken to demonstrate the possible occurrence of a similar "large" AFP in the flounder liver. Hew and Yip (20) have earlier shown that flounder liver contains AFP mRNA activity, and most likely represents the site of AFP synthesis.

An in vitro liver slice incubation procedure was adopted. The optimum conditions for the incorporation of radioactive alanine were determined. The identification of the in vitro biosynthetic products of AFP depends on the following criteria: (i) its solubility in 10% trichloroacetic acid (flounder AFP is soluble in the acid) (ii) incorporation of radioactive alanine into the polypeptide (iii) structural characterization (i.e. amino acid analysis, enzymic digestion). Accordingly, acid-soluble materials from the liver slice incorporation studies were isolated and characterized using various biochemical techniques. The possible precursor-product relationship

of the in vitro biosynthetic product with authentic AFP was investigated.

Results

A. Time Studies

Acid-soluble materials from the liver slice incubation were desalted on a Sephadex G-25 column (1.5 x 40 cm) in 0.05 M NaCl (Figure 6). Protein fractions coming off the void volume (fractions 13-17) were pooled and lyophilized.

The acid-soluble proteins were re-chromatographed on a Sephadex G-75 column in 0.05 M NH_4HCO_3 (Figure 7). Radioactivity was incorporated into two components: a major component of approximately 16,000 daltons, and a minor component which eluted earlier in the column. In this chromatographic system, flounder AFP has an elution volume slightly larger than the cytochrome C marker and would correspond to fraction 50 in the column. As can be seen in Figure 7, no radioactivity was detected corresponding to this elution position. To find out which, if any, of the two components were related to AFP, the materials were lyophilized and subjected to amino acid analysis. Amino acid analysis (p. 69) showed that only the 16,000 dalton component, which eluted before the cytochrome C marker, was similar to the antifreeze protein as revealed by the abundance of alanine residues in the composition. The larger, minor component, however, showed

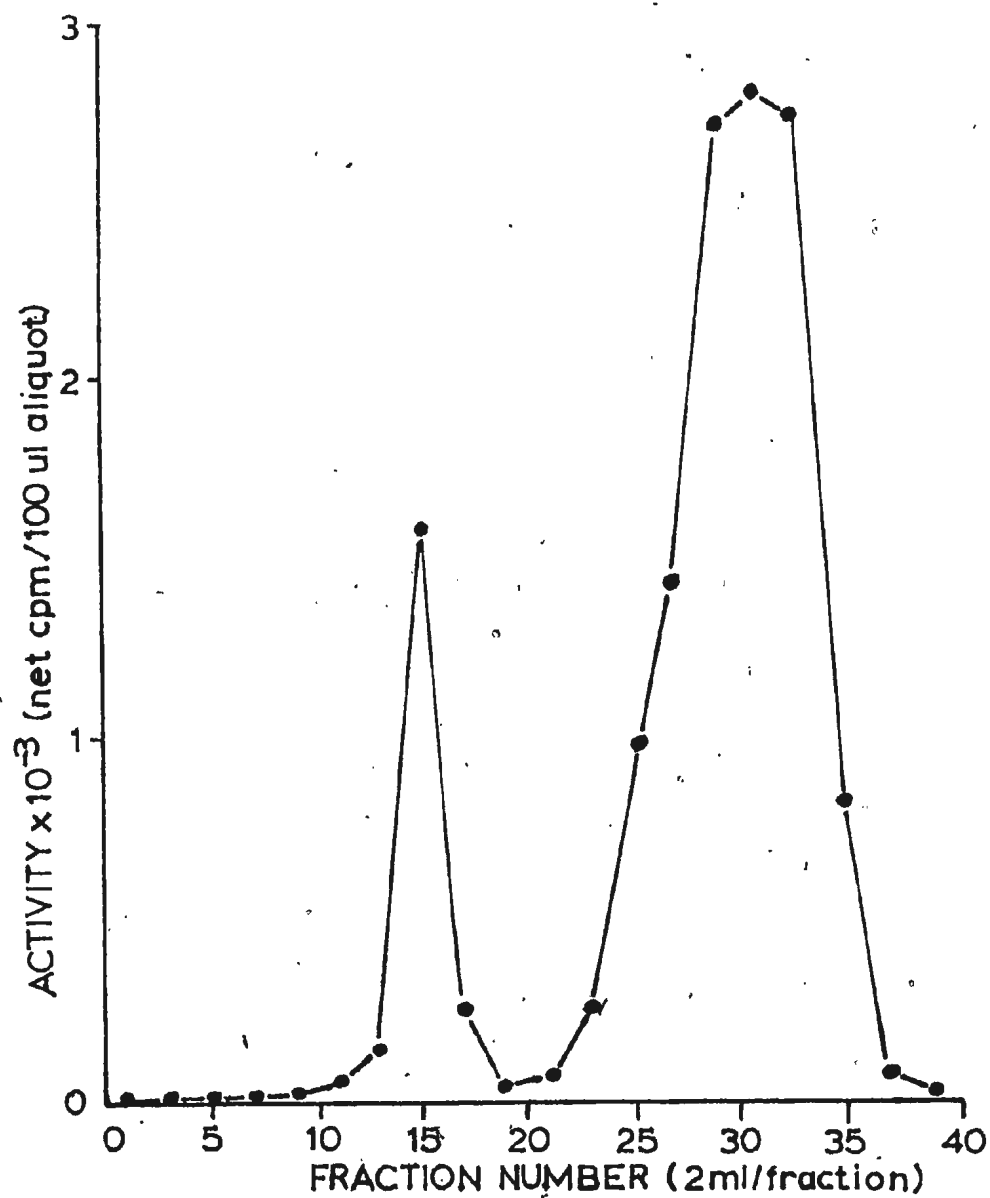


Figure 6 Desalting of 10% TCA-soluble material on a Sephadex G-25 column (1.5 x 40 cm) in 0.05 M NaCl.

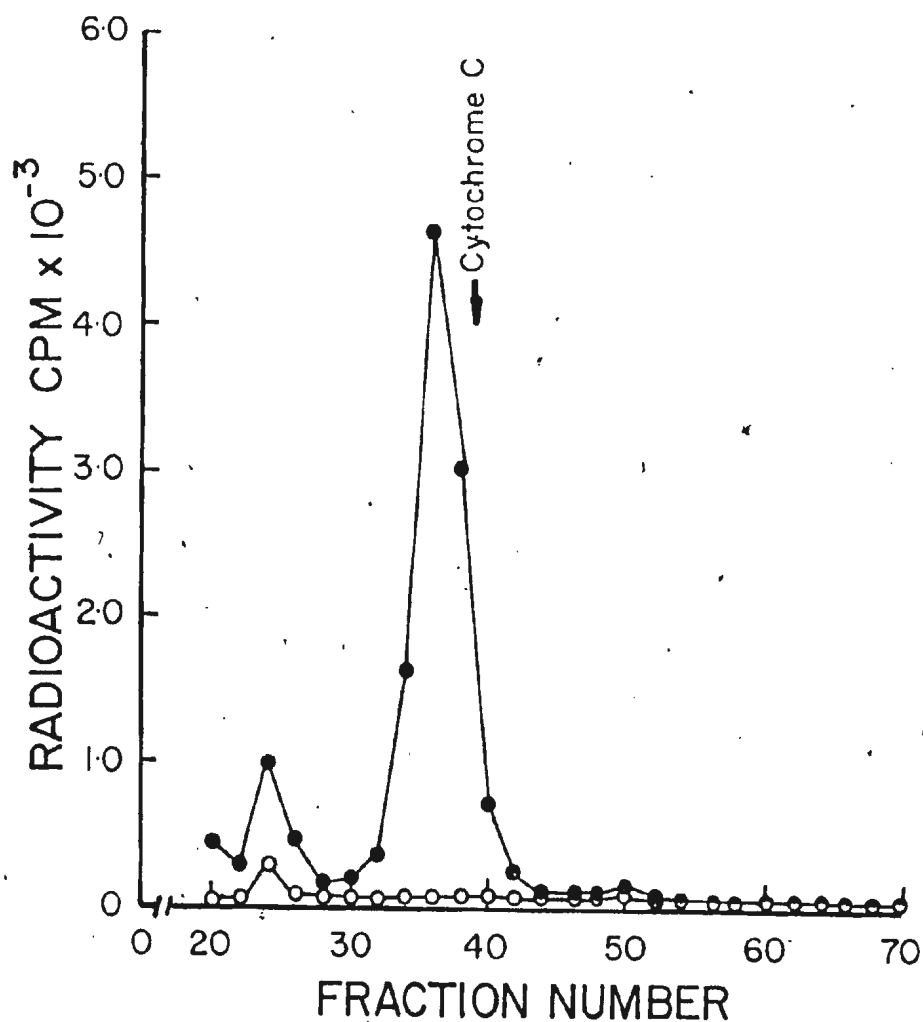


Figure 7 Chromatography of partially purified in vitro biosynthetic product on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M ammonium bicarbonate. Elution profile of a winter fish (February 19, 1978) ●—●. Elution profile of a summer fish (July 18, 1977) ○—○.

no obvious similarity with AFP¹²⁵ and its level of incorporation bears no resemblance with the expected seasonal appearance of AFP. The chemical nature of this material was not further investigated. We have therefore concentrated our studies on the 16,000 dalton component.

Liver slices obtained from one animal were incubated for 1, 2, 3, 4, and 5 hours respectively, at 15°C. The level of synthesis of the in vitro biosynthetic component in the acid-soluble fraction increased approximately linearly for 4 hr. at 15°C (Figure 8). Similarly, the level of synthesis of the acid-soluble proteins increased approximately linearly for 5 hr. at 15°C.

B. Temperature Studies

Liver slices obtained from one animal were incubated at 0°, 5°, 12°, and 15°C respectively for 4 hours. Maximal incorporation of radioactivity into the acid-soluble and acid-insoluble fractions was observed at an incubation temperature of 15°C (Figure 8). The level of synthesis in both the acid-soluble and acid-insoluble fractions increased in an approximately linear fashion with temperature.

C. Recovery of ³H-Antifreeze Protein Using 10% TCA

Approximately 84% of the radioactivity added to the liver slice incubation system in the form of ³H antifreeze protein was recovered from the liver homogenate (Table 2).

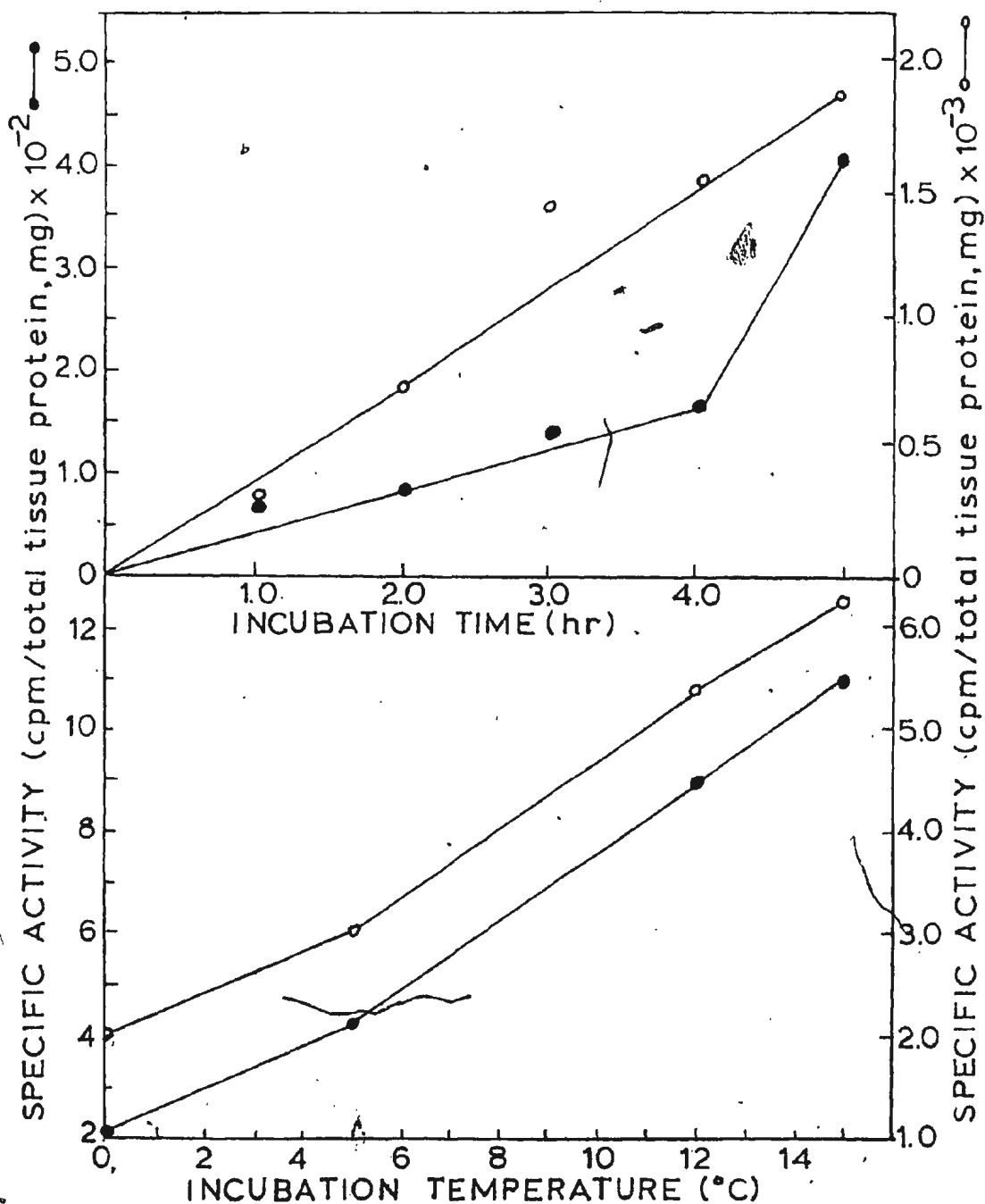


Figure 8 Effect of incubation time and incubation temperature on the *in vitro* incubation system.
 10% TCA-soluble fraction ●—●
 10% TCA-insoluble fraction ○—○

Table 2 Percentage Recovery of Antifreeze Protein
by the 10% Trichloroacetic Acid Procedure

Sample	Activity (net cpmx10 ⁻⁶)	% Recovery
H ³ Antifreeze Protein	4.56	100
Liver Homogenate	3.84	84.21
10% TCA Soluble Fraction	3.22	70.61
10% TCA Insoluble Fraction	.57	12.50

Of this percentage, approximately 71% was recovered in the 10% trichloroacetic acid-soluble fraction, while 13% was recovered in the 10% trichloroacetic acid-insoluble fraction.

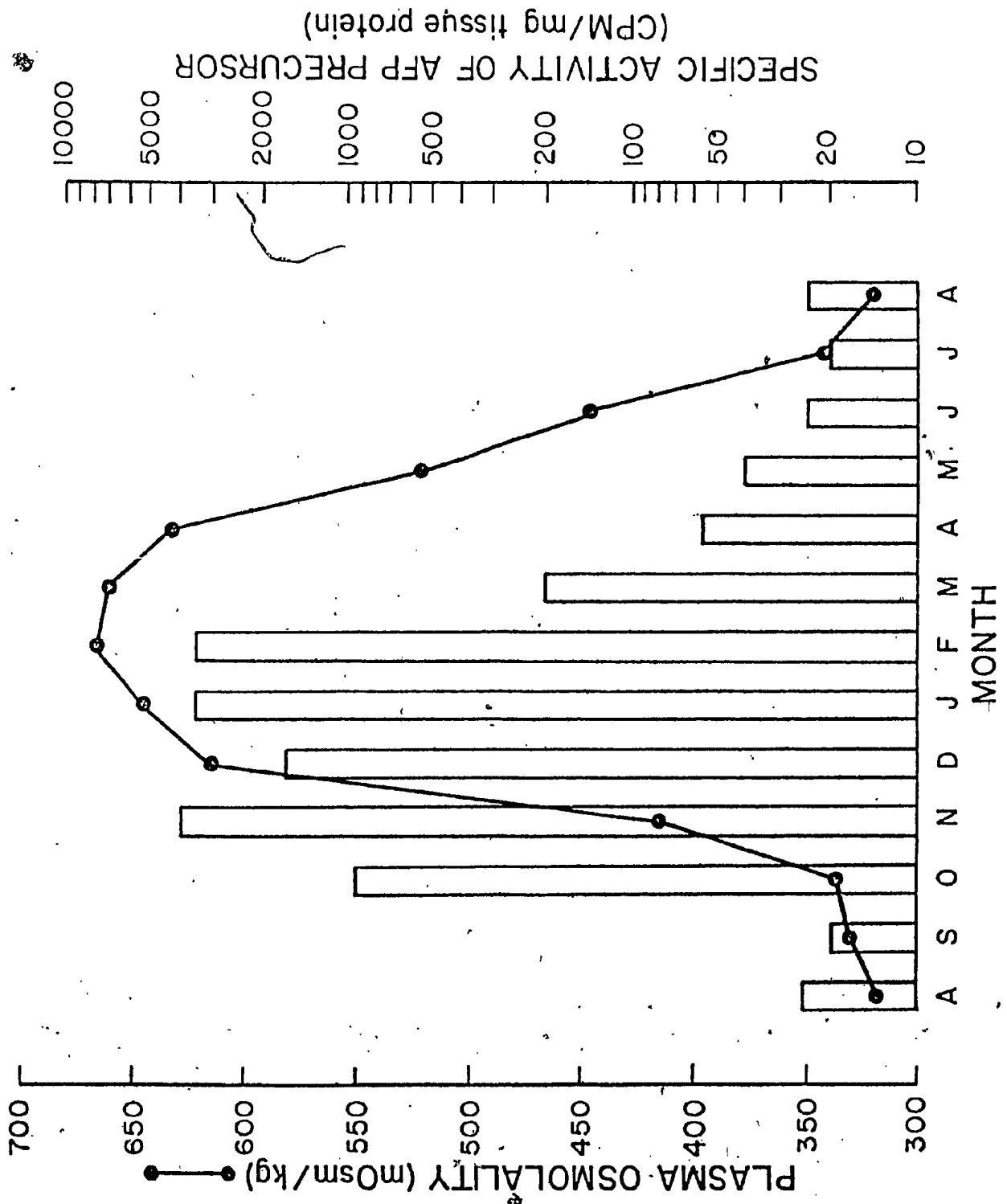
D. Seasonal Appearance of the *In Vitro* Biosynthetic Products

When the *in vitro* incorporation studies were conducted with flounder caught during the summer, no radioactivity was detected in the 16,000 component (Figure 7). This observation was consistent with the absence of the serum antifreeze protein in the summer, and indicated the absence of AFP mRNA activity in the summer. In order to correlate the *in vitro* biosynthetic activities with serum antifreeze levels, a more detailed analysis was carried out. *In vitro* incorporation studies were repeated at monthly intervals with at least two animals per incubation. As shown in Figure 9, the level of synthesis of the *in vitro* biosynthetic component correlates with the increased plasma osmolality in the winter, and slightly precedes the appearance of antifreeze protein in the serum. Maximal activity was observed in November through March. As the serum AFP level started to decline in April, the biosynthetic activity in the liver dropped off rapidly and remained minimal until November.

E. Pulse Chase Experiments

One possibility exists that the absence of a radioactive

Figure 9 The level of synthesis of the in vitro
biosynthetic component (May 1977-May 1978)



component corresponding to authentic AFP in the liver slice incubation study could be due to the slow rate of conversion of the presumptive precursor protein. The effect of a puromycin and cold alanine chase on the biosynthesis of the AFP were examined.

Sephadex G-75 chromatography of the TCA-soluble fraction from liver slice incubations conducted in the presence of excess cold alanine or puromycin resulted in elution profiles similar to that of the control liver slice incubation (Figure 10). After 4 hours of incubation there was no demonstrable conversion of the "large" in vitro biosynthetic component to the 10,000 MW antifreeze protein in the liver slice incubation system.

F. SDS Disc Gel and Urea Tris-Glycine Gel Electrophoresis

The in vitro biosynthetic product was analysed by SDS disc gel electrophoresis and 4 M urea tris-glycine pH 9.2 disc gel electrophoresis (Figure 11). A molecular weight of approximately 16,000 daltons was estimated from the SDS disc gel electrophoresis (Figure 12). Both SDS disc gel electrophoresis, and urea tris-glycine disc gel electrophoresis showed only one major radioactive component (Figure 11).

G. Tryptic Conversion

The in vitro biosynthetic product was further

Figure 10 The result of pulse chase experiments in the presence of cold alanine or puromycin. Chromatography of partially purified in vitro biosynthetic product on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M ammonium bicarbonate.

Elution profile of control ●——●

Elution profile of pulse chase in the presence of excess cold alanine ○-----○

Elution profile of pulse chase in the presence of puromycin ■——■

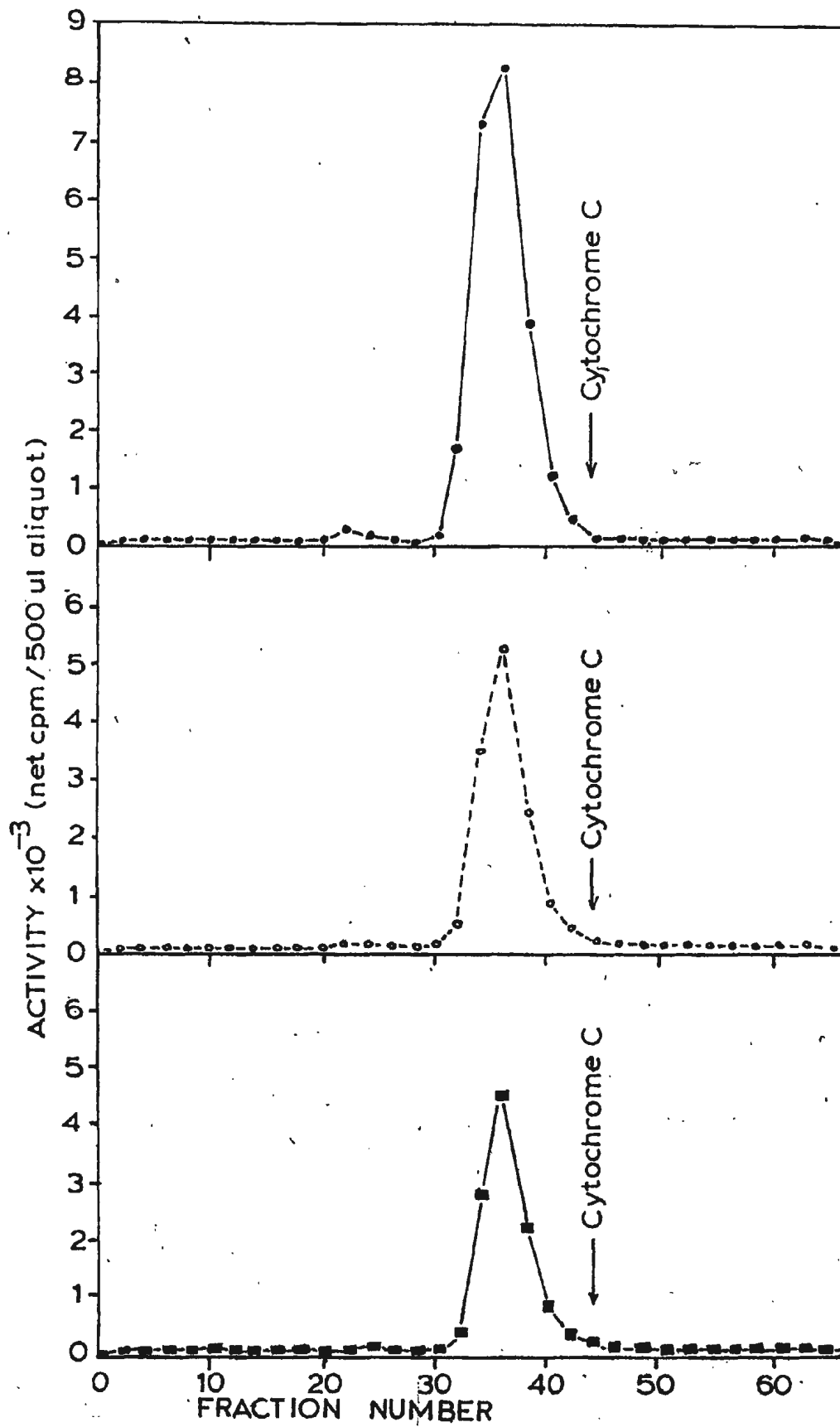
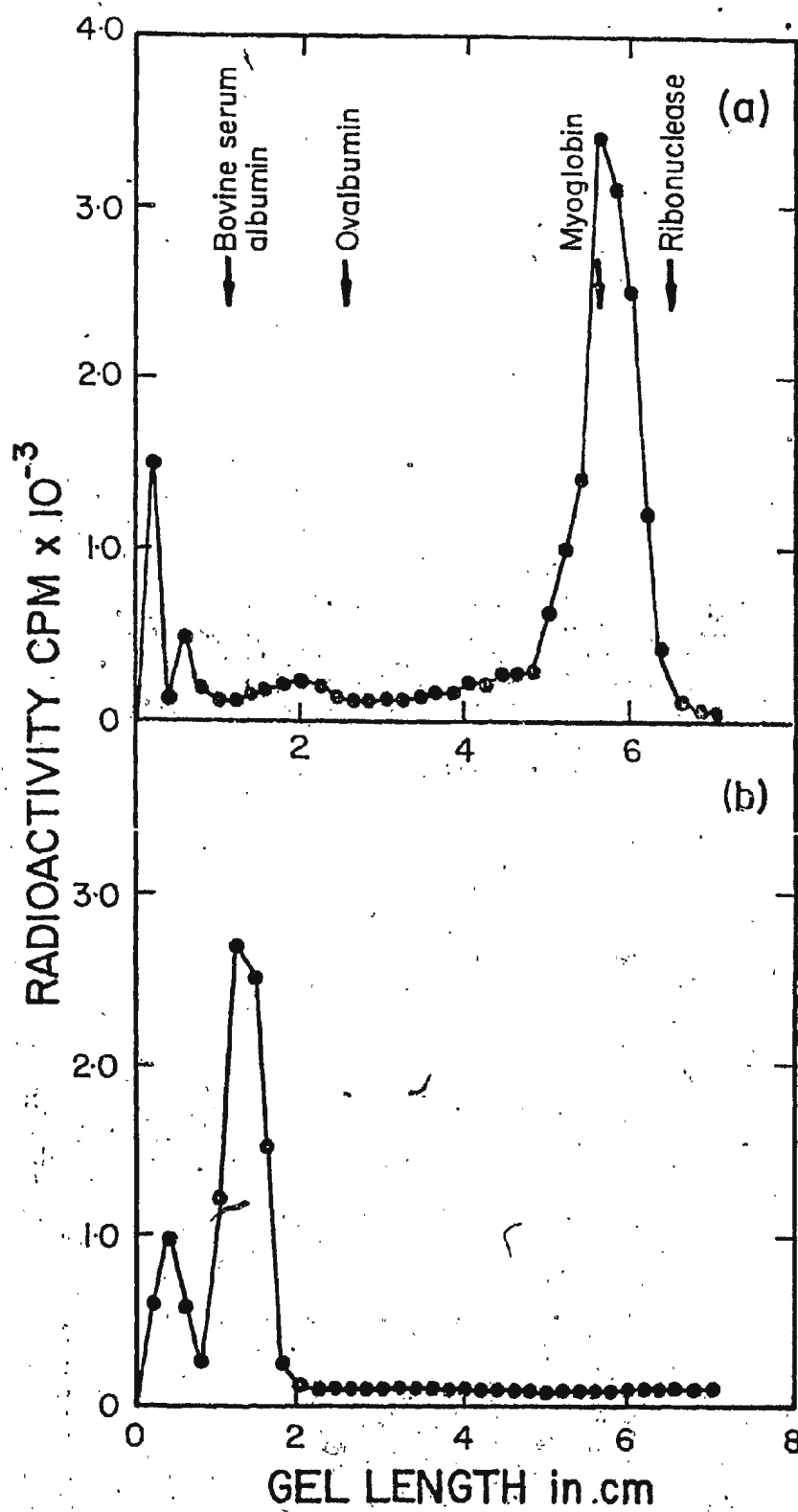


Figure 11 The characterization of the in vitro biosynthetic product by SDS disc gel electrophoresis (a) and 4 M urea tris-glycine pH 9.2 disc gel electrophoresis (b).



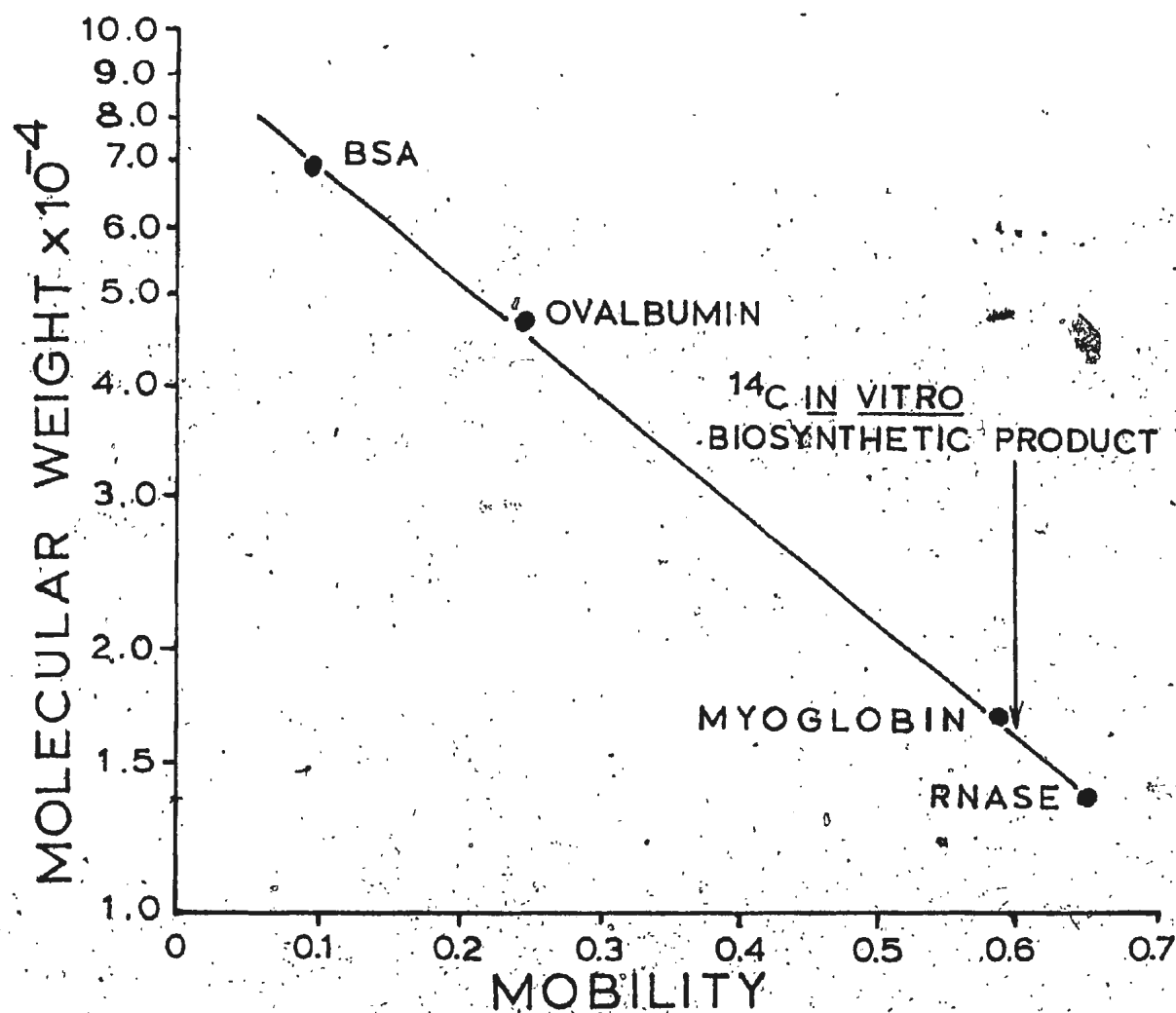


Figure 12. Molecular weight determination of the in vitro biosynthetic product by SDS gel electrophoresis.

analysed on a Sephadex G-75 column in 6 M urea, 0.1 M Tris, 0.5 M glycine, 50mM β -mercaptoethanol. One major radioactive component was observed (Figure 13). A molecular weight of approximately 16,000 daltons was estimated for the in vitro biosynthetic product from the urea Sephadex column (Figure 15). This agreed with the molecular weight determination from SDS disc gel electrophoresis.

Upon mild tryptic digestion the in vitro biosynthetic component could be converted to peptides of 10,000 daltons and 6,000 daltons on the urea Sephadex column (Figure 13). The elution position of the 10,000 MW component corresponded to that of the ^3H antifreeze protein on the urea Sephadex column (Figure 14). In addition, ^3H antifreeze protein was determined to have a molecular weight of approximately 10,000 daltons on the urea Sephadex column (Figure 15).

H. Separation on DEAE Ion Exchange

The partially purified in vitro biosynthetic product, obtained from the Sephadex G-75 column, was re-chromatographed on a DEAE cellulose column (0.9 x 12 cm). The resultant elution profile is illustrated in Figure 16. One minor component eluted from the column before the elution gradient of 0.01 M to 0.5 M NH_4HCO_3 was begun. Both components were subjected to amino acid analysis.

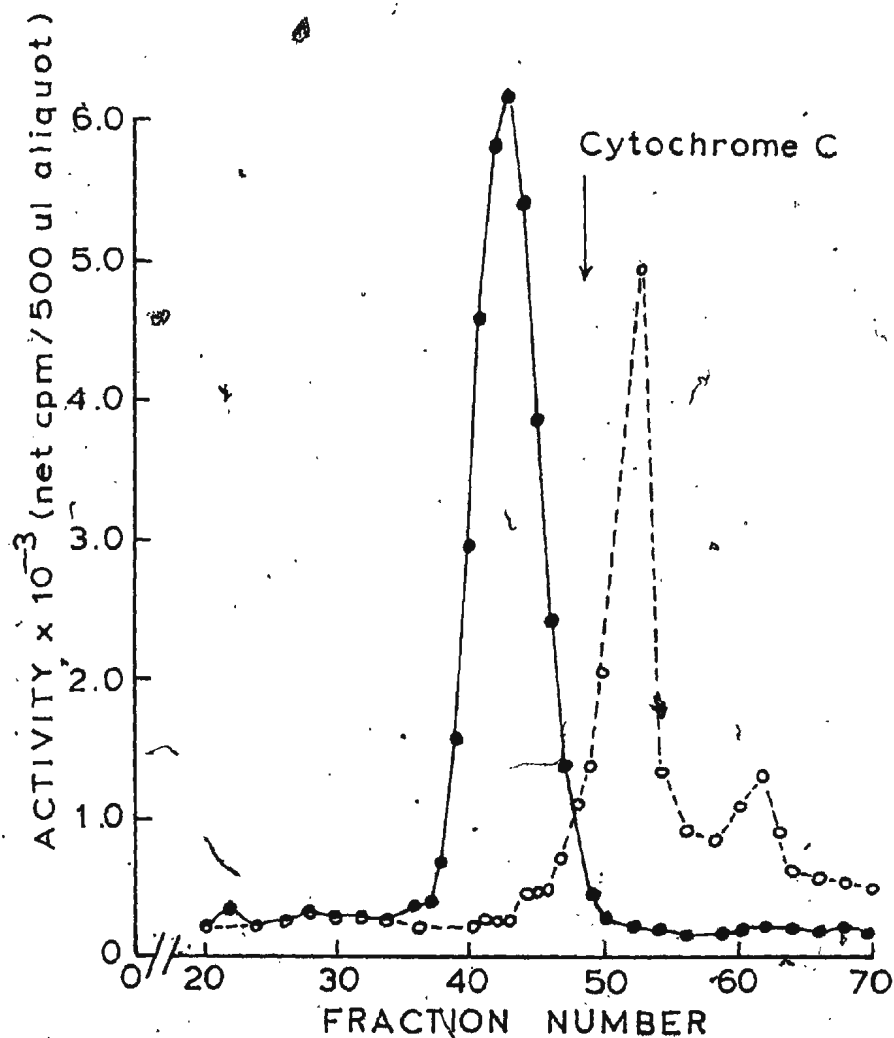


Figure 13

Elution profile for the *in vitro* biosynthetic product on a Sephadex G-75 column (1.6 x 86 cm) in 8 M urea, 0.1 M tris, 0.5 M glycine, 50mM β -mercaptoethanol —●—●—

Elution profile of tryptic digestion of *in vitro* biosynthetic product ○---○

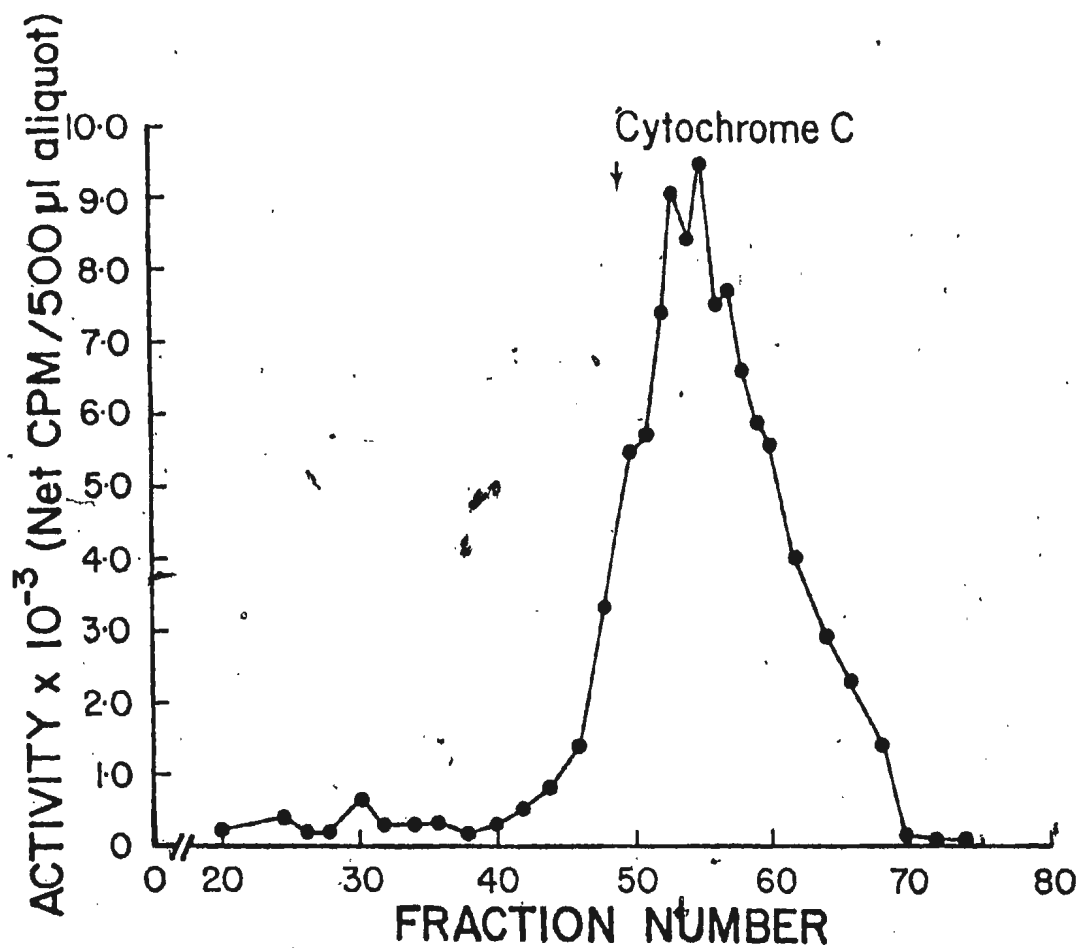


Figure 14 Elution profile of ^3H antifreeze protein on a Sephadex G-75 column (1.6 x 86 cm) in 8 M urea, 0.1 M tris, 0.5 M glycine, 50 mM β -mercaptoethanol.

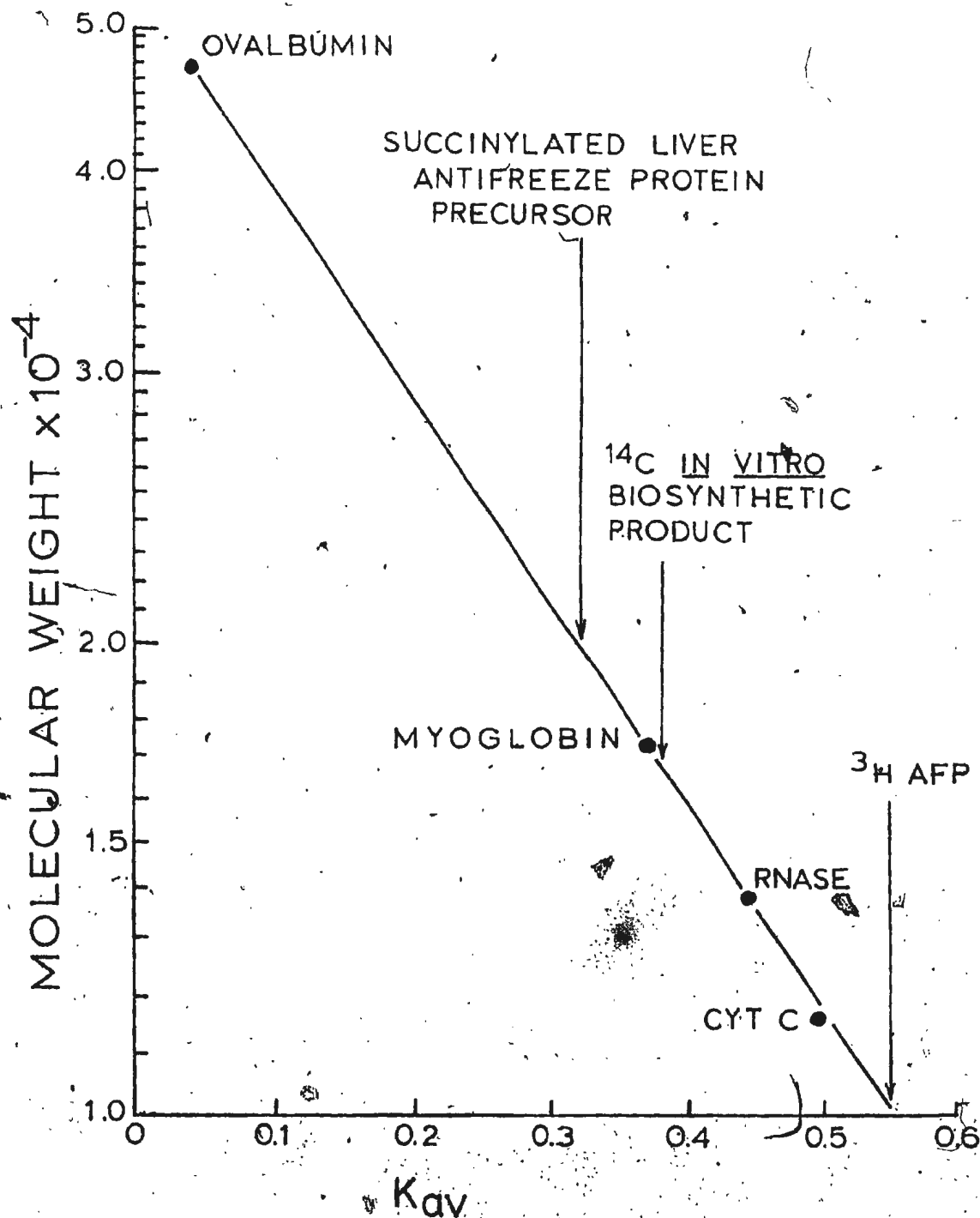


Figure 15 The calibration of the urea tris-glycine Sephadex G-75 column (1.6 x 86 cm) for molecular weight determination

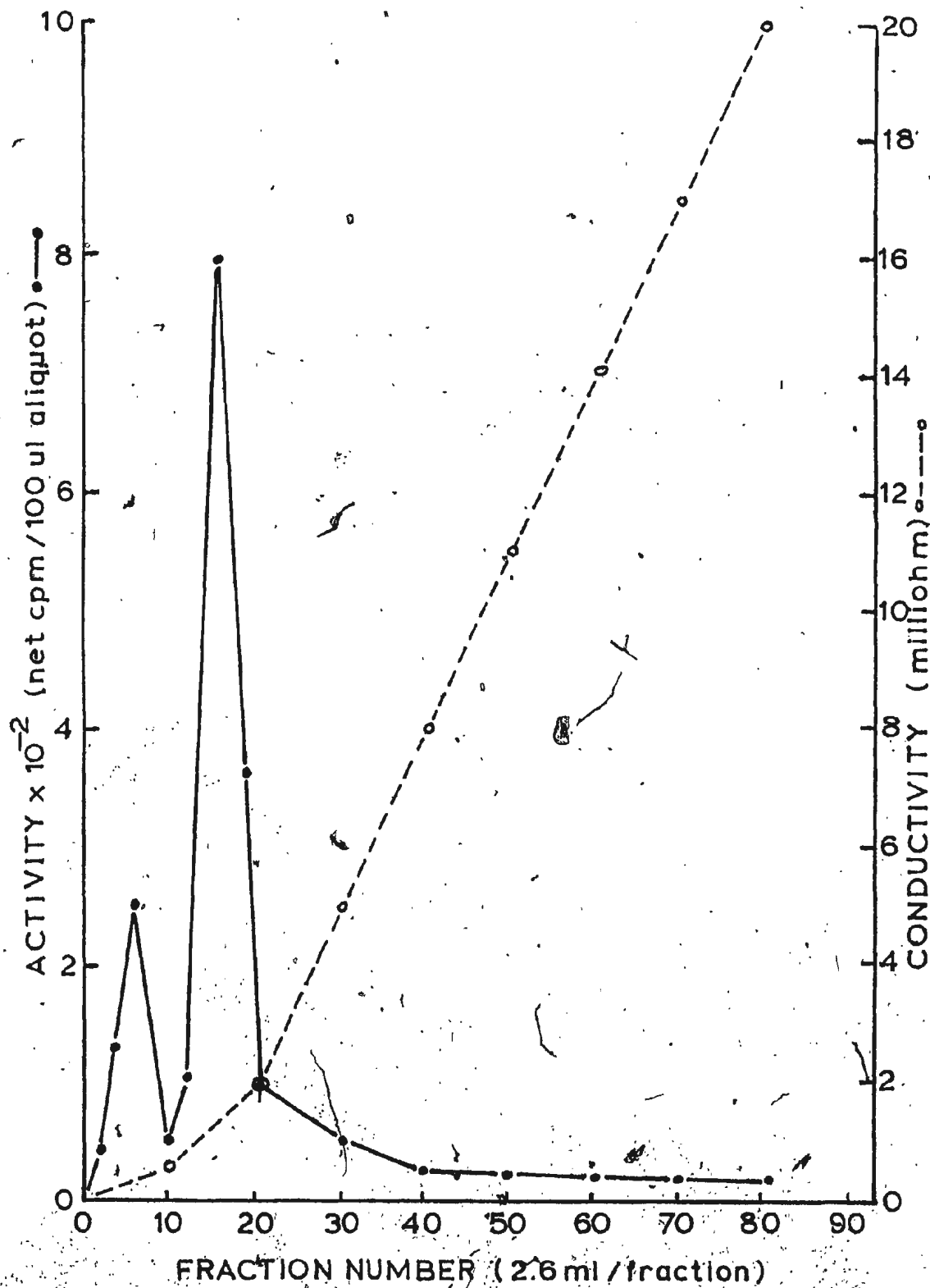


Figure 16 Chromatography of the in vitro biosynthetic product on DEAE ion-exchange

I. Amino Acid Analysis

The amino acid composition of the in vitro bio-synthetic products from DEAE cellulose chromatography is shown in Table 3. Both the minor and major peak showed very similar compositions, both containing an abundance of alanyl residues, indicating that these two components are structurally related (i.e. same size on G-75 chromatography, solubility in TCA, and similarity in amino acid composition). Due to the small amount of material available, we were unable to further characterize these two components. It appears likely that both components are structurally related to AFP, possibly representing the precursors and the modified products (such as phosphorylation or intermediate cleavage products).

In comparison with serum AFP, these in vitro bio-synthetic products are similar in their amino acid composition. However, as to be expected, the biosynthetic materials contain more amino acids, as well as some amino acids that are absent in flounder AFP.

Table 3 Amino Acid Analysis of the In Vitro Biosynthetic Products from DEAE Cellulose Chromatography

Amino Acids	Minor Peak		Major Peak		Serum AFP
	Yield in μ moles	No. of Residues	Yield in μ moles	No. of Residues	No. of Residues
Asp	51.2	15.6	59.6	15.6	14
Thr	35.4	10.6	43.9	11.6	12
Ser	19.2	5.8	28.8	7.6	4
Pro	40.3	12.2	49.0	12.9	0
Glu	9.4	2.8	11.6	3.1	2
Gly	13.8	4.8	20.1	5.2	0
Ala	330.0	100	380.2	100	76
Val	-	-	1.21	0.3	0
Met	0.8	0.3	-	-	0
Ileu	0.8	0.3	1.20	0.3	0
Leu	17.3	5.3	22.1	5.8	7
Tyr	0.4	0.1	0.51	0.2	0
Phe	0.4	0.1	0.51	0.2	0
Lys	19.6	5.9	22.1	5.8	4
Arg	12.8	3.8	12.7	3.4	2
Total Amino Acids		168		176	121
M. Wt.		14,249		15,235	10,450

DISCUSSION

Analysis of the 10% trichloroacetic acid-soluble material from the in vitro liver slice incubation system showed the occurrence of one major radioactive "antifreeze" component. This "large" antifreeze molecule was not present in liver slice incubations conducted on fish in the summer. Incubation of the in vitro liver slice system in the presence of either puromycin or excess cold alanine did not result in the conversion of the "large" in vitro biosynthetic product to the 10,000 antifreeze protein.

When the in vitro biosynthetic product was subjected to SDS disc gel electrophoresis and urea tris-glycine disc gel electrophoresis, one radioactive component of 16,000 daltons was observed. The molecular weight of the in vitro biosynthetic product was determined by chromatography on Sephadex G-75 in the presence of 8 M urea. Relative to standard protein markers, the in vitro biosynthetic product was observed to have a molecular weight of approximately 16,000 daltons, whereas H³ antifreeze protein was observed to have a molecular weight of 10,000 daltons. When subjected to tryptic digestion, the 16,000 MW component isolated from flounder liver could be cleaved to yield two peptides of approximately 10,000 and 6,000 daltons. Amino acid analysis showed close similarity between the in

vitro biosynthetic product and authentic antifreeze protein.

It is known that the freezing point depression of winter flounder blood serum is inversely proportional to the ambient sea water temperature. This increase in osmolality in the flounder's serum in the winter is due mainly to the presence of an antifreeze protein which is found in the serum only during the winter. The osmolality of flounder serum increases from 330 mOsm in October to 650 mOsm in January when the sea water temperature is -1°C . The level of synthesis of the in vitro biosynthetic component correlates with the increased plasma osmolality in the winter and slightly precedes the appearance of antifreeze protein in the serum. The level of synthesis of this component in the in vitro incubation system increased from 37 net cpm/mg protein on October 19 to values ranging from 574-1415 net cpm/mg protein on October 29. It was observed that individuals with the lowest level of serum antifreeze protein had the highest level of synthesis of the in vitro biosynthetic component. The physiological mechanism underlying this 15-40 fold stimulation in the synthesis of the liver "large" antifreeze protein is unknown. The level of synthesis appears to be at its peak during the month of November. It does not seem likely that flounder actively synthesize antifreeze

protein in vivo during the winter months when the seawater temperature is 0 to -1°C . It has been reported that winter flounder cease eating during the winter months (60). Antifreeze protein has been observed to have a half life of approximately 75-90 days in the circulation (61).

This observation provides support for the hypothesis that the bulk of antifreeze protein required for survival during the winter months is synthesized before the freezing water temperatures depress whole body metabolism. The ability to synthesize antifreeze protein until late February is suggestive of a biological fail-safe mechanism which could protect the flounder against a premature degradation of antifreeze protein as a result of sudden prolonged increases in water temperature in shallow coastal areas during the late autumn or early spring.

Undoubtedly, temperature and photoperiod play an important role in the control of the initiation and degradation of antifreeze protein. It has been suggested that an intact pituitary is necessary for the disappearance of antifreeze protein from the plasma of the winter flounder (47). Nevertheless, the underlying regulatory mechanism for the control of the synthesis and degradation of antifreeze protein remains to be determined.

In summary, the in vitro biosynthetic product has been observed to have the following characteristics:

(i) Solubility in 10% trichloroacetic acid (ii) Molecular weight of 15-16,000 daltons as determined on SDS disc gel electrophoresis and urea-Sephadex G-75 chromatography. (iii) Its seasonal appearance correlates with the seasonal increase of serum antifreeze^W protein levels. (iv) Its amino acid composition is similar to that of antifreeze protein. (v) Tryptic digestion of the in vitro biosynthetic product yielded a 10,000 dalton fragment, indicative of structural similarity with the authentic AFP.

Thus, it is tentatively concluded that the in vitro biosynthetic product represents the biosynthetic precursor for AFP.

SECTION II The Bulk Isolation of Liver Antifreeze Protein Precursor

Introduction

In an attempt to further characterize the in vitro biosynthetic product, the isolation procedure was scaled up using pooled livers from flounder processed in the winter months. The materials were subjected to amino acid analysis and peptide fingerprints.

Results

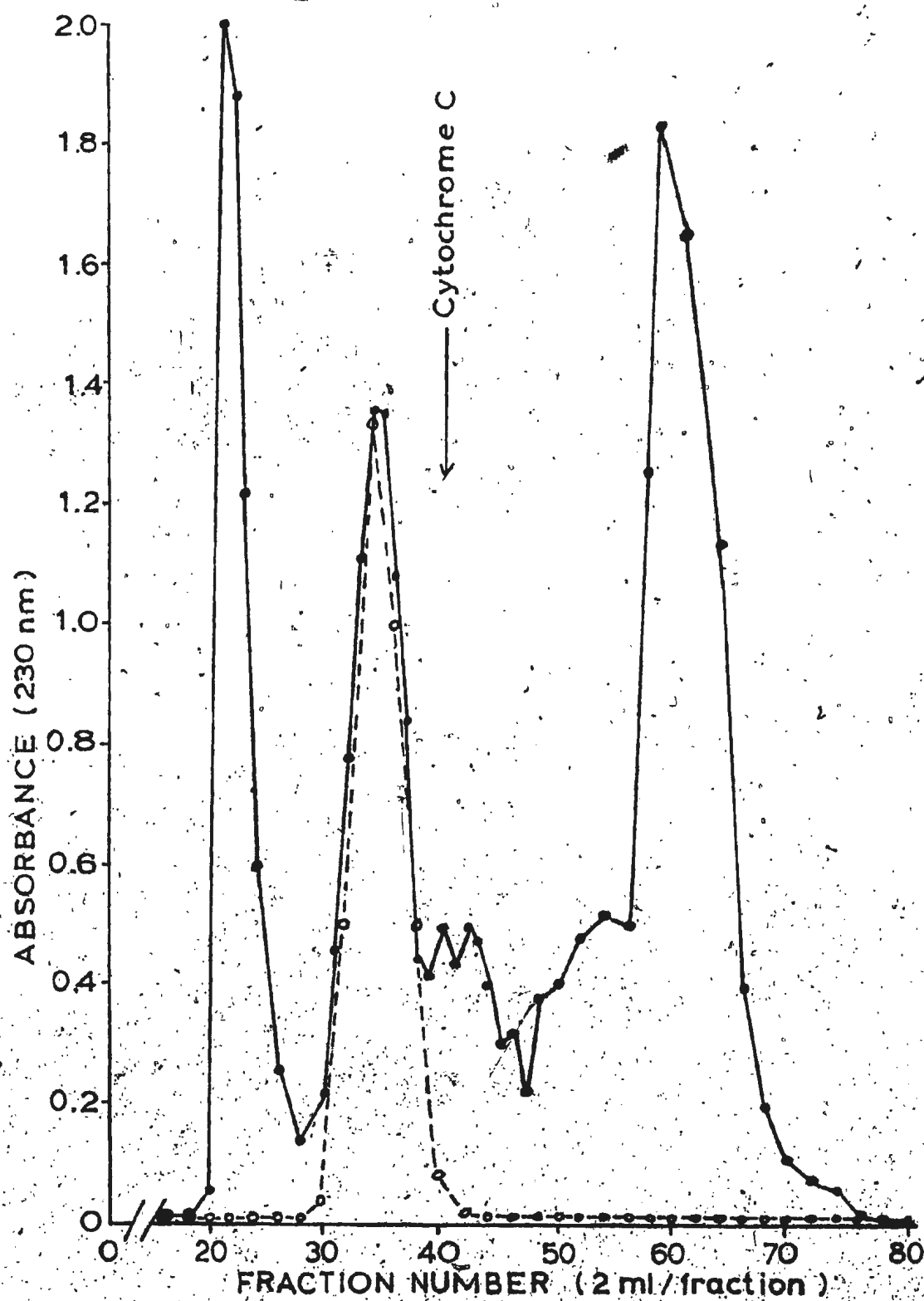
A. Elution Profile on a Sephadex G-75 Column

The previously dialysed and lyophilized acid-soluble material from flounder liver was chromatographed on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M NH_4HCO_3 (Figure 17). The material which eluted before the cytochrome C marker, and which corresponded with the elution position of the in vitro biosynthetic product (fractions 30-38) was pooled, lyophilized, and re-chromatographed on the Sephadex G-75 column (1.6 x 86 cm) in 0.05 M NH_4HCO_3 . The resultant elution profile (Figure 17) showed the presence of one component eluting before the cytochrome C marker.

B. Succinylation and Molecular Weight Estimation

Succinylated flounder liver antifreeze protein precursor was chromatographed on a Sephadex G-75 column (1.6 x 86 cm) in 8 M urea, 0.1 M Tris, 0.5 M glycine, 50 mM β -mercaptoethanol

Figure 17. Isolation of flounder liver antifreeze protein precursor. Chromatography of 10% trichloroacetic acid-soluble fraction from flounder liver on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M ammonium bicarbonate —•—. Elution profile of rechromatographed flounder liver antifreeze protein precursor on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M ammonium bicarbonate ○-----○.



(Figure 18).

The succinylated flounder liver antifreeze protein was determined to have a molecular weight of approximately 19,500 daltons (Figure 15). Subtraction of the contribution of 8 succinic acids added to the protein (7 lysine + 1 N-terminal NH_2) results in a molecular weight of approximately 18,780 daltons for the flounder liver antifreeze protein.

C. Disc Gel Electrophoresis

Flounder liver antifreeze protein precursor was subjected to 4 M urea tris-glycine pH 9.2 disc gel electrophoresis and visualized under a mineral light ultraviolet source after staining with anilinonaphthalene sulfonate (Figure 19). One major band was observed, followed by two minor trailing components.

D. Amino Acid Analysis

The amino acid composition of the liver antifreeze protein precursor is shown in Table 4. The composition of the serum "large" antifreeze protein and antifreeze protein is included for comparison. The amino acid compositions are similar, notably the abundance of alanine in the composition. Based on these compositions, the liver antifreeze protein precursor has a molecular weight and amino acid composition similar, if not identical to the serum large antifreeze protein. However, at present, we cannot conclude that they are identical. It is possible that post-

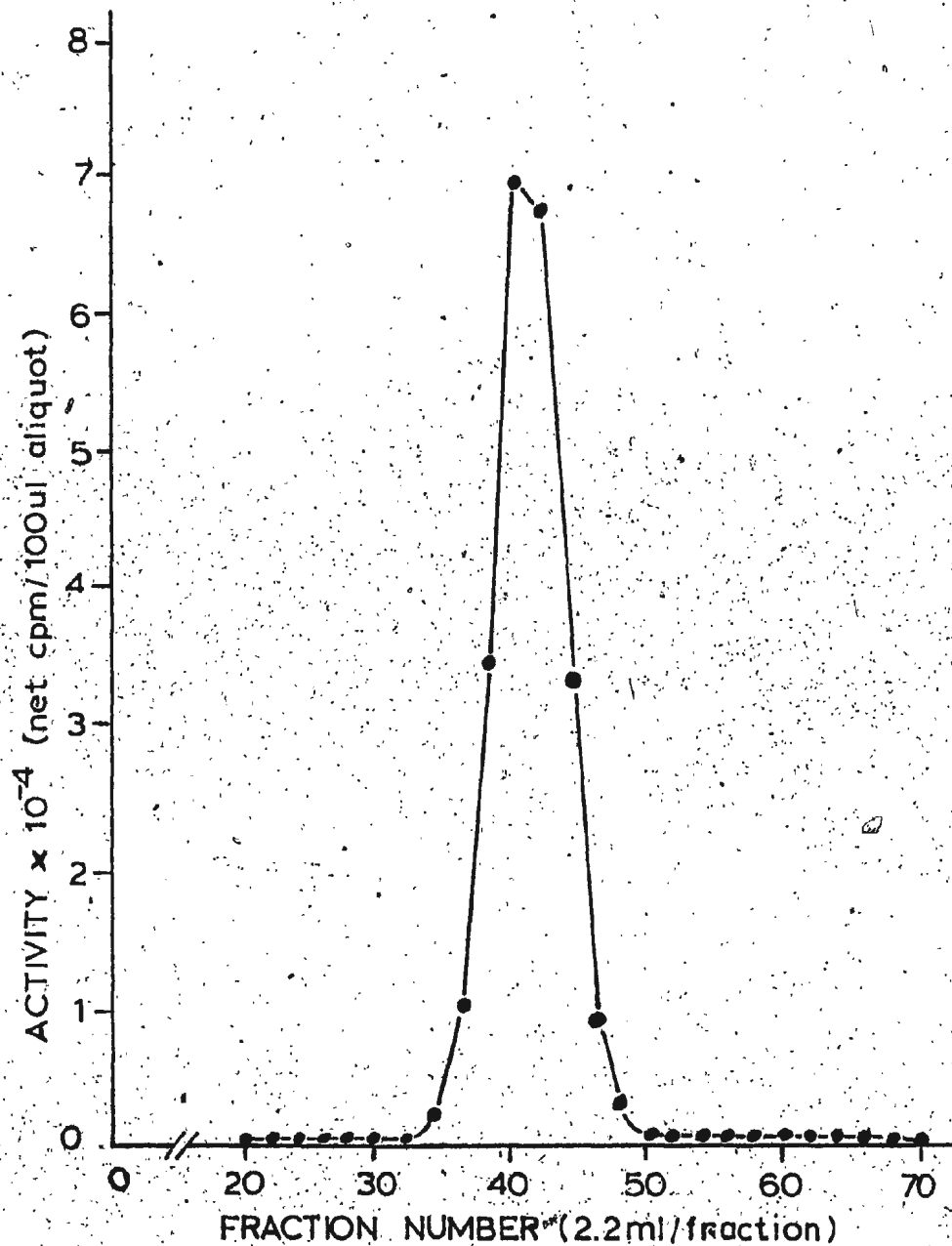


Figure 18 Elution profile of succinylated liver anti-freeze protein precursor on a Sephadex G-75 column (1.6 x 86 cm) in 8 M urea, 0.1 M tris, 0.5 M glycine, 50 mM β -mercaptoethanol.





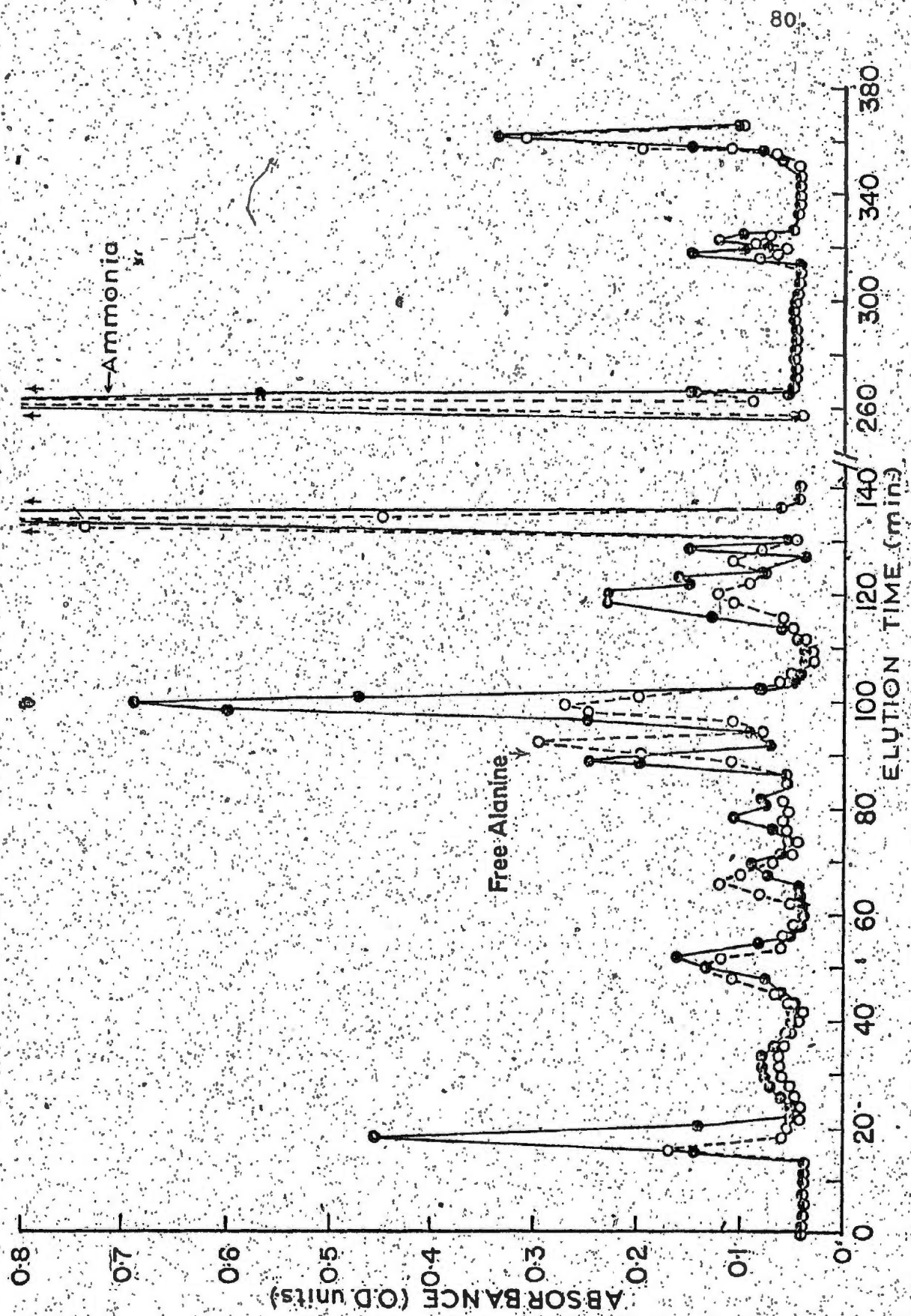
Figure 19 Electrophoretic mobility pattern of flounder liver antifreeze protein precursor in 4 M urea tris-glycine pH 9.2 disc gel electrophoresis as visualized under a mineral light ultraviolet source after staining with anilinonaphthalene sulfonate  Marker dye 

Table 4 Amino Acid Composition of Flounder Liver
Antifreeze Protein Precursor

Liver Antifreeze Protein Precursor				
Amino Acids	Yield in μ moles	No. of Residues	No. of Residues in Serum "Large" Antifreeze Protein *	No. of Residues in Antifreeze Protein *
Asp	.78	17	18.5	14
Thr	.58	13	12	12
Ser	.27	6.1	6	4
Pro	.67	14	10	0
Glu	.19	4	5.5	2
Gly	.16	3.6	2.2	0
Ala	4.43	100	100	76
Val	.04	1	2.1	0
Met	.03	0.68	0	0
Ileu	.03	0.68	0	0
Leu	.29	6.5	7.1	7
Tyr	.01	Low	1.0	0
Phe	.01	Low	1.0	0
Lys	.34	7	5.0	4
Arg	.17	3.2	3.4	2
Total Amino Acids		179	171	121
M.Wt.		15,712	15,500	10,457

* Hew et al., submitted

Figure 20 Peptide mapping of the liver antifreeze protein precursor O--O and serum antifreeze protein with thermolysin.



translational modification might be involved in the secretion and transport of the AFP precursor from the liver to the serum.

E. Peptide Finger Prints

The liver antifreeze protein precursor and flounder antifreeze protein were digested with thermolysin and analysed using a single-column physiological fluid analysis with W-2 resin on a Beckman 121 amino acid analyzer. The resultant peptide maps are shown in Figure 20. The elution profile of the liver antifreeze protein precursor was identical with that obtained with the serum antifreeze protein, thus illustrating their close structural similarity.

DISCUSSION

The liver antifreeze protein precursor was isolated from pooled liver preparations. The molecular weight of this component, as determined from chromatography of the succinylated liver antifreeze protein precursor, and from amino acid analysis, was approximately 16-18,000 daltons. Disc gel electrophoresis (Figure 19) of the material showed one major component. As judged from the amino acid analysis, the biosynthetic precursor of AFP must represent this major component. Otherwise, the amino acid analysis would not show any close similarity with the flounder AFP. This is further confirmed by the results of peptide finger prints.

Though the liver biosynthetic precursor has amino acid composition and molecular weight similar to that of the serum "large" antifreeze protein, more extensive studies have not been done to establish their identity. It is possible that minor differences such as post-translational modifications exist between these two components. The tentative scheme for the biosynthesis and secretion of AFP might be represented as follows:

large biosynthetic precursor	→	large AFP	→	AFP
M.Wt. 16-18,000		M.Wt. 16,000		M.Wt. 10,000
(synthesis in liver)		(occurs in serum)		(occurs in serum)

SECTION III The Production of Specific Anti-Antifreeze Protein Antibodies

Introduction

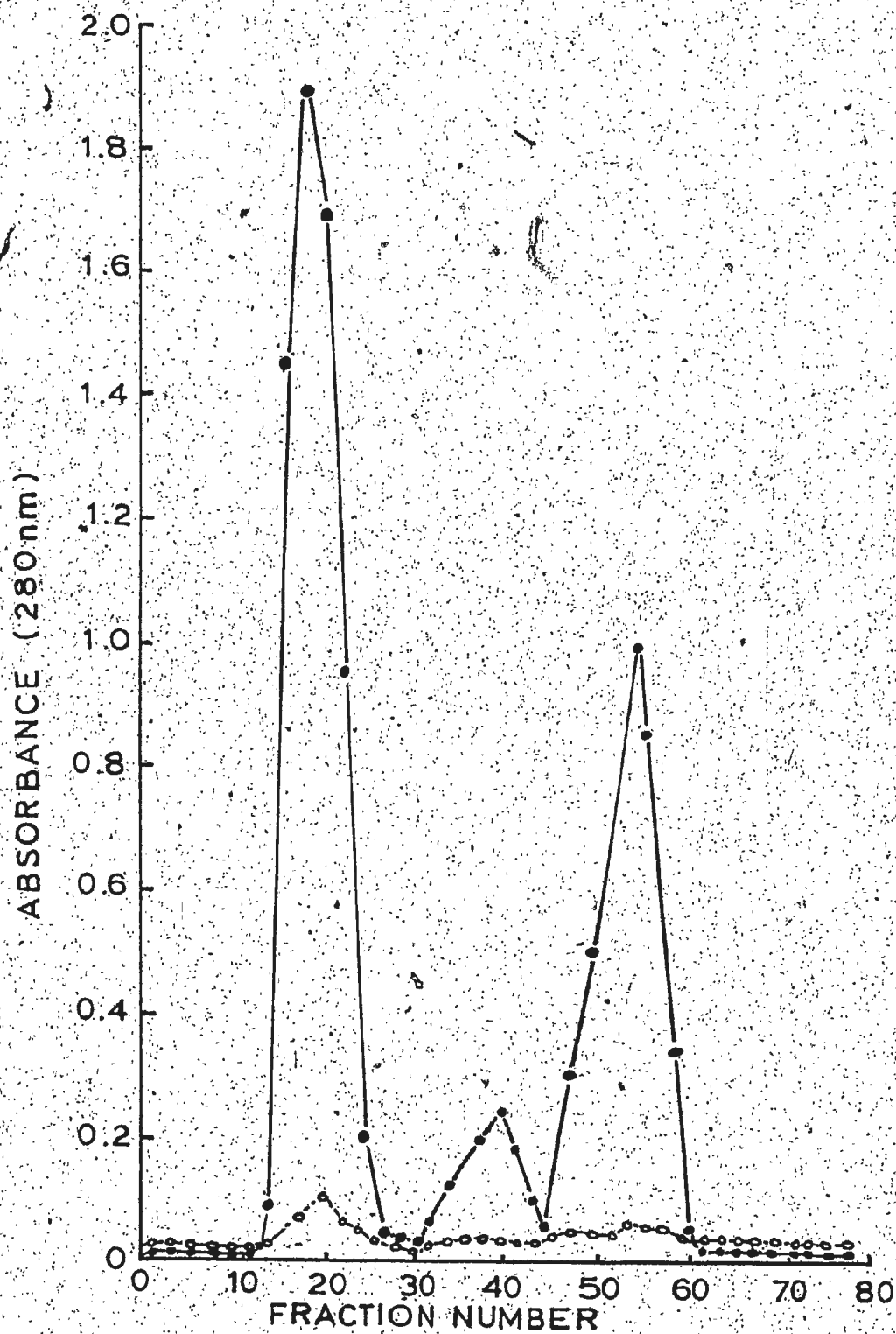
The availability of specific anti-antifreeze protein antibodies would be extremely useful in the study of the biosynthesis and regulation of the AFP. In these studies, two different immunological procedures were used: (i) the immunization of AFP in rabbit and guinea pig (ii) the immunization of AFP-albumin conjugate in rabbit and guinea pig.

Results

The gel filtration profile of antifreeze protein reacted with DFDNB and coupled to albumin is shown in Figure 21. The antifreeze-albumin conjugate elutes in the void volume of the column (fractions 15-25), followed by albumin (fractions 34-45), and antifreeze protein (fractions 50-60). The residual precipitate obtained after the antifreeze-dinitrophenyl-albumin solution was dialysed contained a minimal amount of albumin-conjugate.

Information concerning the chemistry of the antifreeze conjugate was obtained by amino acid analysis. The amino acid composition of a preparation of antifreeze-albumin conjugate is presented in column 3 of Table 5. When the number of residues contributed to the conjugate by albumin (column 2) are subtracted from the conjugate, the resultant

Figure 21 Elution profile of antifreeze protein coupled
to albumin on a Sephadex G-100 column (1.6 x
83 cm) in 0.05 M NH_4HCO_3 . Supernatant fraction
●—● . . . Precipitate fraction ○—○



the resultant amino acid composition (column 4), homologous with that of antifreeze protein (column 5), is suggestive of a 1:1 coupling ratio between antifreeze protein and albumin.

Blood sera was obtained from rabbits and guinea pigs immunized with 10,000 MW serum antifreeze protein. When subjected to an ouchterlony test, neither the control serum nor the immunized serum yielded precipitin bands on the ouchterlony plates. In an attempt to further characterize immunized serum for the presence of an antifreeze protein antibody, control and immunized sera were equilibrated with ^3H antifreeze protein and chromatographed on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M NH_4HCO_3 . As can be seen in Figure 22, there was no significant difference in the elution position of the ^3H antifreeze protein between control and immunized serum. The presence of an antifreeze protein antibody in the immunized serum would have resulted in the formation of a ^3H antifreeze protein-antibody complex. Such a complex would be expected to elute from the column before ^3H antifreeze protein.

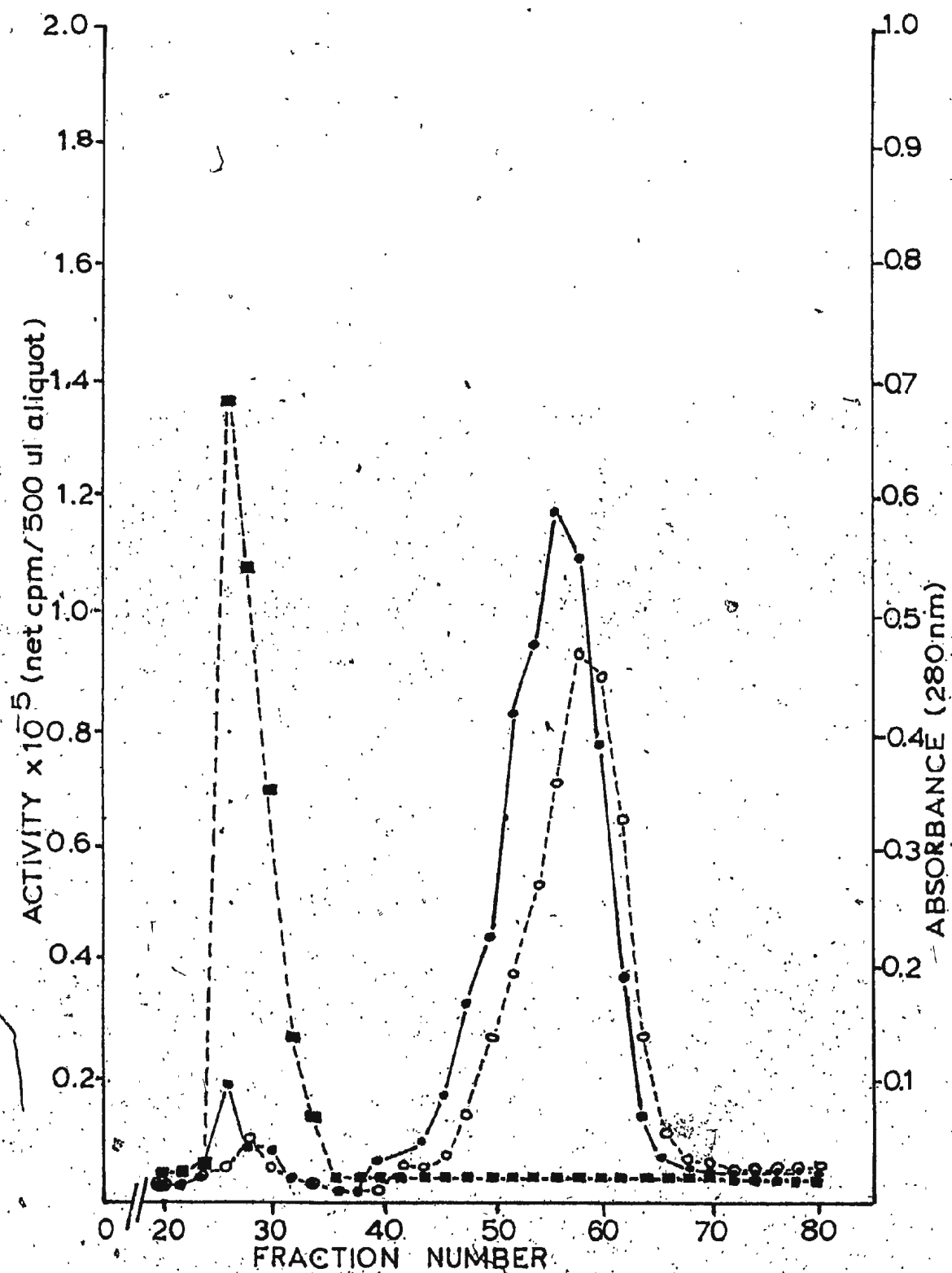
Blood sera obtained from rabbits and guinea pigs immunized with the purified "large" serum antifreeze protein (S1A) was also subjected to characterization by ouchterlony testing and immunoprecipitation utilizing ^3H antifreeze protein. An antifreeze protein antibody could not be detected utilizing these techniques.

Table 5 Amino Acid Compositions of Albumin, Antifreeze-DNP-Albumin Conjugate, and Antifreeze protein.

Amino acids	Albumin (No. of Residues) ^a		Conjugate (No. of Residues)	Conjugate-Albumin (No. of residues)	Antifreeze protein (No. of Residues)
Ala	49	(46)	110	61	76
Arg	23	(23)	24	1	2
Asp	61	(38)	74	13	14
Cys	28	(35)	-	-	-
Glu	80	(59)	87	7	2
Gly	18	(15)	20	2	-
His	16	(17)	15	1	-
Ile	9	(14)	9	1	-
Leu	60	(61)	65	5	7
Lys	58	(59)	47	1	4
Met	5	(4)	5	1	1
Phe	26	(26)	26	1	1
Pro	29	(28)	28	1	1
Ser	31	(28)	35	4	4
Thr	33	(34)	45	12	12
Trp	2	(2)	1	1	1
Tyr	20	(19)	19	1	1
Val	22	(36)	22	1	1

^aThe accepted amino acid composition of bovine serum albumin (59) is given in parentheses.

Figure 22 Characterization of rabbit serum utilizing ^3H antifreeze protein on a Sephadex G-75 column (1.6 x 86 cm) in 0.05 M ammonium bicarbonate.
A₂₈₀ ■---■ Activity of control serum ●---●
Activity of immunized serum ○---○



Analysis of blood sera obtained from animals immunized with the antifreeze-albumin conjugate indicated that antibodies were produced against the DFDNB groups rather than against the proteins. This conclusion was based on the observation that serum from animals immunized with the conjugate did not produce precipitin bands against antifreeze protein, nor albumin, but rather against the conjugate itself. Further characterization of this sera by immunoprecipitation utilizing ^3H antifreeze protein resulted in no shift of the elution position of the radioactivity.

Discussion

The absence of precipitin bands in the ouchterlony test of the antigen and sera could imply: (i) the absence of specific antibodies (ii) the presence of monovalent antigen (i.e. non-precipitating antigen). In order to eliminate the latter possibility, a study was conducted of the binding of ^3H AFP with the immunized sera. As indicated in Figure 22 (p 87), it is obvious that no specific binding occurs. It is concluded that the immunization with the antigen conjugate was not successful. The antifreeze protein, being of small molecular weight and relatively few determinants (only 8 amino acids), appears to be a poor antigen. A similar situation has also been encountered with the Antarctic glycoprotein (R.E. Feeney,

personal communication). Other approaches will be required to overcome these difficulties.

SECTION IV Biosynthesis of Antifreeze Protein: Polysomal Profile Analysis

Introduction

It is generally believed that secretory proteins are synthesized on membrane-bound polysomes, while intracellular proteins are associated with free polysomes (62). In order to further understand the function and secretion of the AFP, an investigation was conducted to determine the distribution of alanine on the free and bound polysomes.

Results

Polyribosomes were isolated from flounder liver slices incubated in the presence of ^{14}C alanine. The distribution of radioactivity in the total polysomes of flounder liver cells is illustrated in Figure 23.

The distribution of radioactivity in the membrane-bound and free polysomes is illustrated in Figure 24. Membrane-bound polysomes isolated from the in vitro incubation system had a specific activity in the range of 1069-1376 net cpm/ A_{260} unit, while free polysomes had a specific activity in the range of 780-820 net cpm/ A_{260} unit (based on the results of 4 separate experiments).

Discussion

There was 1.5 - 2 fold more incorporation of radioactive alanine in the bound polysome fraction as compared to that in the free polysome fraction. This observation

Figure 23 Distribution of radioactivity in total polyribosomes of flounder liver cells. Optical density at 260 nm —•—•— Radioactivity
o---o

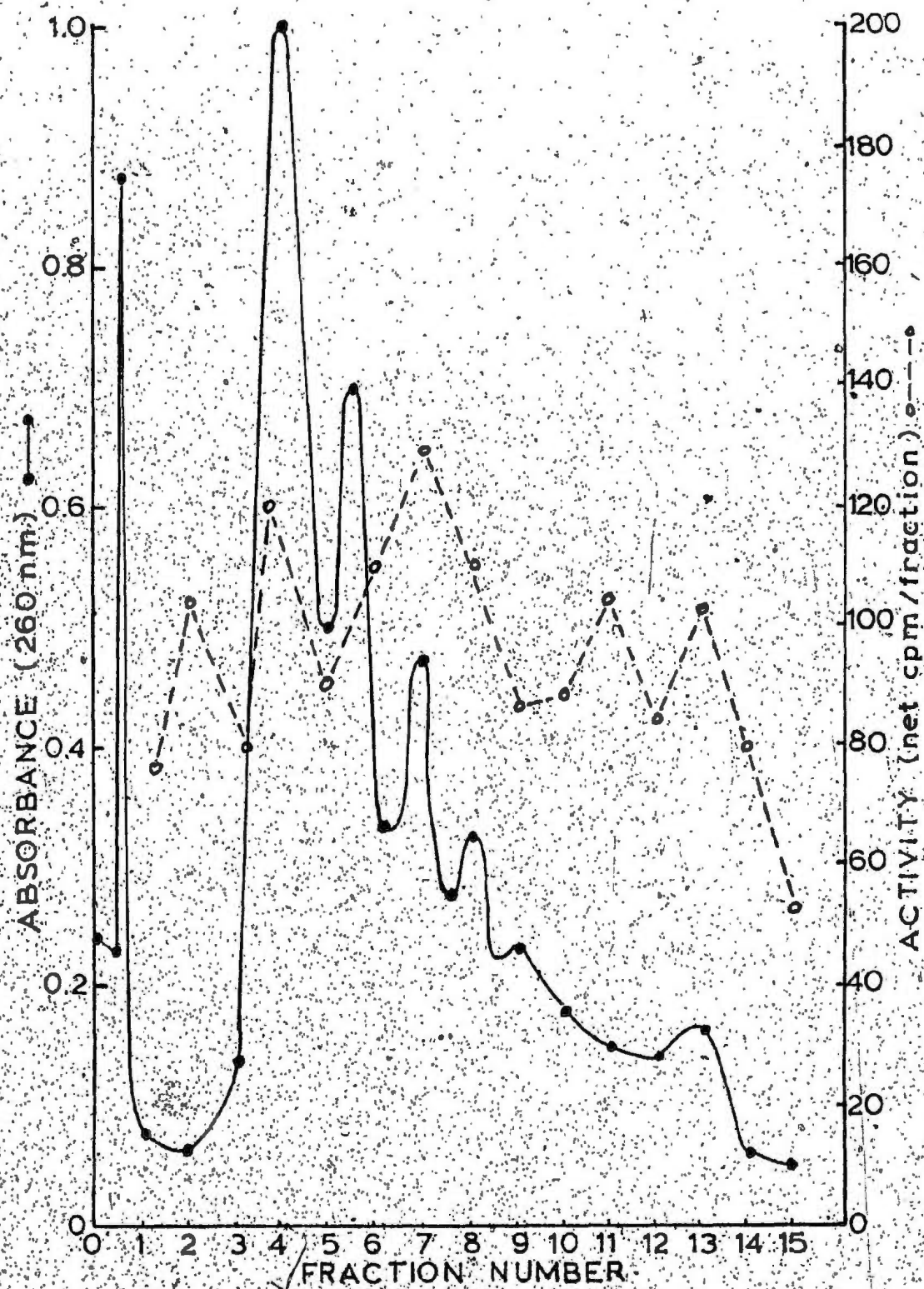
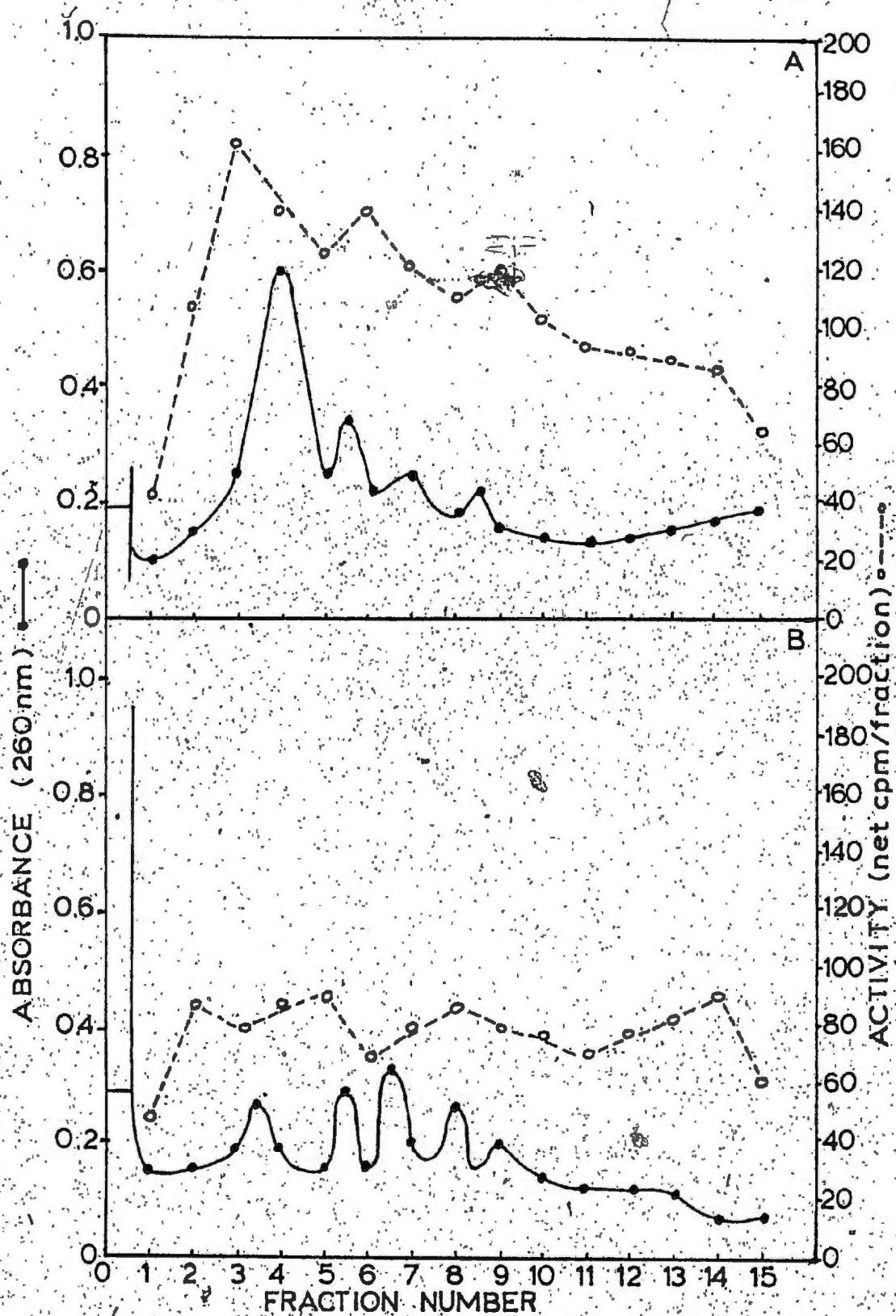


Figure 24 Distribution of radioactivity in membrane-bound and free polyribosomes of flounder liver cells.
A. Membrane-bound polysomes
B. Free polysomes
Optical density at 260 nm —•• Radioactivity
---••



suggests that antifreeze protein may be synthesized on the bound polysome fraction, a notion consistent with its secretory function. More conclusive identification of the polysome fraction which is active in antifreeze protein synthesis could be achieved using immunological precipitation techniques.

The successful isolation of the intact polyribosomes (including both the free and bound polysomes) will be extremely useful for the identification of the AFP-synthesizing polysomes using the immunological procedure and for the eventual isolation of the AFP mRNA.

SUMMARY

Recently it has become apparent that many proteins are synthesized as larger precursors and that these are ultimately converted to physiologically active forms by the selective enzymatic cleavage of peptide bonds. In this report evidence is presented for the existence of a 16,000 MW component in the liver of winter flounder whose level of synthesis corresponded closely with the appearance of the 10,000 MW antifreeze protein in the serum. Experimental results suggest that the in vitro biosynthetic product is related to the serum "large" antifreeze protein and appears to represent the same polypeptide. It appears that flounder liver synthesizes a "large" antifreeze protein which is secreted into the circulation. This 16,000 MW antifreeze protein is then cleaved to yield the 10,000 MW serum antifreeze protein.

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