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KINETICS OF SYNTHESIS AND SECRETION OF PLASMA PROTEINS IN MARINE TELEOSTS

by

ALAN PAUL HUDSON

A dissertation submitted to the graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Alan Hudson

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

December 16, 1977
Date

A. Haschemeyer
Chairman of Examining Committee
Prof. A. Haschemeyer

December 20, 1977
Date

Louis G. Moriber
Executive Officer
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Theodore Peters, Jr. — Prof. T. Peters, Jr. — The Mary Imogene Bassett Hospital, Institution

Arthur L. DeVries — Prof. A. DeVries — University of Illinois, Institution

The City University of New York

ABSTRACT

The synthesis of liver proteins, plasma proteins, fibrinogen, and the antifreeze glycoprotein has been studied *in vivo* at several temperatures in toadfish, Antarctic cod, and various species of the genus *Trematomus*. A kinetic model is developed which permits analysis of incorporation data in terms of three types of reaction: (1) synthesis and degradation; (2) vectorial migration; and (3) collection in secretory vesicles. In toadfish acclimated to $7^{o}C$ and assayed at the same temperature, secretion of plasma proteins follows a sigmoidal time course. This has been described kinetically in terms of the following times: 28 min for synthesis of an average polypeptide chain; 5 hr for vectorial migration; 6 hr of residence in collecting vesicles. Rate constants for concentration-dependent flux and for intrahepatic protein degradation are also derived. Comparison with data at $20^{o}C$ in toadfish indicates a large direct temperature effect on both protein synthesis and secretion in this temperate eurythermal fish. In contrast, results in the Antarctic cod show a highly effective secretory system operating at extremely low temperatures. These results are evaluated in relation to the concept of cold adaptation. The kinetics of synthesis and secretion of the antifreeze glycoprotein have been compared with data for other plasma proteins. The results suggest that this protein utilizes a different secretory pathway and may be synthesized at a site other than liver.

DEDICATION

This dissertation is dedicated to my father, Eldon N. Hudson. Without his encouragement, this work might never have been undertaken; without his patience and support, it certainly would never have been finished.

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I. INTRODUCTION

A. Temperature and Life

The temperature at the surface of the earth is, on the average, 10^{o} to 15^{o}* (Blum, 1968), but ranges from about -2^{o} to $+30^{o}$ in the open ocean and from -88^{o} to $+85^{o}$ in air (Bunt, 1967; Prosser, 1973). From the frigid polar regions to hot-springs organisms have successfully adapted to life within the limitations imposed upon them by their environments, and they survive and reproduce even under the most stringent of conditions. Bacteria living in deep oil well brines function at temperatures approaching 100^{o} (Johnson et al., 1974). Other species of bacteria, algae and fungi show optimal growth at temperatures of 75^{o} or higher (Farrell and Rose, 1967). At the other extreme the Arctic fir carries on photosynthesis at temperatures as low as -40^{o}, and many more organisms can withstand low temperatures without carrying on active life processes (Johnson et al., 1974).

Some of the molecular and physiological mechanisms by which living organisms contend with the exigencies of environmental temperature are now well understood, while others are just coming to light. It is the purpose of the present

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*Unless otherwise indicated, all temperatures quoted in this dissertation will be given in degrees Celsius.

thesis to examine the effect of temperature on one vital biological process in fish — that of synthesis and secretion of proteins. The introductory material presented herein includes a brief examination of current knowledge concerning chemical kinetics and temperature and backround material on (a) the general mechanisms of temperature acclimation in cold-blooded animals; (b) current knowledge concerning the effects of temperature on protein metabolism; and (c) the process of protein secretion in general.

The animals chosen for study are all teleosts, or bony fishes. The particular species were selected on the basis of accessibility, ease of maintenance, and tolerance of experimental procedures. The proteins chosen for study are the liver-synthesized plasma proteins, and a brief discussion of plasma proteins in fishes has been included in the following pages. One further section dealing with the so-called "antifreeze" glycoprotein has been included because this specific protein was examined in addition to the other plasma proteins of Antarctic fish.

B. The Effects of Temperature on Chemical Processes

The foundations for quantitative study of the effects of temperature on chemical systems and living systems were laid long ago. The invention of the "thermoscope" by Galileo Galilei in the early seventeenth century was a major step in the accurate measurement of temperature. In the middle of the nineteenth century, William Thomson (Lord

Kelvin) gave temperature a firm thermodynamic foundation with his scale based on efficiency of a reversible heat engine (Rose, 1967; Shea, 1972). In 1884, J. H. van't Hoff showed that, in a chemical system, the equilibrium constant of a reaction varies with temperature according to the following relationship:

$$\frac{d \ln K_c}{dT} = \frac{\Delta E}{RT^2} \qquad (1)$$

where T is the absolute temperature, R is the gas constant, K_c is the equilibrium constant, and ΔE is the difference in energy between reactants and products. van't Hoff further pointed out that, according to the law of mass-action, the equilibrium constant of a reaction is the ratio of forward rate constant to reverse rate constant:

$$K_c = \frac{k_f}{k_r} \qquad (2)$$

Thus, the van't Hoff equation (1) could be rewritten:

$$\frac{d \ln k_f}{dT} - \frac{d \ln k_r}{dT} = \frac{\Delta E}{RT^2} \qquad (3)$$

and the terms for the forward and reverse reactions could be separated to yield:

$$\frac{d \ln k_f}{dT} = \frac{E_f}{RT^2} + \text{constant} \qquad (4a)$$

and:

$$\frac{d \ln k_b}{dT} = \frac{E_r}{RT^2} + \text{constant} \qquad (4b)$$

where $E_f - E_r$ equals ΔE (Glasstone, 1946).

A few years later, the distinguished Swedish chemist Svante Arrhenius noted that the rate of hydrolysis of sucrose in the presence of various acids varied greatly with temperature. To explain this large temperature effect Arrhenius postulated that any chemical system possessed two kinds of molecules: normal and active. Only active molecules could take part in the chemical reaction. In order to change a normal molecule into an active one, energy, which could be heat, would have to be put into the system. Arrhenius therefore proposed the following equation:

$$\frac{d \ln k}{dT} = \frac{E_a}{RT^2} \qquad (5)$$

where E_a is the "activation energy" of the reaction. Equation (5) has the same form as that derived by van't Hoff in equations (4). Assuming that the activation energy is a constant of the chemical system being described and is independent of temperature, equation (5) yields upon integration:

$$\ln k = -\frac{E_a}{RT} + \ln A \qquad (6)$$

where ln A is the constant of integration. Clearing of logarithms yields:

$$k = Ae^{-E_a/RT} \qquad (7)$$

The constant A is generally agreed to represent the frequency of collisions between two reactant molecules, regardless of whether or not they possess the requisite activation energy, and A is therefore called the "frequency factor". Equation (7) is the well-known Arrhenius equation for the temperature-dependence of the rate constant (Glasstone, 1946; Moore, 1955).

Examination of equation (6) indicates that a plot of ln k vs. 1/T should be linear. In the years following the publication of the Arrhenius equations, the rates of many reactions at various temperatures were shown to be accurately described by the equations. However, some simple reactions showed deviations from linearity by small but consistent amounts in A and E_a (Moore, 1955; Johnson, et al., 1974).

Development of a more complete and theoretically precise reaction rate theory required a better understanding of both the activation energy and the frequency factor of the Arrhenius equations. The first significant attempt in this direction was the so-called "collision theory" developed at the beginning of this century. This theory held that reaction rate should equal the product of the number of collisions per unit time and the proportion of collisions which were effective in producing reaction. Atoms were treated as simple hard spheres, and the energy used in the

calculations was taken as only kinetic energy, specifically, the velocity component of the collisions. This theory was applied to many simple bimolecular reactions involving monatomic gases, and experimental determinations of the rate constant in many cases were in reasonable agreement with theoretical predictions. Values for the activation energy were experimentally determined by application of the Arrhenius equation. In other kinds of reaction, however, the reaction rates calculated from the theory differed by several orders of magnitude from those determined experimentally. In addition, the collision theory provided little or no insight into reaction rates in solution (Moore, 1955; Johnson, et al., 1974).

As advances in statistical mechanics and quantum mechanics led to a clearer understanding of the structure of the atom and the nature of the forces holding atoms together in molecules, an alternative explanation for the kinetics of rate processes emerged. This new approach, the absolute reaction rate theory, postulated that before a chemical reaction can occur, two reactant molecules possessing the requisite energy of activation must collide to form an "activated complex", which subsequently decomposes to form the reaction products. The theory is called the absolute reaction rate theory because, unlike the collision theory, the calculation of reaction rate is based solely on the physical properties of the reacting atoms or molecules.

Moreover, it explains satisfactorily why collision theory was successful for some reactions but not for others, and it provides a precise theoretical basis for both the activation energy and the frequency factor of the Arrhenius equation (Glasstone, 1946; Moore, 1955; Johnson, et al., 1974).

The absolute reaction rate theory is based primarily on quantitative description of both the potential energy and kinetic energy of atoms. In addition, it does not treat atoms as simple hard spheres. It is now well known that two atoms, such as those of a diatomic gas, at their equilibrium distance possess a minimum of potential energy. As the nuclei of these two atoms are forced closer together than the equilibrium distance, the potential energy of the system increases sharply. As the nuclei are separated to distances beyond the equilibrium distance, potential energy again rises from the minimum until it approaches a limiting value corresponding to the dissociation of the atoms (Johnson, et al., 1974).

The formation of the activated complex, or transition state, postulated by the absolute reaction rate theory is best understood in potential energy terms. In a hypothetical reaction such as:

$$A + BC \rightleftharpoons AB + C \qquad (8)$$

the reaction depends upon the approach of reactant A to

reactant BC. As approach occurs (assumed here to be linear), the potential energy of the system increases with decreasing distance between A and BC. This increase forces the atoms in BC to separate slightly. Atom A must have enough kinetic energy to overcome the repulsive forces which separate A from BC. As the approach of A to BC continues, a transition state is formed in which the likelihood of A bonding to B is equal to the likelihood of B remaining bonded to C. In this transition state, all three of the atoms are joined together in a short-lived activated complex A-B-C (Glasstone, 1946; Johnson, et al., 1974).

The activated complex represents the configuration with the highest possible potential energy. AB and C, the products of this reaction, are formed when the activated complex decomposes in the forward direction and the system returns to a lower level of potential energy. Thus, the rate of reaction is equal to the fraction of activated complexes times the rate at which they decompose to form product. The reaction of equation (8) can be rewritten as:

$$A + BC \rightleftharpoons A\text{-}B\text{-}C \rightleftharpoons AB + C \qquad (9)$$

The changes in the potential energy are represented as in Figure 1. The activated complex formed in either the forward reaction or the reverse reaction is the same, and the number of activated complexes which proceed from the reactant to the product side of the potential energy barrier is determined

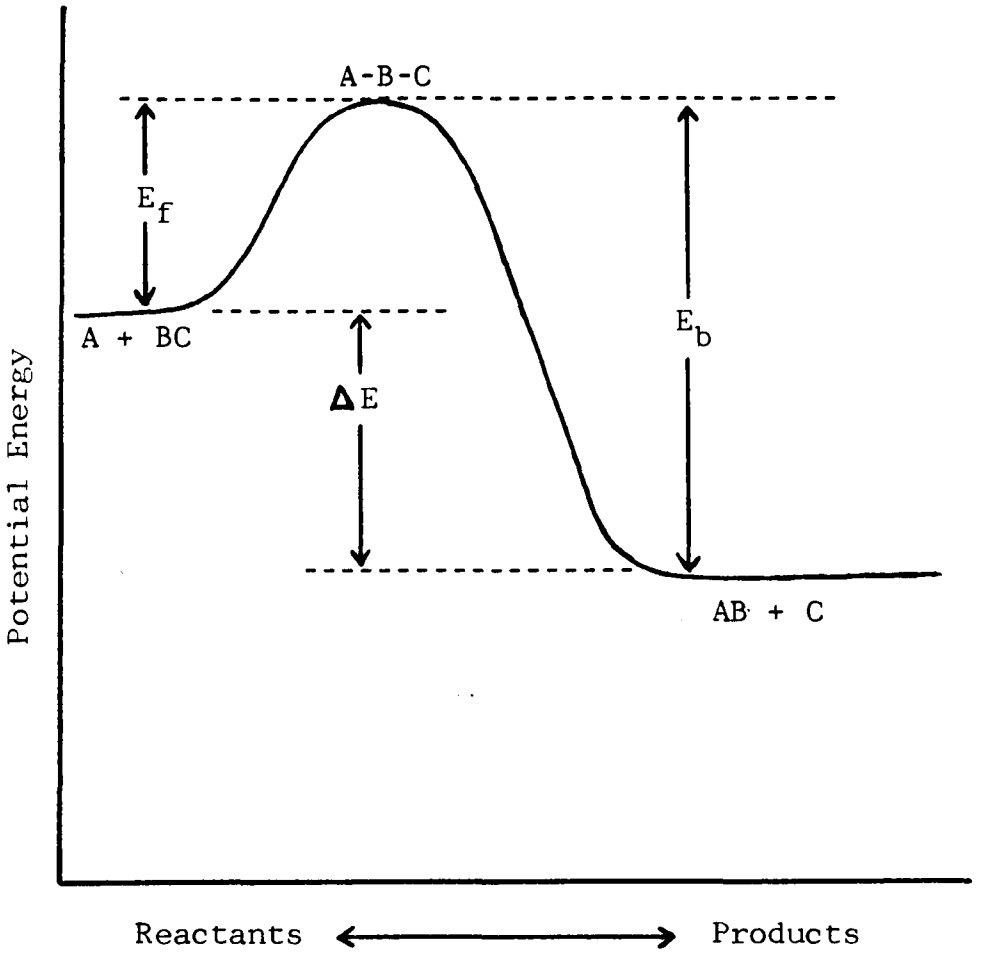


FIGURE 1

The changes in potential energy in the hypothetical chemical reaction:

$$A + BC \rightleftharpoons A\text{-}B\text{-}C \rightleftharpoons AB + C$$

where: E_f = activation energy (E_a) for forward reaction

E_r = activation energy for reverse reaction

ΔE = difference in energy between reactants and products

(Adapted from Glasstone, 1946; Johnson et al., 1974)

quantum mechanically. Although the activated complex formed in either the forward or reverse reaction is the same, in an exothermic reaction, such as that represented in Figure 1, the energy of activation for the forward reaction is less than that for the reverse reaction.

In practice, calculation of potential energy barriers for a chemical reaction involves accounting for energy distribution in all the translational and vibrational states of the reacting molecules. Three-dimensional energy diagrams are required in which several pathways may be present that lead to reaction. The majority of crossings of the barrier, however, will be at the barrier's lowest point.

Although absolute calculation of reaction rates from molecular properties remains a formidable problem, the evaluation of temperature dependency is straightforward. This is apparent from the more familiar reaction rate equation, in which activation energy E_a is replaced by the Gibbs free energy of activation $\Delta G^{\ddagger}$:

$$k = \kappa \frac{k_b T}{h} e^{-\Delta G^{\ddagger}/RT} \qquad (10)$$

where k is the rate constant, κ is the probability that decomposition of the activated complex will occur in the forward direction (usually taken as 1), k_b is the Boltzmann constant, T is absolute temperature, h is Planck's constant, and R is the gas constant. The free energy of activation is made up of the usual thermodynamic components:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \qquad (11)$$

Comparison of equations (10) and (11) with the Arrhenius equation (7) indicates that the components which determine temperature dependency are related by the following:

$$E_a = \Delta H^{\ddagger} + RT \qquad (12)$$

At room temperature, the term RT amounts to only 0.6 Kcal/mole.

Examination of many biochemical processes (simple covalent bond breaking or forming reactions) has indicated a great similarity in their activation energies. Similar findings are made when respiration or other functions of whole organisms are measured. These results are more typically expressed in terms of Q_{10}, i.e., the factor by which the rate of a reaction is increased in response to a 10^{o} rise in temperature. Thus:

$$Q_{10} = \frac{k_{T+10}}{k_T} \qquad (13)$$

where k_T is the reaction rate at temperature T, and k_{T+10} is the reaction rate at a temperature 10^{o} higher (Hoar, 1966; Prosser, 1973). Q_{10} may be approximated from the relationship:

$$Q_{10} = \left(\frac{k_1}{k_2}\right)^{10/(T_1-T_2)} \qquad (14)$$

where k_1 and k_2 are the reaction rate constants at T_1 and T_2, respectively. The temperature range in which the measurements

are made must be specified.

Q_{10} values have been determined for many physiological processes, and the majority of these processes show Q_{10}'s of from 2.0 to 3.0 over the range of biological temperatures (Prosser, 1973). The Q_{10}'s for a number of enzymatic reactions also fall into this range. For photochemical reactions and for physical processes such as diffusion, values are generally less than 1.5 (Hoar, 1966).

C. Adaptations of Poikilothermic Organisms

Poikilotherms ("many temperatures") are organisms which do not regulate internal body temperature, and this temperature is therefore more or less determined by the temperature of the environment. These organisms have evolved many complex adaptations to the varying temperatures of their habitats, and these adaptations allow them to acclimatize and function over a wide range of environmental temperatures. Poikilotherms can be found in nearly all the habitats of the earth. Homeotherms ("same temperature"), mammals and birds, have evolved a neurally-mediated regulation of body metabolism which allows them to maintain a constant high internal body temperature. This allows more effective and continuous control of nervous coordination of body activity, and this is of obvious advantage in any environment. It is therefore not surprising to find a widespread distribution of birds and mammals, including man (Hoar, 1966).

The temperature adaptations of poikilotherms can be seen as various means to compensate for the direct effect of temperature on biochemical reaction rates, as just discussed. Three types of adaptation phenomena are generally distinguished (Bullock, 1955; Prosser, 1967):

Adaptation: Adjustment of metabolism over the course of evolution of an organism in order to optimize function under a given set of climatic conditions. Cold adaptation, for example, would be evidenced by a standard metabolic rate which is higher than that predicted from the Arrhenius relationship and the rates observed in species which function at higher temperatures.

Acclimation: The compensation of metabolism in individuals occurring in response to changing temperature. This process, requiring times of the order of weeks, permits an organism to maintain a more constant level of activity over the range of temperatures typical of its habitat. In the laboratory, the process is measured by following metabolic rates as a function of time after a temperature change.

Acclimatization: This refers to the process of acclimation as it occurs in nature, typically in response to a variety of climatic changes including temperature.

One of the classic studies of temperature adaptation is that of Scholander et al. (1953) on a variety of species of fish, arachnids, crustaceans, and insects. Animals of tropical origin assayed at 30^{o} showed oxygen consumption rates

which were three to ten times higher than the rates of Arctic animals assayed at 0^{o}. The difference was significantly less than expected for the large temperature difference. At temperatures near the normal habitat temperatures of the organisms, Q_{10} for respiration was about 2.5. From this one can calculate that Arctic species should show oxygen consumption rates of 15-fold below that of the tropical organisms. It was concluded that the species indigenous to polar regions were cold-adapted. Wohlschlag (1964) obtained similar data for several Antarctic fishes, which are extremely stenothermal, i.e., tolerate only a narrow temperature range. The nototheniid fishes studied, Trematomus borchgrevinki, T. bernacchii, T. lönnbergi, and T. hansoni all showed metabolic rates consistent with high levels of cold adaptation. The zoarcid fish Rhigophila dearborni showed only slight cold adaptation.

Recently, the results of the experiments summarized above have been challenged by Holeton (1974). In a study of eleven species of Arctic fishes, Holeton was unable to duplicate the high rates of oxygen consumption obtained by Scholander in 1953. With the exception of the Arctic cod Boreogradus saida, all species assayed showed values for oxygen consumption which were about one-third the previous values. Thus, the difference between Arctic and tropical species was 15-fold as predicted for a Q_{10} of 2.5. If Q_{10} of standard metabolism is larger, however, the polar fish would be considered cold-adapted. The best value for Q_{10} is

still in doubt. However, it is likely that cold adaptation in polar fishes is less than previously thought.

Far more attention has been given to cold acclimation in eurythermal fishes, those capable of functioning over a wide range of temperatures. Compensation of standard metabolism may be complete in some species such that constant activity in acclimated individuals is achieved over a 10^{o} or more temperature range (Roberts, 1967). A great variety of biochemical changes also have been reported. In general, enzymes involved in energy production, such as glycolysis, those associated with the tricarboxylic acid cycle, and those involved in electron transport show elevated levels in cold-acclimated individuals, whereas enzymes associated with degradative pathways show the reverse effect or no change (Hazel and Prosser, 1970, 1974). Houston and co-workers (1976) have shown that the components of the hemoglobin system in teleosts are affected by temperature acclimation. In goldfish, Wilson (1973) found that activity changes associated with various enzymes during temperature acclimation are a result of alterations in the amounts of enzyme present, rather than of changes in the properties of the enzymes. The latter is considered to play a role in evolutionary adaptation but not in individual acclimation or acclimatization responses (Hazel, 1972).

In summary, there is a good deal of evidence to suggest that protein metabolism may be important in the responses of organisms to changing temperature. Work in this area is

reviewed in the following section.

D. The Effects of Temperature on Protein Metabolism

Current information indicates that protein metabolism, like other metabolic processes in poikilotherms, shows a temperature-dependency much like that of chemical processes and biochemical processes in general. Incorporation of radioactive tyrosine into goldfish brain protein has been studied *in vivo* using high doses of the amino acid for stabilization of specific radioactivity in the tissue (Lajtha and Sershen, 1976). These studies showed a fractional synthesis rate of 12% per day at 34°, not far below the value of 17% obtained in rat brain (Waterlow and Garlick, 1975). In the temperature range of 24° to 34°, Q_{10} for incorporation was 2.7. A similar value of Q_{10} was obtained from ordinary amino acid incorporation data in toadfish liver (10° to 20°) *in vivo* and in liver homogenates *in vitro* (Haschemeyer, 1968; 1969a). Jackim and LaRoche (1973) have also obtained this result in studies of amino acid incorporation into muscle of killifish.

Other organisms show Q_{10} values comparable to those above when protein synthesis measurements are carried out at room temperatures near the normal habitat temperature. For example, in *E. coli* Q_{10} is in the range of 2 to 3 for temperatures of 25° to 37° (Landau, 1966); at 8°, however, synthesis stops almost completely (Das and Goldstein, 1968;

Friedman et al., 1969 and 1971). This effect has been traced to defects in both the transcription of messenger RNA and in translation (Anderson, 1975). A similar breakdown in synthetic function occurs in bacteria upon shift-up in temperature from 23° to 40° (Chaloner-Larsson and Yamazaki, 1977). Effects of sudden temperature change may be reversible in some cases: Shaw and Ingraham (1967) have found that protein synthesis in E. coli resumes 4 hr after a temperature drop from 37° to 10°. These results are in contrast with those in psychrophilic microorganisms which function well at 0°, but show greatly increased heat-sensitivity (Inniss, 1975).

Temperature dependence of protein synthesis has been examined in a number of eukaryotic cells in culture. In rabbit reticulocytes, incorporation of ^{3}H-valine into globin is strongly temperature-dependent (Conconi et al., 1966); the rate of elongation of the α and β polypeptide chains in the temperature range of 15° to 25° shows a Q_{10} of 4.6 (Hunt et al., 1969). Craig (1976) has determined Arrhenius activation energies for both total protein synthesis and for elongation rate alone in reticulocytes and mouse L-cells. In the L-cells, polyribosome breakdown occurred below 25°, suggesting a defect in initiation of mRNA translation. In reticulocytes, polyribosome structure remained intact at all temperatures studied, but a sharp break in temperature-dependency of synthesis associated with elongation occurred

at 24°. Converting his results to Q_{10}'s gives the following: for the temperature range 24° to 40° Q_{10} of amino acid incorporation was 3.2 and that for elongation rate was 1.9; for the range 10° to 24° Q_{10} for incorporation was 8.3 and that for elongation was 7.8. A similar break in Arrhenius plot was observed at 25° in E. coli (Goldstein et al., 1964). No effect of this type, however, was found in a study of plasma protein synthesis in turtles over the range 11° to 33°; a linear Arrhenius plot with Q_{10} = 3.9 was obtained in this case, identical to that found in cooled rabbits in the range of 24° to 40° (Green and Anker, 1955).

Several workers have noted that cold acclimation of poikilotherms enhances the level of amino acid incorporation into protein. Das and Prosser (1967) found an increased level of amino acid incorporation into protein in gills and liver of cold acclimated goldfish several hours after injection. Cold acclimation also increases incorporation of amino acids into proteins in the intestinal mucosa of of goldfish (Morris and Smith, 1967) and in liver of trout (Dean and Berlin, 1969). In toadfish, incorporation levels were 75% higher in fish acclimated for two weeks to 10° compared to fish acclimated to 22°, when both groups were assayed at 22° (Haschemeyer, 1968). McCarthy et al. (1976) have found a similar effect in the tissues of the intermolt lobster Homerus americanus in culture.

This increase in the level of amino acid incorporation into protein as a result of cold acclimation has been

correlated with an increase in the rate of polypeptide chain elongation (Haschemeyer, 1969b). The effect can be observed at both high and low temperatures of measurement (Haschemeyer and Persell, 1973). The increase in elongation rate is about 60% in cold-acclimated fish compared to warm-acclimated fish. A similar change in tissue concentration of polypeptide elongation factor 1 is found, and this has been proposed to be the rate-limiting factor in the system (Nielsen et al., 1977).

In summary, the results available in the literature indicate a wide range of temperature responses of the protein synthetic system. Within the range at which some level of function of the organism occurs, Q_{10} for protein synthesis may range from about 2.5 to 8. Cut-off temperatures occur at both high and low extremes where protein synthesis is severely or completely inhibited. These limits are species-specific. Cold acclimation appears to be a process by which an organism shifts its protein synthetic activity in order to elevate its metabolic rate and activity at low temperatures. The possibility exists that a similar process may have occurred over the course of evolution to produce cold adaptation; no studies have been reported in this area.

E. Protein Secretion

Current knowledge of the mechanism of protein secretion, as recently reviewed by both Peters (1975) and Palade (1975), has been derived to a great extent from studies of the rat pancreatic acinar cell (Jamieson and Palade, 1967, 1968a, 1968b, 1971a, 1971b,; Caro and Palade, 1964; Tartakoff *et al.*, 1974). Proteins which are destined for secretion are synthesized on membrane-bound ribosomes and after synthesis are released into cisternae of the endoplasmic reticulum. They then migrate through small smooth-surfaced vesicles into the Golgi complex, where they are concentrated and stored in zymogen granules. These granules migrate to the periphery of the cell, and the contents of the granules are released to the extracellular compartment by means of exocytosis.

Generally, this series of events has been confirmed in other systems, e.g., in the exocrine pancreas of the frog *Rana esculenta* (Slot *et al.*, 1974, 1976), the parotid acinar cell of the rabbit (Castle *et al.*, 1972) and for melanocyte-stimulating hormone secretion by the pars intermedia of *Xenopus laevis* (Hopkins, 1972). Rothman (1975), however, has argued that digestive enzyme transport may not be the simple unidirectional mass-transit process described in the classical studies. Moreover, Rothman asserts that not all secretory proteins made on the rough endoplasmic reticulum pass directly into the cisternal space. This difference of

view remains to be reconciled.

Several factors have been shown to affect the secretory process in various organs. In the glandular lobe of the corpus cardiacum of Locusta migratoria, it has been shown that secretion of the adipokinetic hormone is increased by flight and is probably under nervous control (Rademakers and Beenakkers, 1977). Secretion of previously-made vs. newly-made exportable proteins in the rat parotid is controlled by neurotransmitters (Sharoni et al., 1976). Thus, isopreterenol is found to randomize secretion of old and new protein, whereas, under normal circumstances, the old protein is secreted preferentially. Secretion of thyroxin is increased sharply in the young pig in response to a drop in the environmental temperature (Evans and Ingram, 1977). An agent which has proven useful in the study of secretory processes is cerulein. This peptide has been shown to affect both the rate of protein synthesis and the overall process of protein secretion in the rat exocrine pancreas (Völkl et al., 1976; Bieger et al., 1976a, 1976b). Cerulein dramatically increases secretion rate, apparently by increasing the rate of all steps in the secretory process.

The process of secretion of protein in liver has been shown to be similar to that in the exocrine pancreas, although no storage granules are present and secretion takes place directly from the Golgi apparatus (Glaumann and Ericsson, 1970; Redman and Cherian, 1972; Peters, 1975).

Considerable chemical modification of proteins takes place within the secretory system in liver. Albumin, the plasma protein in highest concentration in mammals, is synthesized in the form of proalbumin (Russell and Geller, 1973; Quinn et al., 1975). Conversion to albumin occurs in the secretory vesicles (Ikehara et al., 1976). The glycosylation of glycoproteins also takes place within the secretory system at several different sites (Schachter, 1974; Lawford and Schachter, 1966; Molnar and Sy, 1967).

There have been numerous studies of the synthesis and secretion of various proteins, particularly the plasma proteins albumin, fibrinogen, and transferrin in mammalian systems (e.g., Katz et al., 1967; Judah and Nicholls, 1971; Morgan and Peters, 1971; Crane and Miller, 1977). Normal and diseased states have been compared in several cases (Schreiber et al., 1971; Peters and Peters, 1972), and the role of hormones examined (Feldhoff et al., 1977). There have been very few attempts, however, to characterize the actual kinetics of synthesis and/or secretion of any of the secretory proteins. A short-term kinetic study of secretion of luteinizing hormone from pituitary cells in culture showed that the release of the hormone followed a biphasic timecourse (Hopkins, 1977). No actual kinetic analysis was performed on these data, however. This lack of knowledge concerning the kinetics of synthesis and secretion of proteins can be traced to limitations in the radioisotope techniques

used, as reviewed recently by Haschemeyer (1976).

A kinetic study has been performed on the synthesis and secretion of plasma proteins in a poikilotherm, the toadfish Opsanus tau (Haschemeyer, 1973). In this study, comparing the rates of synthesis and secretion at two different temperatures, rate constants were derived for the various steps involved in synthesis and secretion, and an overall schema was proposed to describe these processes. This treatment of synthesis and secretion assumed that the processes could be described in terms of simple reversible monomolecular reactions. Computer-generated rate constants were derived which yielded theoretical curves consistent with the observed temperature-dependency and thermal acclimation effects. This approach formed the starting point for the kinetic equations developed in this thesis.

F. Plasma Proteins in Fishes

In adult vertebrates, the liver has been shown to be the site of synthesis of many plasma proteins, including albumin, haptoglobin, transferrin, fibrinogen, prothrombin, the high-density and low-density lipoproteins, and the α_1-acid-glycoprotein (Schultze and Heremans, 1966). In addition, the liver is the source of a substantial proportion of the plasma α-globulins and β-globulins. Other α- and β-globulins in the plasma, as well as all of the γ-globulins, are synthesized in differentiated tissues of

the reticulo-endothelial system.

Only a few plasma proteins of fishes have been isolated and studied in detail. In most cases, electrophoretic mobilities of fish plasma proteins do not correspond closely with those of the mammalian plasma proteins used as standards (e.g., Deutsch and McShan, 1949) thus introducing ambiguity in identification. Perrier et al. (1973a) have suggested that fish plasma proteins be classified on the basis of their physical properties alone, rather than by comparison with mammalian plasma proteins. With these difficulties in mind, current information about plasma proteins in fishes will be briefly reviewed.

Early studies of plasma proteins in marine fishes indicated great variation in the quantity of protein present in the blood (Denis, 1913-14; Lepovsky, 1929-30; Turner, 1937). This was found to be true of freshwater fish as well (Wilson and Adolph, 1917; Vars, 1934). Qualitative distribution of the types of plasma protein also varied widely. For example, albumin to globulin ratio is 4:1 in the carp Cyprinus carpio and 2:1 in the trout Salvelinus fontinalis (Field et al., 1943). Large variation in both type and amount of plasma protein is also found in closely related species of trout (Deutsch and McShan, 1949) and in Tilapia (Badawi, 1971).

Generally, there is an increase in the amount and the number of different types of plasma proteins from the lower fishes to the more specialized higher fishes (Turner, 1937;

Gunter et al., 1961). All species of fish thus far studied show lower total plasma protein concentration than higher vertebrates (Deutsch and Goodloe, 1945; Hamoir, 1955; Gunter et al., 1961; Sulya et al., 1961). Albumin, a protein found in high concentration in mammalian plasma, appears to be absent in the blood of many lower fishes, particularly the elasmobranchs. Irisawa and Irisawa (1954) showed that both the skate Raja kenojei and the shark Heterodontus japonicus lack albumin, or an albumin-like component, in their blood. The shark Squalus acanthius also lacks albumin, as do several garfish and a few teleosts (Bueker, 1961; Gunter et al., 1961).

Most teleosts have albumin or an albumin-like component, but the relative amount varies. In the brown trout Salmo fario only 9% of the plasma protein is albumin, whereas in the rainbow trout Salmo irideus 50% albumin is found (Morris, 1959). Among marine fish the wirrah Acanthistius serratus showed a 10% albumin concentration, while the bonito Sarda sarda had an albumin concentration of 55%. In the king salmon and in the Antarctic fishes Trematomus borchgrevinki and Dissostichus mawsoni, albumin is present but at considerably lower concentrations than in human plasma (Komatsu et al., 1970 a). Albumin has also been identified in several species of carp (Chandresekhar, 1959), in the eel (Fine et al., 1963), the rainbow trout (Snieszko, et al., 1966), and the dace Leuciscus leuciscus (Harris, 1974). A

serum albumin-like component of 59,000 daltons molecular weight from C. carpio has been identified and partially characterized (Nagano et al., 1975). The concentration of this albumin-like component in carp plasma is 1 to 2 mg/ml. A component which appears to be prealbumin has been identified in the dace (Harris, 1974).

In the blood of most fishes, including the elasmobranchs, a substantial portion of the plasma protein is made up of the globulins (Lepovsky 1929-30; Field et al., 1943; Sulya et al., 1960), usually occurring in three to five components. The most common, and most constant, globulin found in fish plasma is α-globulin (Deutsch and Goodloe, 1945; Deutsch and McShan, 1949). β-globulins are usually present but vary widely in concentration. γ-globulins are absent, or nearly absent, in all fishes studied (Deutsch and McShan, 1949). Of eleven species of teleosts studied by Engle et al. (1958) only one (the bonito Sarda sarda) had a γ-globulin fraction. In sea herring, γ-globulin is nearly absent (Sindermann and Mairo, 1958). The dace L. leuciscus also has a γ-globulin fraction (Harris, 1974), as does the eel (Fine et al., 1963). The catfish Ameirus melas appears to be an exception to the rule, as it shows a γ-globulin concentration of 37% in blood serum (Bueker, 1961).

Fibrinogen, the plasma protein responsible for blood clotting, has not been extensively studied in teleosts. Field et al. (1943) identified a fibrinogen in the plasma of

both C. carpio and S. fontinalis, and Doolittle and Surgenor (1962) identified this protein in the blackfish Tautoga onitis. In S. gairdneri Richardson, Perrier et al. (1974) found a fibrinogen which does not have any sugar associated with it.

Fibrinogen has been relatively well studied in the elasmobranchs and the cyclostomes. Doolittle (1963) found that the fibrinogen of the dogfish Mustelis canis shows characteristics similar to human fibrinogen in terms of its reactions to various inhibitors of the clotting reaction. Human and dogfish fibrinogen, however, react differently to increasing ionic strength in the clotting medium: fish clotting time is shortened by increasing ionic strength, while that of human is lengthened. Fibrinogen of the lamprey eel Petromyzon marinus can be clotted by bovine thrombin (Doolittle, 1965a). Sedimentation coefficient of lamprey fibrinogen was close to that of human fibrinogen, and the B fibrinopeptides were similar (Doolittle, 1965b). Fibrinopeptide A, however, was released from lamprey fibrinogen only by lamprey thrombin and not by bovine thrombin. The A fibrinopeptide of lamprey is eight amino acids long, whereas the B peptide is forty residues long. In general, fish fibrinogens are capable of being clotted by bovine thrombin (Doolittle et al., 1962; Doolittle and Surgenor, 1962), indicating similarity in the biochemistry of this final step in clot formation. However, the conversion of prothrombin

to thrombin may be quite different in fishes (Doolittle and Surgenor, 1962). Further, it is found that elasmobranch blood, unlike teleost blood, clots extremely slowly, and thus may be deficient in clotting components (Lewis, 1972).

Serum transferrins, proteins which bind iron, have been studied in several fishes. The transferrin of the snapper Lutjamus vaigiensis, as identified by a radioiron binding assay and starch gel electrophoresis, is of a single type (Blumberg, 1960). In carp, there are three transferrin variants, and these variants may be related, as in higher vertebrates, to a genetic polymorphism (Creysell et al., 1966). Transferrins from king salmon, T. borchgrevinki, and D. mawsoni are more acidic than human transferrins (Komatsu et al., 1970a). Transferrins have also been identified in the blood of the goosefish Lophius piscatorius, as well as in several other species (Jimenez and Planas, 1973).

Various other plasma proteins have been identified in fishes. Haptoglobins have been found in several species (Blumberg, 1960; Jimenez and Planas, 1973), and a plasma transport protein for 25-hydroxycholecalciferol has been shown to exist in both teleosts and elasmobranchs (Hay and Watson, 1976). Perrier et al. (1976) have identified an iodide-binding protein in the plasma of rainbow trout S. gairdneri Richardson. A serum vitellin component has been shown in female coho salmon Oncorhynchus kisutch (Vanstone and Ho, 1961), and possibly in rainbow

trout as well (Thurston, 1967). Plasma high density lipoproteins in the pink salmon Oncorhynchus gorbuscha have been shown to be generally similar to those of higher vertebrates (Nelson and Shore, 1974). No low-density lipoproteins could be identified in this study. In the shark Centrophorus squamosus, Mills et al. (1977) have found that low-density and very-low-density lipoproteins are similar to those of higher vertebrates; high-density lipoproteins were shown to be somewhat different, however. Preproinsulin, a protein of non-hepatic origin, has recently been shown to exist in both the goosefish Lophius americanus and the sea raven Hemitripterus americanus (Shields and Blobel, 1977).

Several species of Arctic and Antarctic fishes have been shown to possess plasma proteins or plasma glycoproteins which are active in freezing point depression. Since one of these substances has been studied in this thesis, these "antifreeze" compounds will be reviewed separately, in the following section.

Hormonal effects and environmental effects on the plasma proteins of fishes have been investigated. Perrier et al. (1973b) found a small increase in total plasma protein, as well as in total plasma lipid, after the administration of adrenaline in S. gairdneri Richardson. In a study of seasonal variation in serum proteins in the same fish, Denton and Yousef (1975) found that overall concentration varied from a low in March to a high in July. A seasonal difference in

plasma protein concentration was not found in the goosefish L. piscatorius, however (Jimenez and Planas, 1973). Various forms of stress, such as capture and tagging of fish, have been shown to affect levels of total plasma protein (Thurston, 1967; Cardwell et al., 1971; Hattingh and van Pletzen, 1974).

In the cichlid fish Tilapia mossambica Solomon and Allanson (1968) found that extended exposure to low temperatures lowered overall plasma protein concentration, although the concentration of albumin-like component increased. In another species, Tilapia zilli, it was found that albumin concentrations in plasma increased with increasing temperature, while α- and β-globulin concentrations decreased (Farghaly et al., 1973). In S. gairdneri, Meisner and Hickman (1962) have shown that cold acclimation results in an increase in albumin concentration and a decrease in the α- and β-globulins.

G. Freezing Resistance and the Antifreeze Glycoprotein

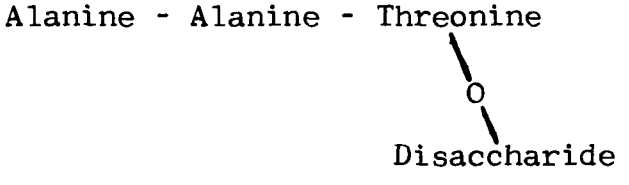
Adaptation of poikilotherms to extremely cold environments includes the development of freezing resistance (Wohlschlag, 1964; Rose, 1967; Brattstrom, 1970; Hazel and Prosser, 1974). "Antifreeze" glycoproteins (AFGP) have been identified in Antarctic fishes of family Nototheniidae (Feeney et al., 1972; DeVries, 1974; Holeton, 1974), and a simple peptide "antifreeze" has been found in

the winter flounder Pseudopleuronectes americanus (Duman and DeVries, 1974a, 1974b). Similar antifreeze compounds have been found in the Alaskan fishes Eleginus gracilis (the saffron cod) and the sculpin Myoxocephalus verrucosus (Raymond et al., 1975).

The search for these compounds began with the work of P.F. Scholander and his colleagues in Arctic fishes off northern Labrador and Baffinland. The freezing point of blood serum of winter fishes in shallow waters was significantly less than that for the same species in summer (Scholander et al., 1957). Deep water fishes at nearly constant temperatures of -1.7^{o} also showed a depressed freezing point compared with teleosts of temperate zones (Black, 1951; Scholander et al., 1957). Sodium chloride, the electrolyte responsible for 80% to 90% of body osmolality in teleosts was higher in the Arctic fishes but accounted for only half of the observed freezing point depression (Scholander et al., 1957). Similarly, in the fjord cod Gadus ogac winter electrolyte levels account for 79% of freezing point depression, and for 62% in the Arctic sculpin Myoxocephalus scorpius (Gordon et al., 1962). In P. americanus, sodium chloride accounts for 57% of freezing point depression observed during winter (Duman and DeVries, 1974a). In Antarctic species, electrolytes account for about half of observed freezing point depression and show little seasonal variation (DeVries, 1974).

The substances responsible for the remaining "antifreeze" activity first came to light when DeVries and Wohlschlag (1969) found that the supernatant from acid and heat treated serum of Trematomus borchgrevinki had a freezing point within 0.05^{o} of the value for whole serum. A family of glycoproteins was isolated by molecular exclusion chromatography with molecular weights in the range of 10,000 to 50,000 daltons. Digestion with proteolytic enzymes (pronase and subtilisin) destroyed antifreeze activity. Amino acid analysis showed the presence of only two amino acids: alanine and threonine. Galactose was found to account for a substantial portion of the molecule (DeVries and Wohlschlag, 1969).

Extensive biochemical analysis has since shown that the AFGP comprises eight related glycoproteins (DeVries et al., 1970; Lin et al., 1972). Molecular weights range from 33,000 daltons (glycoprotein #1) to 2,600 daltons (glycoprotein #8), and all members of the family are composed of polymers of varying length of the simple monomeric unit:



(DeVries et al., 1970; Komatsu et al., 1970b; DeVries et al., 1971). Glycoproteins 6, 7, and 8, the ones in smallest concentration in blood serum, have proline residues replacing some alanine residues (Lin et al., 1972). The sugars

composing the disaccharide units are galactose and N-acetylgalactosamine (DeVries and Wohlschlag, 1969; DeVries et al., 1970). The internal linkage between these two sugars is $\beta(1\rightarrow3)$ (Shier et al., 1972; Vandenheede et al., 1972; Shier et al., 1975). The linkage between the threonyl residue and N-acetylgalactosamine is $\alpha(1\rightarrow3)$ (Shier et al., 1972; Vandenheede et al., 1972).

Not all of the eight glycoprotein species show equal effectiveness in depressing the freezing point of blood serum or of water solutions. Glycoproteins 3, 4, and 5 (molecular weights 10,500, 17,000, and 21,500 daltons, respectively) appear to be the most effective in this activity. Glycoproteins 7 and 8 show less freezing point depression activity (DeVries et al., 1970; Lin et al., 1972). A synthetic random copolymer composed of 65% alanine and 35% aspartic acid shows antifreeze activity also (Ananthanarayanan and Hew, 1977).

Studies of secondary structure by circular dichroism and viscosity measurements indicate that these antifreeze glycoproteins are in an extended conformation (DeVries et al., 1970). A similar conclusion has been drawn from quasi-electric light scattering experiments (Ahmed et al., 1975). The mechanism by which these glycoproteins confer freezing resistance, however, remains elusive. On a molar basis, the AFGP's effectiveness in lowering freezing point is an order of magnitude greater than that of sodium chloride or ordinary proteins (DeVries and Wohlschlag, 1969;

DeVries, 1971; Raymond and DeVries, 1972). Proteolytic digestion causes complete loss of activity (DeVries and Wohlschlag, 1969). Modification of the disaccharide portion of the molecule by periodate oxidation of the galactose residues, β-elimination in NaOH, or acetylation of one third of the hydroxyl groups also destroys anti-freeze activity (Komatsu et al., 1970b). Oxidation of the C-6 hydroxyl groups to the aldehyde by means of galactose oxidase causes slight loss of activity; complete loss occurs when a negatively-charged group is placed at this position (Vandenheede et al., 1972). Native glycoproteins are inactive below pH 2 (Vandenheede et al., 1972). Acetonated AFGP is also inactive (Shier et al., 1972).

Aqueous solutions of AFGP show thermal hysteresis, i.e., melting and freezing points differ (DeVries, 1971; Feeney and Hofmann, 1973). This effect is concentration-dependent, and is due to the fact that AFGP depresses freezing point but not melting point. The effect is observed in blood serum of Arctic and Antarctic fishes as well (DeVries, 1971; DeVries, 1974).

Several explanations of the mechanism of action in freezing point depression of the AFGP have been advanced. The sugar modification experiments suggested that the hydroxyl groups might be important in structuring water molecules, thereby making them less readily available for ice formation (Komatsu et al., 1970b; DeVries, 1971; Feeney and Hofmann, 1973). DeVries (1971) has suggested that

the AFGP acts as a "surface deactivator" at the ice-water interface; by binding to the surface of the ice crystal, it prevents further addition of water molecules. Ice crystals do not grow, even over long periods of time, in a solution of AFGP kept at a temperature 0.05^{o} above the freezing point of the solution. When freezing occurs, AFGP molecules become incorporated into the crystal lattice (Raymond and DeVries, 1972). It does not, however, interfere with the normal compartmentation of sodium chloride (Lin et al., 1976).

Studies of quasi-electric light scattering indicate that the AFGP do not undergo conformational changes in water solutions at various temperatures or in an ice-water mixture at -0.2^{o} (Ahmed et al., 1975). Proton magnetic resonance measurements in frozen solutions indicate that the amount of bound water in AFGP solutions is small, but significantly greater than with ordinary proteins (Haschemeyer et al., 1977). The extended structure of the AFGP is thought to play a role in maximizing contact of the AFGP and its bound water with the surface of the ice crystal.

The AFGP have also been shown to be effective in preventing hemaglutination of human type-O and sheep erythrocytes by the Osage Orange lectin (Chuba et al., 1973). Glycoproteins 1-5, which give the most freezing point depression, show the most anti-lectin activity. Anti-lectin activity, like antifreeze activity, depends on the

integrity of the intact molecule (Ahmed et al., 1973). Recent experiments have shown that the AFGP is immunologically similar to the T-antigen (Thomsen-Friedenreich antigen), which occurs on cell surfaces of mouse and human mammary adenocarcinoma (Glöckner et al., 1975). In view of these findings, the AFGP may prove to be an important model compound in investigations of malignant transformation.

H. Rationale of Thesis

The objectives of this thesis were twofold:

(1) to develop experimental and theoretical methods for analysis of liver protein synthesis and secretion in vivo as a means to understand these processes as they operate throughout the vertebrates;

(2) to examine the direct effect of low temperatures on liver protein metabolism in the toadfish, a eurythermal marine fish with a wide temperature tolerance, and in the Antarctic cod, a stenothermal species adapted to a constant very low temperature.

These techniques provide a new approach to examination of the phenomena of cold acclimation and cold adaptation in teleost fish and to the study of the synthesis of a specialized protein such as the antifreeze glycoprotein.

II. MATERIALS AND METHODS

A. Fish

Specimens of the toadfish, Opsanus tau, were obtained from the Marine Biological Laboratories in Woods Hole, Massachusetts. Animals of both sexes were selected, and all animals used were in the weight range of 300-600 gm. Previous to experimentation, all toadfish were kept in large holding tanks with running seawater. They were fed ad libitum with live Fundulus heteroclitus and/or mussels, depending on availability. Immediately prior to experimentation, experimental subjects were isolated in smaller running seawater tanks, and they were starved for 48 hr. All experimental animals were isolated from those not involved in experiments, and the temperature of the running seawater in the experimental tanks was constantly monitored. Experimental subjects were not fed during the experiment.

With fish which were being used for temperature acclimation experiments, the acclimation was accomplished previous to the start of the experiment by placing the subjects in insulated running seawater tanks ten days prior to the start of the experiment. Desired temperature was maintained in the water by means of a Neslab Heat Exchanger. During the winter, fish that were used were assumed to be acclimatized to the ambient seawater temperature, and they were maintained at that temperature in the holding tanks in

the laboratory.

The giant Antarctic cod, Dissostichus mawsoni, was obtained by fishing with steel set lines in roughly 400 meters of water in McMurdo Sound, Antarctica. The weight range of specimens caught was 9.1 to 61.2 kg, but the specimens used for experimentation were confined to the weight range 9.1-18.1 kg. Both sexes were used. After catching, specimens that appeared to be in good condition physically, that is, specimens which had only minor hook wounds and which were normally colored and lively, were transported back to McMurdo Station in specially-constructed water-tight boxes. In each of these boxes, the water was constantly aerated by means of a battery-driven air pump. Upon arrival at McMurdo Station, all fish were immediately transferred to a large swimming pool with running seawater, in which the water temperature was constantly monitored. A fish's behavior was observed for several days prior to being used in any experiment.

As with the toadfish, Antarctic cod which were used in experiments were isolated prior to the start of the experiment, and each fish was starved for two weeks before experimentation began. Fish involved in an experiment were totally isolated from fish not under experimentation. All the isolation tanks were well aerated, had constant temperature monitoring, and were large enough for these fish to swim actively. All tanks had running seawater. As with

the toadfish, Antarctic cod were not fed while involved in an experiment. Fishes of genus Trematomus were obtained by fishing with wire-mesh traps, baited with seal meat, in roughly 400 meters of water. All specimens were caught near McMurdo Station in McMurdo Sound, Antarctica. Trematomus bernacchii and T. hansoni were the most commonly caught species and were therefore used for experiments on Antarctic fish which required small specimens. After bringing up the traps, healthy specimens weighing about 0.5 kg were selected and transported back to McMurdo Station in the same water-tight boxes used for the transportation of D. mawsoni. Once back at McMurdo Station, these fishes were kept in small swimming pools with running seawater. All swimming pools used either for holding or experimental isolation had adequate aeration and were carefully monitored as to temperature. Before and during experimentation, these animals were treated in the manner described for D. mawsoni.

B. Radioisotopes

The following radioisotopes were obtained from New England Nuclear Corp.: L-[^{14}C(U)]-leucine, 284 mCi/mmole; L-[3,4,5,-^{3}H(N)]-leucine, 100 Ci/mmole; D-[^{14}C(U)]-glucosamine, 10 mCi/mmole; L-[^{14}C(U)]-alanine, 154 mCi/mmole. [1-^{3}H]-D-galactose, 2.8 Ci/mmole, and N-acetyl-D-galactosamine [acetyl-^{3}H], 86.5 mCi/mmole, were obtained from ICN Pharmaceutical Co.

C. Anesthetic

For both the toadfish and all the Antarctic fishes, ethyl-m-aminobenzoate methanesulfonic acid (MS-222, Sigma Chemical Company or Eastman Chemical Company) was used as anesthetic. For purposes of blood sampling during an experiment, toadfish were lightly anesthetized by immersing them in aerated seawater containing 0.2 gm/l MS-222. Usually, these fish remained in the anesthetic bath for no more than three to four minutes in preparation for a blood sample, and they were completely awake and active within 5 min of being returned to the experimental isolation tank.

For the Antarctic cod and the fishes of genus Trematomus, no anesthetic was required for taking a blood sample due to the use of a caudal vein cannula. This cannula and the technique for implanting it are described later.

For both the toadfish and the Antarctic cod, anesthesia was required before the surgical procedure for injection of radioisotopes was undertaken. Immediately prior to surgery, toadfish at 20^{o} were anesthetized by immersing them in an aerated seawater bath containing 0.3 gm/l MS-222. During this anesthetizing procedure, the fish usually remained in the bath for 5 to 10 min to insure complete anesthesia. In toadfish acclimated to 7^{o} a slightly stronger anesthetic bath was required. In these experiments, the concentration of MS-222 was raised to 0.5 gm/l in aerated seawater at 7^{o}.

During the surgery on fishes at both temperatures,

aerated seawater containing 0.1 gm/l MS-222 was allowed to flow over the gill arches. For toadfish at both temperatures it usually required no more than 15 to 20 min back in the experimental isolation tank for a fish to become normal and active again.

The Antarctic cod was anesthetized in a similar manner for the surgical procedure. Experimental subjects were captured in a large plastic tube, rather than a bath, and the MS-222 was added in a solution which, when mixed with the seawater already in the tube, yielded a final concentration of 0.12 gm/l. The water in the tube was well aerated, and the fish was usually ready to undergo surgery about 15 min after the MS-222 was added to the water. During the surgical procedure running seawater containing 0.1 gm/l MS-222 was allowed to run over the gills. It usually took no more than 20 to 25 min for the Antarctic cod to become active again after being returned to the experimental isolation tank.

In both the toadfish and the Antarctic cod, great care was taken during the anesthetizing and surgical procedures to insure that operculation was constant and regular.

Administration of MS-222 in preparation for implanting the caudal vein cannulas in Antarctic cod and fishes of genus Trematomus was done in the same fashion as that used for surgical preparation. Administration of anesthetic to fishes of genus Trematomus was in an aerated bath; concentrations of MS-222 were the same as those described for D.

mawsoni.

D. Injection Solutions

Injection solutions were routinely made up such that they were of the smallest practical volume. For injection solutions used in toadfish experiments, total volume was usually 0.1 ml in pH 7.4 phosphate buffered saline. Injection solutions used in experiments on the Antarctic cod were usually no more than 4.0 ml total volume in a balanced salt solution specific for this animal (Dobbs and DeVries, 1975). Injection volumes for T. hansoni and T. bernacchii were 0.1 ml total volume in the same balanced salt solution used for the Antarctic cod.

Doses of radioactivity given to fish during experiments were: for toadfish, 0.75 μCi/gm body weight; for the Antarctic cod, 200 μCi/fish; and for the smaller Trematomus fishes, 5 μCi/fish. For experiments involving fibrinogen isolation in toadfish, the dose of ^{14}C-leucine was 15 μCi/fish. For the protein synthesis inhibition experiments on T. bernacchii, cycloheximide (Sigma Chemical Company) was given at a dose of 5 mg/kg body weight 2 hr prior to the start of the experiment.

E. Blood Sampling Techniques and Caudal Vein Cannulation

In the toadfish, all blood samples were taken by means of a 1 ml tuberculin syringe with #26 gauge (short) needle from the arteries of the gill arches immediately beneath the opercular covering. All samples, except the one taken at the termination of the experiment, were 0.3 ml, and no more than three blood samples were taken from any single fish. The terminal blood sample was usually 1 ml.

In experiments involving the Antarctic cod and the Trematomus fishes, all blood samples were taken by means of a cannula placed in the caudal vein of the animal. Caudal cannulas were implanted in experimental subjects immediately prior to the surgical procedure described in the next section, thus allowing both implantation of cannula and surgery to be performed with a single application of anesthetic.

Implantation of the cannula in D. mawsoni was performed by placing the anesthetized animal ventral side up on an operating table consisting of a plastic stretcher supported by two wooden rods. A six-inch human spinal needle fixed to a 5 ml syringe was inserted into the fish's muscle along the ventral midline, immediately posterior to the animal's anus. The spinal needle was pushed downward at a slight angle until the large caudal vein was reached. While holding the tip of the spinal needle in the vein, the syringe was carefully removed, and a length of PE50 tubing

was passed down the needle until it encountered the vein. The total length of the PE50 tubing was approximately 1 meter, and it contained 0.5% heparin in saline. The cannula was then worked into the vein to a length of about 1.5 cm, and the spinal needle was freed from the vein leaving the cannula still in place. After removal of the needle, the tubing was secured to the skin of the fish in two places using #000 silk suture. The cannula's free end was stoppered with a common straightpin.

Implantation of the caudal cannula in the smaller Trematomus specimens was done by exactly the same procedure as that described for the Antarctic cod, except that a two-inch #18 gauge needle was used in place of the spinal needle, and PE10 tubing, instead of PE50 tubing, was used for the cannula.

To take a blood sample from a cannulated fish, the straightpin was first removed from the free end of the cannula, and a #18 gauge (short) needle, with a 1 ml tuberculin syringe, was inserted in the pin's place. The heparin solution was then withdrawn from the tubing, the syringe containing the heparin removed from the needle, and a new 1 ml syringe attached to the needle. An 0.25 ml blood sample for plasma protein analysis was drawn into the syringe. Periodically, blood samples of 4.0 ml were taken from D. mawsoni for antifreeze glycoprotein analysis. After blood had been withdrawn, the blood in the cannula was forced back into the caudal vein by refilling the cannula

with fresh heparin solution. Both syringe and needle were then removed from the end of the cannula, and the straightpin was replaced. During the entire procedure of taking a blood sample, the fish was totally awake and actively swimming.

F. Surgical Procedure

The object of the surgical procedure, both in toadfish and in the Antarctic fishes, was to inject radioactively-labelled amino acids (or in some cases, radioactively-labelled sugars) into the hepatic portal circulation of the animal. The hepatic portal vein ultimately supplies the main venous drainage for the intestines (Satchell, 1971) and as such, when blood in the hepatic portal circulation passes through the liver, any nutrients picked up in passage around the intestines will be given up to the liver. Injection of radioisotopes into the hepatic portal vein of the fish insures that much of that labelled compound will be taken up by the fish's liver.

The surgical procedure involved in exposing the hepatic portal vein for direct injection of radioisotopes was similar in both the toadfish and the Antarctic cod. The anesthetized animal was placed ventral side up on an operating rack, and seawater of the proper temperature was allowed to flow over the gills. An incision was then made in the ventral body wall, slightly to the left of the ventral midline, directly over the body cavity. The incision was retracted slightly.

In toadfish, the hepatic portal vein runs to the liver within the same mesentery that contains the gall bladder duct. Thus, by moving the fish's gall bladder upwards and anteriorly the hepatic portal vein can be clearly exposed. In this position, the vein rests securely on the airbladder. Injection of radioisotopes was accomplished by using a 1 ml tuberculin syringe with #32 gauge (short) needle. Injections were always performed such that the inflow of injection solution followed the direction of blood flow. After the completion of injection in toadfish, a small square of gauze was placed over the point where the needle entered the vein, and the needle was carefully withdrawn. The gall bladder was then returned to its normal anatomical position.

In the Antarctic cod, there is no air bladder, and the anatomy of the hepatic portal system is slightly different than that of the toadfish. Thus, the injection procedure in Antarctic cod was slightly different. Rather than a single large hepatic portal vein, as was found in toadfish, formed directly from the confluence of all the large mesenteric veins coming from the intestines, the hepatic portal circulation in cod is in two parts. The larger, and the least easily accessible to our hands during the surgical procedure, was the section formed by the intestinal vein confluence. A smaller section, a vein which entered the portal circulation from the stomach, was accessible, however.

The wall of this vessel was found to be extremely

fragile, and it was found during practice operations that it tore frequently when the injection needle was removed. A method was therefore devised for cannulating this vessel which allowed radioisotope injection with no losses to the body cavity. A #18 gauge needle (long) on a 1 ml tuberculin syringe was inserted into the vessel, and a short length of PE10 tubing (total length about 30 cm) was passed down the needle into the vein. The tubing was filled with isotonic salt solution. As in the caudal vein cannulation, the insertion needle was then carefully removed, leaving the tubing in the vein. The tubing was sewed into the wall of the stomach so as to insure that no leakage occurred around the edge of the cannula. Injection of radioisotopes was accomplished by inserting a #32 gauge (short) needle with 5 ml syringe into the free end of the cannula and injecting the required amount of injection solution into the vein. The cannula was then flushed with fresh saline to insure that all of the isotope had been put into the vein. The vein was tied off just anterior to the point of entrance of the cannula, and the cannula was removed.

After completion of injection in both the toadfish and the Antarctic cod, the body wall was sutured using #000 silk suture. The fish was returned to an isolated holding tank and allowed to awaken. During the surgical procedure on the Antarctic cod, the body of the fish was periodically wet with cheesecloth soaked in ambient temperature seawater.

For toadfish, total time on the operating table was usually about 10 min. For the Antarctic cod, total time for the caudal vein cannulation and the entire surgical procedure was usually about 30 min. During the surgical procedure on both toadfish and cod, there was no significant blood loss from the incision made in the body wall or from any other source, and both species seemed to act normally throughout the course of the experiment, after the surgical procedure. Coloration was normal in both species after the operation.

Since specimens of the genus Trematomus were quite small compared to the much larger D. mawsoni, the surgical procedure on these animals proved to be extremely difficult. Therefore, for all experimental injections on these animals, the injection route was through the caudal cannula.

G. Plasma Protein Analysis of Blood Samples

Blood samples taken from toadfish or the Antarctic fishes were spun at 2000 rpm on a clinical, table-top centrifuge for 3 min in order to separate the cells from the plasma. 100λ of the clear, straw-colored plasma was then pipetted off by means of a Lang-Levy micropipette (Fisher Scientific Company) and placed on a 2.4 cm Whatman #1 filter disc. The plasma on the filter was allowed to dry for a few moments, and it was then placed in about 20 ml of cold trichloroacetic acid (TCA) for 15 min. Filters

were washed, for 10 min each, in each of the following solutions: 20 ml cold 10% TCA; twice in 20 ml cold 3% perchloric acid; twice in 20 ml cold 95% ethanol; and twice in 10 ml room-temperature anhydrous ether. After the final ether wash, the ether was poured off and the filters allowed to dry overnight. All filters from blood samples were subjected to scintillation counting in scintillation grade toluene (Amend Drug and Chemical Company) containing the following: PPO (2, 5 diphenyloxazole, Packard Instrument Company) and POPOP (1,4-bis-[2-(4-methyl-5-phenyloxazole)]-benzene, Packard Instrument Company). All counting was performed on either a Beckman LS-100C or a Packard model 3320 Tri-Carb scintillation counter. All counting was done in duplicate for 10 min each time.

H. Quantitation of Fibrinogen in Toadfish Plasma

Quantitation of fibrinogen in toadfish plasma was done by taking advantage of its biological activity; that is, a clotting assay was devised by means of which fibrinogen could be quantitatively separated from the other plasma proteins. In experiments involving fibrinogen quantitation, 0.4 ml blood samples were taken from toadfish in a 1 ml tuberculin syringe, wetted with 0.1 M EDTA, with #26 gauge (short) needle. The blood was centrifuged, as described above, to separate cells from plasma, and 0.1 ml of the plasma was added to a 5 ml beaker containing: 0.2 ml sodium

phosphate buffer (pH 6.4, with 0.075 M NaCl), 10λ 1 M $CaCl_2$, and 0.1 ml bovine thrombin (1 unit/0.1 ml, Parke-Davis Pharmaceutical Company). The clotting reaction was allowed to proceed for 60 min at room temperature. The resulting clot was then dumped onto a piece of filter paper, washed twice with phosphate buffered saline, and allowed to synerize for about 4 min, after which it was placed in 0.9 ml 8 M urea. This was allowed to stand at room temperature overnight. The following morning, the clot and urea were boiled for 60 min at 105° and allowed to cool. A_{282} was read, and the dissolved clot was subjected to scintillation counting in 10 ml Aquasol (New England Nuclear Corporation). The concentration of fibrinogen was calculated from the following (Blombäck, 1958):

$$mg/ml = 0.606 \times A_{282}$$

I. Quantitation of AFGP in Antarctic Fish Plasma

For quantitation of AFGP from Antarctic fishes, blood samples of 4.0 ml were taken from D. mawsoni at predetermined intervals during the experiments. In the smaller Trematomus specimens, samples of about 3 ml were taken just before the experimental subject was sacrificed. These samples were centrifuged, as described above, and 2 ml of plasma was dialyzed in Spectrapore #1 membrane tubing

(Spectrum Medical Industries, Inc.) for 24 hr against distilled water in the cold. The dialysate was then loaded on a column of DEAE-32 cellulose (Whatman, W. & R. Balston, Ltd.) equilibrated with 0.0025 M Tris, pH 9; AFGP species #1-5 and #7-8 were sequentially eluted in 0.0025 M Tris and 0.10 M Tris, respectively. The remainder of the plasma proteins were eluted from the column with 0.50 M Tris, 0.10 M NaCl. All fractions from the column were lyophilized overnight to reduce their volume, and they were then dialyzed for 48 hr in the cold against distilled water in Spectrapore #3 membrane tubing; the water was changed four times during this dialysis. At the end of 48 hr, all dialysates were lyophilized to dryness. The purified AFGP species and the plasma protein fraction were then carefully weighed on a Mettler model H20 balance, redissolved in 1 ml of distilled water, and subjected to scintillation counting in 10 ml Aquasol (DeVries et al., 1970, 1971).

J. Determination of Free Radioactivity in Plasma

In order to determine how much of the injected dose of radioisotope had remained free in the plasma of both toadfish and Antarctic fishes, 100λ of clear plasma was drawn off from a regular blood sample. This aliquot was precipitated with an equal volume of cold 10% TCA, and the precipitation was allowed to take place in the cold overnight to insure completeness. The next day, the precipitated

plasma was centrifuged at 2000 rpm in a clinical centrifuge for 10 min, and 100λ of the supernatant was removed. This supernatant aliquot was subjected to scintillation counting in 10 ml Aquasol.

K. Liver Homogenization, Liver Protein Determination, and Liver Free Radioactivity Determination

Incorporation of radioactivity into liver proteins and the total amount of free radioactivity in liver were determined from a homogenate of the entire liver. In toadfish, the entire liver was quickly excised from the body cavity at the end of any given experiment, and the liver was then weighed and homogenized in two volumes of Medium A (0.35 M sucrose, 0.05 M Tris, pH 7.4, 0.025 M KCl, 0.01 M $MgCl_2$; Haschemeyer, 1973). Homogenization was performed in the cold for 3 min at top speed on a Sorvall Omnimixer (Sorvall Instrument Company). Homogenates were then poured into 40 ml oak ridge tubes and allowed to settle for about 1 hr on ice. After settling, 100λ of the homogenate was pipetted off onto a 2.4 cm Whatman #1 filter disc. The homogenate on the filter was allowed to dry for 2 to 3 min and subsequently subjected to the same washing procedure as described for plasma protein filters. After drying, the filters were subjected to scintillation counting in toluene containing PPO and POPOP. The procedure for making liver homogenates in all specimens of the genus

Trematomus was the same as the procedure described for toadfish.

In experiments on Antarctic cod, the liver was always far too large to allow the rapid preparation of a complete-liver homogenate. Unlike toadfish, whose livers usually weighed between 5 gm and 15 gm, and Trematomus specimens, whose livers usually weighed between 3 gm and 10 gm, the livers of D. mawsoni specimens usually weighed anywhere from 150 gm to 300 gm. This large liver prohibited, given the equipment at hand, the rapid homogenization of the entire organ. Thus, the preparation of liver homogenates in D. mawsoni had to be slightly modified. At the termination of experiments involving Antarctic cod, the entire liver was quickly excised from its attachments in the body cavity, and it was then removed to a tray containing aluminium foil-covered ice. The entire organ, while on ice, was thoroughly and quickly mashed by hand; as the mashing was going on, the liver was also being thoroughly mixed together in order to avoid selecting extremely radioactive (or relatively non-radioactive) portions for homogenization (see Distribution Study, Appendix II). After being mashed and mixed, portions of the organ were thoroughly homogenized in the manner described above for toadfish and Trematomus specimens. No less than 20% by weight of the liver was ever homogenized.

Liver free radioactivity was determined from the liver homogenates of all fishes studied by precipitating 0.5 ml of the homogenate with an equal volume of cold

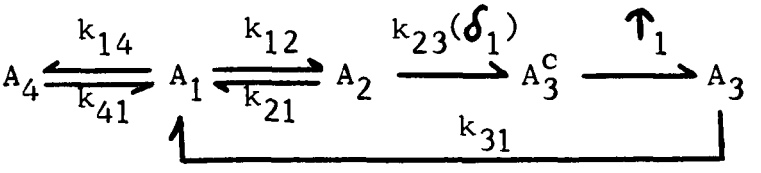
10% TCA. Precipitated homogenates were allowed to sit overnight in the cold to insure complete precipitation. The following day, the precipitates were centrifuged at 2000 rpm for 10 min in a clinical centrifuge. 100λ of the supernatant was pipetted into 10 ml Aquasol and subjected to scintillation counting.

L. Analysis of Data

Most preliminary data analysis was performed by computer, as was the entire kinetic analysis. The computer employed for these purposes was the IBM 370 computer of The City University of New York. All programs used in data analysis will be found in Appendix I.

M. Leucine Kinetic Analyses

Analysis of leucine incorporation data, both in toadfish and in the Antarctic fishes, was based on the following reaction scheme:



(15)

The A_i denote total amino acid residues, in the case of flux analysis, or fraction of recovered radioactivity, in the case of labelling kinetics, in the following compartments: A_1, intrahepatic amino acid pool; A_2, intrahepatic plasma proteins at the ribosomal synthetic site; A_3^c, intrahepatic

plasma proteins after completion of migration through the secretory system; A_3, plasma proteins released into the circulation following passage through a collecting vesicle; and A_4, intrahepatic proteins not subject to secretion.

The rate constants designated k_{12} and k_{14} are first-order rate constants for synthesis of plasma proteins and intrahepatic proteins, respectively. k_{21} and k_{41} are the corresponding rate constants for degradation. The constant designated k_{23} is used to describe the final forward flux of plasma proteins through the secretory system. If no degradation of newly-synthesized plasma protein occurs, that is, if $k_{21} = 0$, then the secretory flux $k_{23}A_2$ will be the same as the synthetic flux $k_{12}A_1$. k_{31} describes the normal degradation of circulating plasma proteins. In the analyses of toadfish data at 7^{o} and all Antarctic fishes at -1.5^{o}, it was assumed that there was no significant degradation during the timecourse of the experiment. Thus, at these temperatures k_{31} was set to zero. In the leucine kinetic analysis of toadfish at 20^{o}, a value of $0.0032\ hr^{-1}$ was used to describe the degradation of plasma proteins (Haschemeyer, 1973).

The first time constant δ_1 refers to a period of vectorial migration through the secretory system. In the actual analyses, it was obtained experimentally from the value of the absolute lag time between hepatic portal vein injection of radioactively-labelled amino acid and the first

appearance of radioactive protein in the blood. The term τ_1 represents the residence time for plasma proteins in a collecting vesicle prior to secretion. This time constant was derived from the well-stirred chemical reactor approximation in which each increment of influx q_o into a closed reactor space produces a time-dependent efflux described by the equation:

$$q(t) = q_o e^{-t/\tau} \tag{16}$$

(Denbigh and Turner, 1971). In a previous analysis of data in summer toadfish (Haschemeyer, 1973), this term was not included. It was found to be obligatory in the present analyses, especially for the description of kinetics at low temperatures. Without τ_1, as will be described later, the pronounced sigmoidicity of experimental data could not be fitted by theoretical curves.

Actual calculation of rate constants was carried out by means of the differential equations, based on the reaction scheme in equation (15):

$$\begin{aligned}
&(a) \quad dA_1/dt = -(k_{12} + k_{14})A_1 + k_{21}A_2 + k_{41}A_4 \\
&(b) \quad dA_2/dt = k_{12}A_1 - (k_{21} + k_{23})A_2 \\
&(c) \quad dA_3^c/dt = k_{23}A_2 - (1/\tau)\Sigma q_i(t) \\
&(d) \quad dA_3/dt = (1/\tau)\Sigma q_i(t) \\
&(e) \quad dA_4/dt = k_{14}A_1 - k_{41}A_4
\end{aligned} \tag{17}$$

where the $q_i(t)$ represent increments of efflux from the vesicle derived from each earlier increment of influx $dA_2(t)$. Equation (17a) describes the overall synthesis of both plasma proteins and intrahepatic proteins, and includes degradation terms for newly-synthesized proteins. Equation (17b) describes the flux of plasma proteins at their ribosomal synthetic site and includes terms describing both degradation of newly-synthesized plasma protein ($k_{21} \neq 0$) and movement of these proteins into a collecting vesicle from the synthetic site. Equation (17c) describes the flux within the vesicle, and equation (17d) represents the efflux from the vesicle into the circulation. Equation (17e) describes the flux of intrahepatic proteins not subject to secretion. In analysis of 20° toadfish data, where k_{31} was included to describe turnover of plasma proteins in circulation, equation (17d) was modified in the following manner:

$$dA_3/dt = (1/\tau)\Sigma q_i(t) - k_{31}A_3 \qquad (18)$$

Solution of these equations was performed by numerical integration using Fortran programs VES90 and DEGV90, which will be found in Appendix I.

All calculations were carried out with an initial value of 1.0 for compartment A_1 and all other compartments set at zero. Experimental data were expressed in a manner similar to that generated by the computer solutions, that is, as fractions of recovered radioactivity such that ΣA_i at

all times was equal to 1.0. This method of analysis excludes any radioactive amino acid that might have been available for protein synthesis in liver at the beginning of the experiment, but which was not recovered in protein or as free amino acid at the end.

N. Alanine Kinetic Analyses

The reaction scheme used to describe the alanine kinetics in the Antarctic species includes the reactions shown in equation (15) in which the A_i refer to alanine residues or fractional radioactivity in alanine in the various compartments. In addition, it was assumed that synthesis of the antifreeze glycoprotein drew upon the same amino acid pool A_1 and involved a similar secretion pathway designated as follows:

$$A_1 \underset{k_{51}}{\overset{k_{15}}{\rightleftharpoons}} A_5 \xrightarrow{k_{56}(\delta_2)} A_6^c \xrightarrow{\tau_2} A_6 \qquad (19)$$

The additional compartments included are: A_5, intrahepatic AFGP polypeptide at the ribosomal synthetic site; A_6^c, intrahepatic AFGP after completion of migration; and A_6, AFGP released to the circulation after passage through a collecting vesicle. k_{15} represents the synthetic rate constant for AFGP, and k_{51} represents a constant for degradation, if any, at the ribosomal site. k_{56} is a rate constant for the forward secretory flux. δ_2 is the migration time and is analogous to the δ_1 described in

the previous section. τ_2 is the collecting vesicle residence time, as described above. Differential equations similar to those described in equation (17) and based on equations (15) and (19) were solved simultaneously on the computer to obtain a fit of the alanine data. The computer program in Fortran language used for these analyses was designated ALAV90 and will be found in Appendix I. Other reactions of alanine metabolism have been neglected in this analysis, since the experimental data obtained represent only the radioactivity recovered as free alanine or in protein-bound alanine at the end of the experiment. As in the analyses of leucine kinetics, all calculations were carried out beginning with a value of 1.0 for A_1 and all other compartments set to zero. Experimental data were expressed as fractions of recovered radioactivity such that ΣA_i at all times was equal to 1.0.

III. TOADFISH RESULTS

A. Plasma Protein Synthesis at 7°

1. Experimental Results

The study of plasma protein synthesis and secretion in toadfish at low temperatures was carried out in winter at the ambient temperature (1974-1975) of 7°. As shown in Figure 2, radioactive plasma protein begins to appear in the circulation at about 4 to 5 hr following hepatic portal vein injection of radioactive leucine. Accumulation, as indicated by the experimental points, proceeds sigmoidally over the next two days. A theoretical curve, derived by the kinetic analysis described below, is also shown.

Table 1 summarizes the experimental time constants for plasma protein appearance and the final (48 hr) compartment data at 7°. The former were obtained by exponential analysis of the early part of the rise curve (Figure 2), as previously described (Haschemeyer, 1973). The recovery of radioisotope in total liver protein, total plasma protein, and the liver acid-soluble pool averaged 15% of dose. In contrast to earlier studies in toadfish, these fish showed a high proportion of free radioactivity in liver that could not be accounted for as leucine. This was evident both in the kinetic analysis of the data and in examination of plasma to tissue ratio of free radioactivity.

FIGURE 2

Time course of appearance of radioactive protein in plasma of toadfish following hepatic portal vein injection of ^{3}H-leucine. Experimental temperature is 7^{o}. Radioactivity (A) has been corrected for blood loss due to sampling. The curve shown is based on kinetic analysis as described in the text.

FIGURE 2

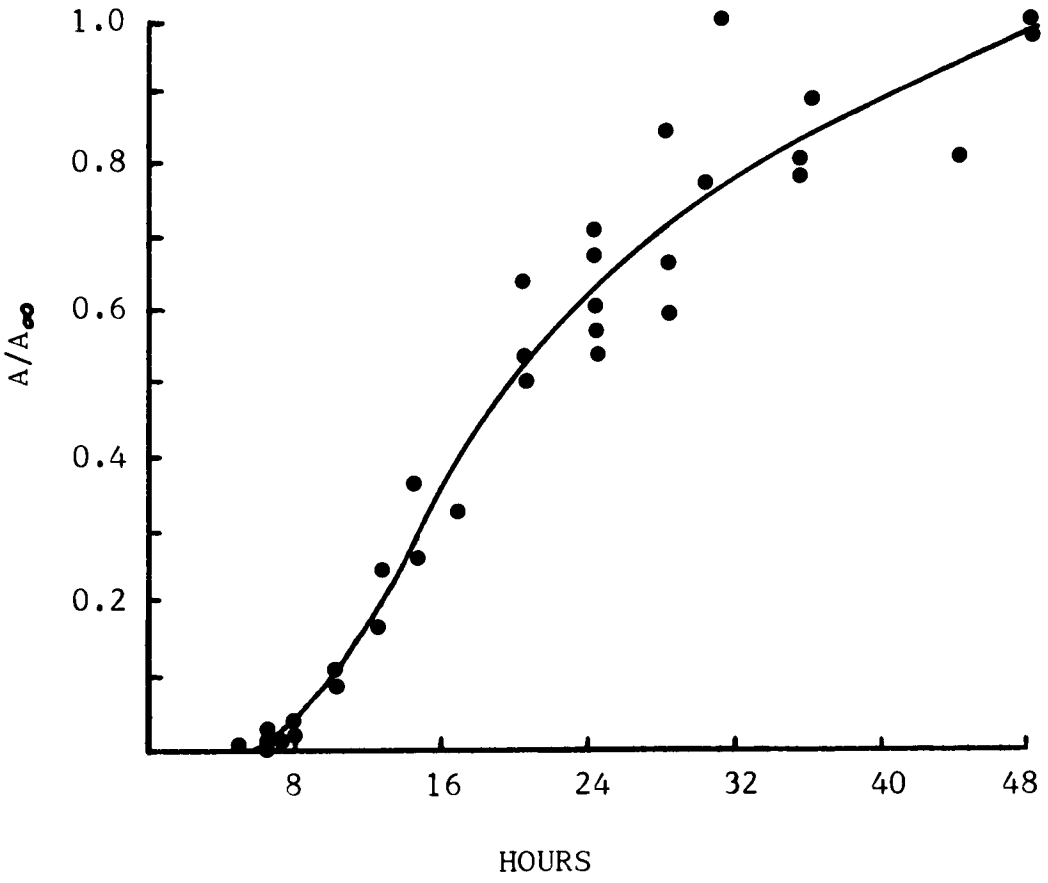


TABLE 1

Recovery of radioactivity in various compartments of liver and plasma of toadfish following hepatic portal vein injection of ^{14}C-leucine at 7°. Estimated experimental time constants at this temperature were: lag time = 5.0 hr; $t_{\frac{1}{2}}$ = 12.4 hr.

		Final Compartments Fraction of Recovery		
	Recovery (% of dose)	Liver Free Pool	Liver Protein	Plasma Protein
without correction	15 ± 3 (15)	0.20 ± 0.15	0.64 ± 0.04	0.16 ± 0.04
with correction for leucine metabolism	13 ± 3 (15)	0.05 ± 0.03	0.76 ± 0.04	0.19 ± 0.05

$$\text{Average } \frac{\text{Liver Free Radioactivity}}{\text{Plasma Free Radioactivity}} = 3.9 \pm 1.5 \ (28)$$

$$\text{(Plasma Protein)/(Liver Protein + Plasma Protein)} = 0.20 \pm 0.03 \ (15)$$

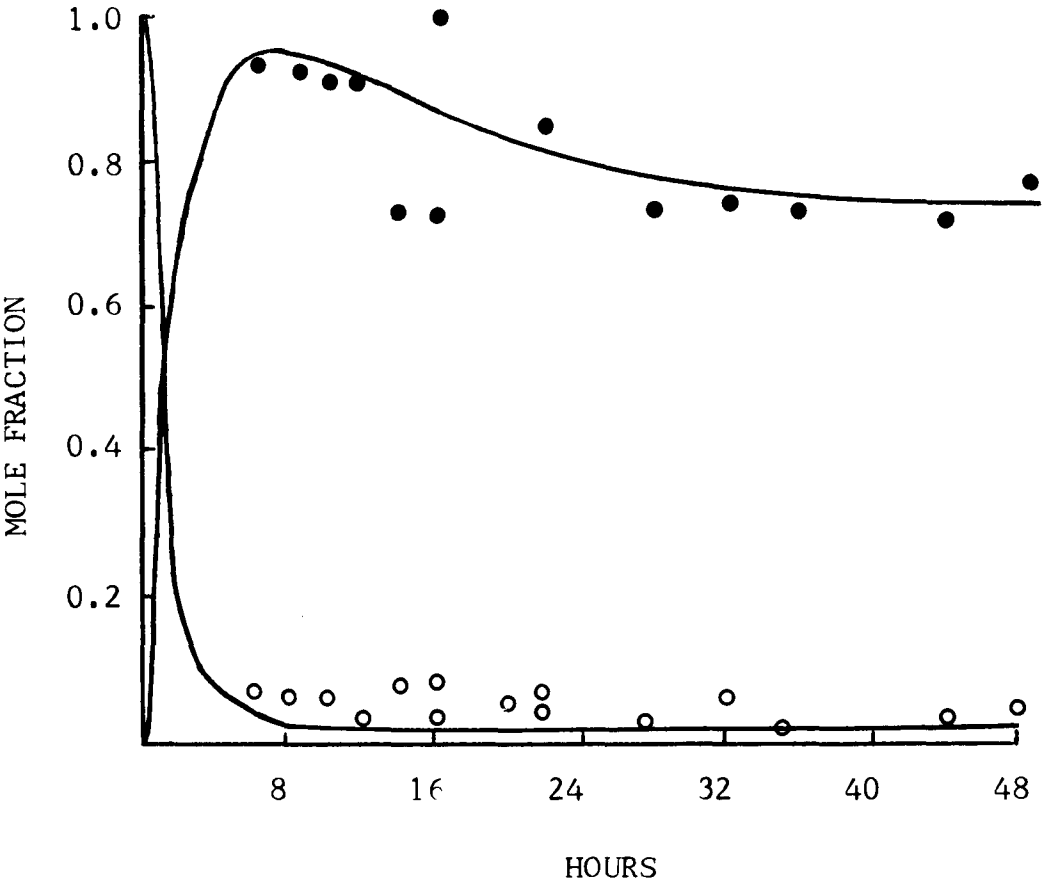
Studies of leucine transport in toadfish (Persell and Haschemeyer, 1976) have shown only a slight accumulation of free leucine in liver relative to plasma, whereas in the present studies free radioactivity ratios (liver:plasma) averaged 3.9 ± 1.5. Correction for the presence of radioactive metabolites of leucine in liver, based on plasma free radioactivity, yielded the set of normalized compartment values given in the second line of Table 1.

In a second series of experiments, toadfish were periodically sacrificed at various times after injection of radioactive tracer in order to determine the time course of labelling of endogenous liver proteins. These data are illustrated by the experimental points in Figure 3, which represent normalized recoveries of radioactivity in liver protein and free leucine calculated from plasma levels. In addition, theoretical curves based on the best-fitting kinetic constants derived below are shown. The decline in the proportion of label in liver protein may be accounted for by the secretion of labelled plasma protein and by a small component of intrahepatic protein degradation. Results for free radioactivity in liver are still higher than can be accounted for kinetically; this is an indication that an additional fraction of this isotope is not available for incorporation into protein. It is difficult to account for this, since other studies in which chromatography was used to identify radioactive leucine also showed higher than anticipated

FIGURE 3

Time course of appearance of radioactivity in liver protein (closed symbols) and as liver free radioactivity (open symbols) in toadfish following hepatic portal vein injection of ^{14}C-leucine. Experimental temperature is 7^{o}. The curve shown is based on kinetic analysis as described in the text.

FIGURE 3



levels in the acid-soluble fraction of liver (Haschemeyer, 1973).

The results of chemical analyses and protein synthetic rate determinations in winter toadfish are summarized in Table 2. These data were used to calculate kinetic constants for synthesis, as presented below.

2. Kinetic Analysis for Toadfish at 7^{o}

a. Total protein synthetic rate ($k_{12} + k_{14}$)

Analysis for total liver RNA in winter toadfish (Table 2) indicates a ribosome concentration in the tissue of about 1.25 nmole/gm, based on a ribosomal RNA molecular weight of 2.7×10^{6} daltons and the estimate that 80% of liver RNA occurs in active ribosomes. Polyribosome profiles in toadfish liver have generally supported the conclusion that most or all liver ribosomes are active in normally-fed fish (Haschemeyer, 1969a), although degradation artifacts have limited the use of this technique. Average polypeptide chain assembly time at 7^{o} in toadfish liver is estimated at 28 min, based on an Arrhenius plot of published data for winter toadfish (Nielsen et al., 1977). The rate of leucine incorporation into polypeptide chains may then be calculated from the following equation:

$$\text{nmole leu/min/gm} = \frac{(1.25 \text{ nmole Rs/gm})(44 \text{ leu/chain})}{28 \text{ min}} \qquad (20)$$

TABLE 2

Chemical analyses of liver and plasma of toadfish.

Liver	
RNA (mg/gm liver)[a]	4.2 ± 1.0 (16)
Protein (mg/gm liver)[a]	115 ± 15 (12)
Leucine, free (μmole/gm)	0.17 ± 0.04 (5)
Plasma	
Protein (not including fibrinogen, mg/ml)	26 ± 3 (5)
Fibrinogen (mg/ml)	1.3 ± 0.7 (45)
Liver Protein Synthesis	
Average polypeptide chain[b] assembly time (minutes)	
at 7°	28
at 20°	4.0

[a] from Nielsen et al., 1977

[b] extrapolated from measurements at 9° and 19° (Nielsen et al., 1977)

The analysis of protein in plasma was done by biuret method.

where a 10% leucine content by weight is taken for liver-synthesized proteins, estimated from data in rat liver (Peters and Peters, 1972). The result is 2.0 nmole/gm/min or 0.12 μmole/gm/hr.

Leucine incorporation may also be expressed by the product of the synthetic rate constant and free tissue leucine concentration. The latter, from Table 2, is about 0.17 μmole/gm liver. When combined with the leucine protein synthetic flux calculated above, $(k_{12} + k_{14})$ is found to be 0.69 hr^{-1}. This value corresponds to a half-life for turnover in the intracellular leucine pool $[\ln 2/k]$ of 1 hr.

b. Plasma protein synthetic rate (k_{12})

The ratio of label recovered in plasma protein compared to total liver plus plasma protein (P/L+P) provides an estimate of the proportion of liver protein synthesis directed toward plasma proteins. In the present study a value of 20% was found (Table 1), similar to that obtained in summer toadfish studied at 20° (Haschemeyer, 1973) and in rat (Peters and Peters, 1972). The true proportion of synthesis, however, could be lower if degradation of newly-synthesized intrahepatic protein occurs during the 2-day course of the experiment. A minimum value for this parameter can be determined by consideration of the size of the total plasma protein pool and the half-life of protein in circulation. A previous calculation in toadfish

(Haschemeyer, 1973) indicated that this minimum is 16% of liver protein synthesis. In terms of the value of $(k_{12} + k_{14})$ derived above, a minimum value for k_{12} is 0.10 hr^{-1}. This value proved to give a satisfactory fit of the compartment data at 7°.

The value of k_{12} can be used to calculate the half-life of plasma protein in the circulation at 7°. Total leucine flux into plasma protein will be given by:

$$(0.10\ hr^{-1})(0.17\ \mu mole/gm\ liver) = 0.017\ \mu mole/gm/hr \quad (21)$$

or 0.41 μmole/gm/day. The total body pool of plasma protein relative to liver weight may be obtained from the following:

$$\frac{(28\ mg\ protein/ml\ plasma)(1.7\ ml\ plasma/100\ gm\ body\ weight)}{2.6\ gm\ liver/100\ gm\ body\ weight}$$

If plasma protein is assumed to contain 11% leucine by weight as in rat (Peters and Peters, 1972), one finds total leucine in plasma protein to be 18 μmole/gm liver. Together with the replacement rate derived above, one calculates a degradation rate constant of 0.023 day^{-1}. Conversion to half-life by the standard equation for a first-order reaction $\left[t_{\frac{1}{2}} = \ln 2/k\right]$ yields a result of 30 days or about 4 weeks.

c. Other rate constants

The constant for degradation of newly-synthesized plasma protein within the liver (k_{21}) was taken to be zero. Although degradation could occur, providing synthetic capacity is increased proportionately, the present data cannot distinguish these possibilities. The degradation constant for intrahepatic proteins (k_{41}) was varied in order to obtain the best fit of Figure 3 and the final compartment values (Table 1). The constants associated with the secretion process (k_{23}, τ) were varied to fit the plasma protein accumulation curve of Figure 2. The constant τ is primarily responsible for generating the early sigmoidal time dependency of the secretion curve. Finally, the constant δ which describes the absolute lag period or migration time in secretion was determined by transposing the theoretical curve along the abcissa in Figure 2.

d. Kinetic results

The first column of Table 3 presents the kinetic constants that yielded the best fit of the combined data of Figures 2 and 3 and Table 1. These results show the following important features:

(1) Plasma protein synthesis is taken to account for 16% of total liver protein synthesis. In order to generate the observed P/L+P ratio of labelling of 20%, a proportion of

TABLE 3

Kinetic constants and final calculated compartment values in 7^{o} winter toadfish (t = 48 hr).

	Best Fit	Reduced k_{41}
k_{12} (hr^{-1})	0.1	0.1
k_{14} (hr^{-1})	0.51	0.51
k_{41} (hr^{-1})	0.010	0.00175
k_{23} (hr^{-1})	0.15	0.15
τ_1 (hr)	6.0	6.0
δ_1 (hr)	4.2	4.2
A_1	0.02	0.002
A_3	0.20	0.17
Liver Protein	0.78	0.83

The compartment designated Liver Protein is the sum of A_2, A_3^c, and A_4.

intrahepatic labelled protein must be degraded. An exact fit of this ratio is obtained with $k_{41} = 0.01\ hr^{-1}$. This value may be compared with the minimum value of k_{41} estimated from net synthetic rate of intrahepatic proteins:

$$k_{41}^{minimum} = \frac{0.10\ \mu mole\ leu/gm/hr}{100\ \mu mole\ protein\text{-}bound\ leu/gm} \qquad (22)$$

$$= 0.0010\ hr^{-1}$$

where the denominator represents the amount of protein-bound leucine residues in liver protein per gm tissue (calculated from the data of Table 2). This calculation assumes that all proteins turn over at the same rate. The value of k_{41} derived from the kinetic analysis is higher, as expected in view of the wide spectrum of half-lives observed for liver proteins (Schimke, 1973). Proteins with short half-lives will make up a larger proportion of synthesis than will long-lived proteins, and their degradation will contribute to loss of labelled protein during the 2-day experiments. The choice of $k_{41} = 0.01$, however, did not give the best fit to the time course of liver protein labelling (Figure 3). This curve was best approximated by $k_{41} = 0.016$ with an increase in P/L+P ratio to 0.22. In view of the experimental error in Figure 3, such an increase in k_{41} is probably not justified. Results are also shown for a lower value of k_{41}, to be discussed in the following section.

(2) A consistent set of secretion constants could be generated to give a very accurate fit of the sigmoidal time course for plasma protein in Figure 2. Of particular importance here has been the introduction of the residence time τ. Previous kinetic analysis (Haschemeyer, 1973) could not account for the sigmoidal shape which is observed in low temperature secretion curves. At higher temperatures the reactions are too fast to clearly delineate these processes, although the sigmoidal shape can be discerned in rat data as well (Peters and Peters, 1972).

B. Plasma Protein and Fibrinogen Synthesis at 20°

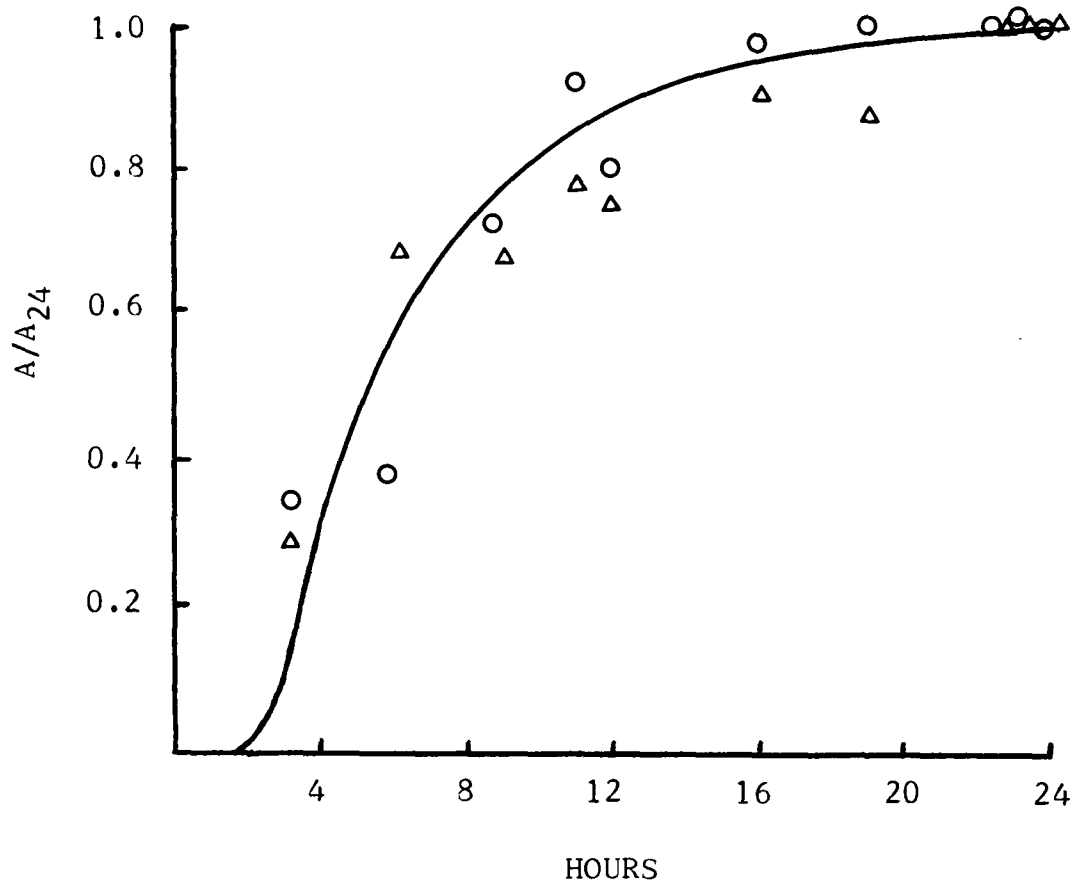
1. Experimental Results

Figure 4 shows the combined results of several experiments designed to monitor the appearance of a specific protein, fibrinogen, in the plasma as a function of time at 20°. The radioactive tracer used in these experiments was ^{14}C-leucine, and the dose was 15 μCi/fish. Figure 4 shows the time course of appearance of label in TCA-precipitable plasma protein in the blood in this series of experiments. Absolute lag time for the appearance of label in total plasma protein and in fibrinogen is estimated in the range of 1-1.5 hr, as in previous studies at this temperature. The half-time for accumulation of label (based on exponential analysis was about 5 hr. The plateau level for

FIGURE 4

Time course of appearance of radioactivity in total plasma protein, including fibrinogen, (▵) and fibrinogen alone (o) in toadfish following hepatic portal vein injection of ^{14}C-leucine. Experimental temperature is 20^{o}. The curve shown is based on kinetic analysis as described in the text.

FIGURE 4



incorporation of radioactive isotope was reached after about 16 hr for total plasma protein and 22 to 24 hr for fibrinogen.

Table 4 presents the final compartment data (t = 24 hr) obtained in the 20° experiments. Free pool values for leucine have been corrected by use of tissue:plasma ratio in order to exclude metabolic products of labelled leucine which accumulate in liver. The distribution of incorporation between plasma and liver protein is similar to that obtained at 7°; i.e., P/L+P = 0.19. The proportion of plasma protein synthesis identifiable as fibrinogen was 0.008/0.18, or about 4.4%. This is very close to the result obtained by analysis of total clottable protein in plasma. Thus, from Table 2, fibrinogen was found to account for 4.7% of total plasma protein synthesis in toadfish, based on analyses in 45 fish.

2. Kinetic Analysis at 20°

a. Total protein synthetic rate ($k_{12} + k_{14}$)

Liver synthetic rate at 20° can be estimated as described in the 7° kinetic analysis by use of an average polypeptide chain assembly time of 4.0 min, based on published results in winter toadfish (Nielsen et al., 1977). Component concentrations are identical to those used previously, and yield a synthetic rate constant ($k_{12} + k_{14}$) = 4.9 hr^{-1}.

TABLE 4

Recovery of radioactivity in various compartments of liver and plasma of toadfish following hepatic portal vein injection of ^{14}C-leucine at 20°. (N = 6)

			Fraction of Recovery		
	Lag (hr)	$t_{1/2}$ (hr)	Liver Free Pool	Liver Protein	Plasma
Total Plasma Protein	1.0	5.3	0.03 ± 0.01	0.79 ± 0.08	0.18 ± 0.08
Fibrinogen	1.5	4.9	0.03 ± 0.01	0.79 ± 0.08	0.008 ± 0.003

Percent Recovery of Dose = 11 ± 2 (6)

b. Plasma protein and fibrinogen synthetic rates

The minimum value for k_{12} based on 16% of synthesis directed toward plasma protein is 0.8 hr^{-1} for this temperature. This value may be broken down into a constant for fibrinogen synthesis (about 0.035 hr^{-1}) and the remainder (0.76 hr^{-1}) representing all other plasma proteins.

c. Other rate constants

For this analysis, the intrahepatic degradation of plasma protein was assumed to be negligible as before. With k_{12} and k_{14} determined as above, k_{41} was the only remaining parameter which significantly affected the final compartment values, in particular the P/L+P ratio. The time course of both plasma protein and fibrinogen secretion was used to obtain estimates of the secretion parameters. Insufficient data were available to evaluate fibrinogen secretion separately, although there was some indication of a slower process for fibrinogen. This would be consistent with a more complicated system for this large protein (MW = 330,000 daltons) compared to albumin (MW = 67,000 daltons) and other secreted proteins.

d. Kinetic results

Table 5 presents the final kinetic parameters for synthesis and secretion at 20^{o}. The results show agreement with the observed P/L+P ratio, i.e., 0.18. This, however,

TABLE 5

Kinetic constants and final calculated compartment values in 20^{o}-acclimated winter toadfish (t = 24 hr).

k_{12} (hr^{-1})	0.80
k_{14} (hr^{-1})	4.1
k_{41} (hr^{-1})	0.012
k_{23} (hr^{-1})	0.40
τ_1 (hr)	1.0
δ_1 (hr)	2.0
k_{15} (hr^{-1})	0.035
A_1	0.002
A_3	0.18
A_6	0.008
Liver Protein	0.83

The compartment designated Liver Protein is the sum of A_2, A_3^c, and A_4.

necessitated the choice of a rather low value for k_{41} (0.012 hr^{-1}), close to the best-fitting value obtained in the 7° analysis. The minimum value of k_{41} at this temperature, based on the synthetic flux for intrahepatic proteins (80% of total synthesis) is 0.0067 hr^{-1}. Thus, turnover rate for the labelled proteins is only about twice that of the tissue average in this analysis, compared with a ratio of 10:1 in the 7° analysis. Recalculation of the 7° kinetics using a proportionately lower value of k_{41} yielded results (see column headed "Reduced k_{41}", Table 3) with a somewhat low P/L+P ratio. This could be corrected, however, by increasing k_{12}, without falling outside one standard deviation in the data. Thus, the reduced value of k_{41} is acceptable at 7° and is consistent with the 20° results in which synthesis and degradation are taken to have the same temperature dependency.

The secretion parameters in this analysis can be considered only a rough approximation. Previous data at 20° in summer toadfish indicate that migration (δ) is about 1 hr at this temperature. The value of 1 hr obtained for the residence time (τ) is consistent with the value of 6 hr at 7°. The final constant k_{23} is greater at 20° than at 7°, as anticipated. This result is not sufficiently reliable to provide a good measure of temperature dependency. The data for fibrinogen indicate that this method can be applied to a single protein synthesized by the liver,

however much greater amounts of radioactive amino acid must be used to obtain more reliable data. The agreement between proportion of label in fibrinogen (compared to total plasma protein) and the concentration of fibrinogen in plasma confirms the assumption that these proteins are synthesized from the same amino acid pool, i.e., of liver. In contrast, as discussed in the following sections, such agreement in specific radioactivity was not found between total plasma protein and AFGP in the Antarctic fish.

IV. ANTARCTIC RESULTS

A. Plasma Protein Secretion - ^{14}C-leucine Labelling

1. Experimental Results

The first experiments in the Antarctic species were carried out with radioactive leucine in order to permit comparison with the results just described for toadfish. The timecourse for appearance of ^{14}C-leucine-labelled plasma protein in D. mawsoni is indicated by the experimental points in Figure 5. The best-fitting theoretical curve, derived as described below, is also shown. A sigmoidal time dependency is evident, as was observed in toadfish at 7^{o}. Table 6 presents the distribution of radioactivity among the various compartments at 96 hr and the time constants for plasma protein accumulation, estimated as described previously (Haschemeyer, 1973). The lag before rapid accumulation began was 10 hr, with half-maximal levels reached after a further 20 hr. Radioactivity in plasma protein was unusually high, about 40% of the total incorporation attributed to liver protein synthesis.

Table 7 summarizes data from chemical analyses of D. mawsoni required for evaluation of the protein synthetic rate constants. Because of limitations in the supply of fish and isotopes, average polypeptide chain assembly time

FIGURE 5

Time course of appearance of radioactive protein in plasma (excluding antifreeze glycoprotein) following hepatic portal vein injection of ^{14}C-leucine. Experimental temperature = -1.5°. Radioactivity (A) has been corrected for blood loss due to sampling and divided by the steady state value (A_∞) reached at 96 hr. The curve shown is based on kinetic analysis as described in text of Section IV.

FIGURE 5

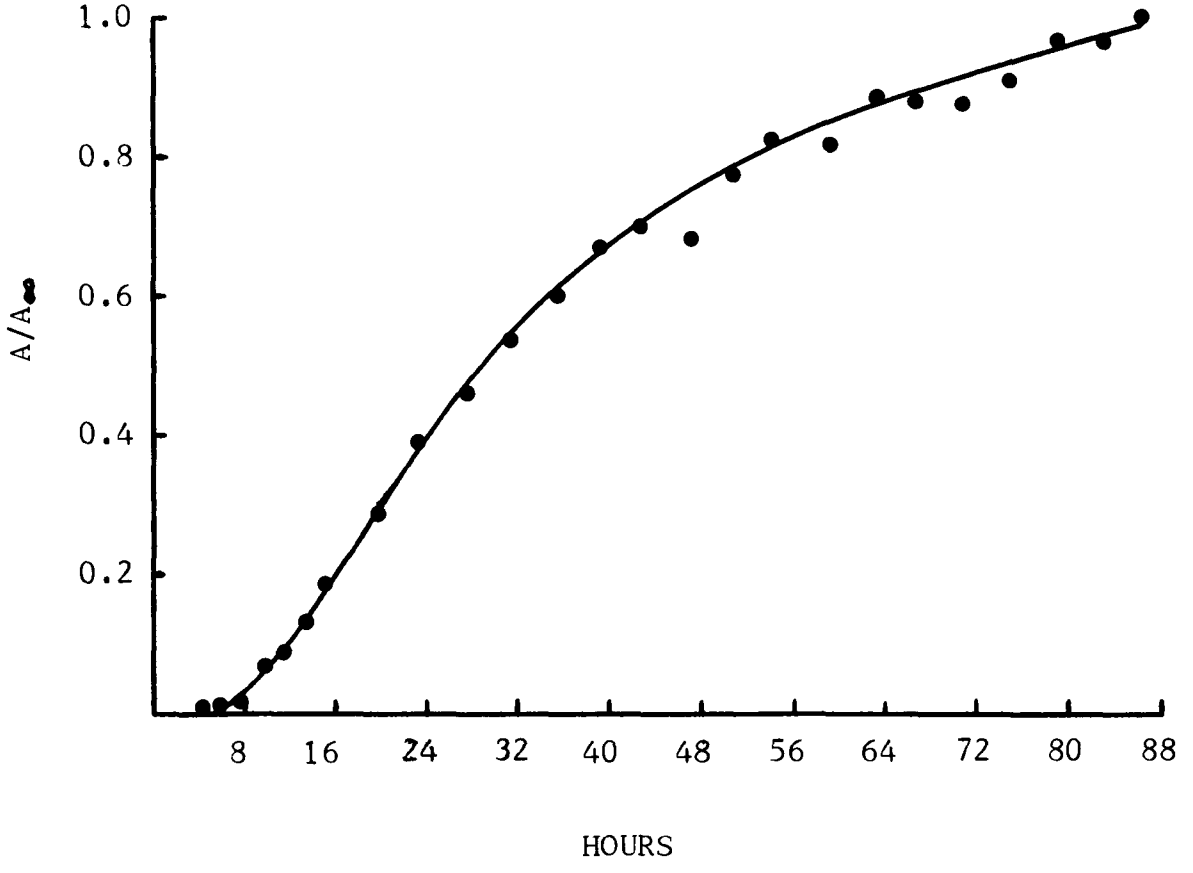


TABLE 6

Recovery of radioactivity and time constants in D. mawsoni at -1.5°.

Radioactive Amino Acid	Fractional Recovery				Time Constants			
					Plasma Protein		AFGP	
	Liver Free Amino Acid	Liver Protein	Plasma Protein	AFGP	Lag (hr)	$t_{1/2}$ (hr)	Lag (hr)	$t_{1/2}$ (hr)
Leucine	0.03	0.57	0.40	0.00	10	20	--	--
Alanine	0.03	0.47	0.40	0.10	8	10	26	13

Data are based on five specimens of D. mawsoni injected by hepatic portal vein with final analysis at t = 96 hr. Secretion lag time was determined by linear extrapolation to zero radioactivity from the period of most rapid accumulation. Accumulation half-time ($t_{1/2}$) was estimated from exponential analysis of the accumulation curve (Haschemeyer, 1973).

TABLE 7

Chemical analyses and polypeptide chain assembly time for Antarctic fish.

Liver	
DNA (mg/gm liver)	1.8 ± 0.5 (5)
RNA (mg/gm liver)	5.5 ± 1.0 (5)
Protein (mg/gm liver)	85 ± 15 (5)
Leucine, free (μmole/gm)	0.45 ± 0.20 (11)
Alanine, free (μmole/gm)	2.0 ± 1.0 (9)
Plasma	
Protein (excluding AFGP) (mg/ml)	38 ± 5 (5)
AFGP (mg/ml)	26 ± 4 (4)
Liver Protein Synthesis	
Average polypeptide chain assembly time (min)	17 ± 4 (5)

Analysis of liver and plasma was performed in *Dissostichus mawsoni*; polypeptide chain assembly time was determined in *Trematomus hansoni* at -1.5°. Results are presented with SD and number of animals. Determination of DNA, RNA, and protein was performed by the methods of Haschemeyer (1968) in liver. Plasma protein analysis was done by the biuret method.

was determined only in the small fish T. hansoni. These data were used to obtain the leucine rate constants.

2. Kinetic Analysis

a. Total protein synthetic rate ($k_{12} + k_{14}$)

Ribosome concentration can be estimated from RNA content at about 1.6 nmole/gm. If 75% of ribosomes are active (or synthesize leucine-containing proteins), leucine incorporation rate may be calculated from the equation

$$\text{nmole leu/min/gm} = \frac{(1.2 \text{ nmole/gm})(44 \text{ leu/chain})}{17 \text{ min}} \qquad (23)$$

where 17 min represents polypeptide chain assembly time (Table 7) for an average chain of 50,000 daltons containing 10% leucine by weight. Total amino acid flux in protein synthesis is thus 0.19 μmole/gm/hr and is equal to ($k_{12} + k_{14}$) times leucine concentration. The latter for the fish of Figure 5 was 0.48 μmole/gm; hence, $(k_{12} + k_{14}) = 0.40 \text{ hr}^{-1}$.

b. Plasma protein synthetic rate (k_{12})

The half-life for plasma protein in D. mawsoni may be estimated at 3 weeks, based on data in toadfish (Haschemeyer, 1973) and observations on AFGP degradation (DeVries, 1974). The total pool of leucine in plasma protein relative to liver weight is 50 μmole/gm liver. This is multiplied by the rate constant for degradation ($1.38 \times 10^{-3} \text{ hr}^{-1}$), obtained from $t_{\frac{1}{2}} = 3$ weeks, to obtain minimum synthetic flux

of plasma protein, 0.069 μmole leucine/gm/hr. Synthesis is also expressed by k_{12} [leu] ; for the fish of Figure 5, as above, one obtains $k_{12} = 0.14\ hr^{-1}$. By difference, $k_{14} = 0.26\ hr^{-1}$.

c. Other rate constants

The constant for degradation of newly-synthesized plasma protein (k_{21}) was initially taken as zero. The remaining constants (k_{41}, k_{23}, and τ_1) were varied to fit the final compartment values (Table 6) and the plasma protein labelling curve (Figure 5). The intrahepatic degradation constant k_{41} influences the distribution of radioactivity in the three compartments as assayed at the end of the experiment; k_{23} and τ_1 affect the rate of rise of radioactivity in plasma protein (A_3) and the sigmoidal shape of the curve. The time constant δ_1 determines absolute lag period.

A satisfactory fit of the experimental data, as evidenced by the theoretical curve shown in Figure 5, was obtained with the rate constants summarized in Table 8 for leucine. These constants also gave agreement between calculated and experimental values for the compartments. The component for plasma protein synthesis (k_{12}) could be increased above the minimum level required for replacement if a corresponding degradation term (k_{21}) was added. This, however, would require that an even greater proportion of ribosomes be engaged in plasma protein synthesis.

TABLE 8

Kinetic constants and calculated fractional recoveries of radioactive amino acids.

	Leucine	Alanine
k_{12} (hr^{-1})	0.14	0.044
k_{14} (hr^{-1})	0.26	0.076
k_{41} (hr^{-1})	0.085	0.007
k_{23} (hr^{-1})	0.05	0.20
δ_1 (hr)	2	2
τ_1 (hr)	6	6
k_{15} (hr^{-1})	--	0.011
k_{56} (hr^{-1})	--	0.05
δ_2 (hr)	--	14
τ_2 (hr)	--	6
A_1	0.01	0.02
A_3	0.40	0.41
A_6	--	0.10
Liver Protein	0.59	0.47

Results represent the best-fitting solution of equation (15) for leucine and equations (15) and (19) for alanine. Degradation constants k_{21} and k_{51} were set equal to zero. The compartment designated liver protein is the sum of A_2, A_3^c, and A_4 for leucine; the sum of A_2, A_3^c, A_4, A_5, and A_6^c for alanine.

B. Plasma Protein and AFGP — ^{14}C-alanine Labelling

1. Experimental Results

Figure 6 presents data from four D. mawsoni on the time course of appearance of radioactive alanine in plasma protein (not including AFGP) and in the antifreeze glycoprotein. Theoretical curves based on the reactions of equations (15) and (19) are also shown. No attempt was made to take into account the slight diurnal variation observed in the data beyond 40 hr.

2. Kinetic Analysis

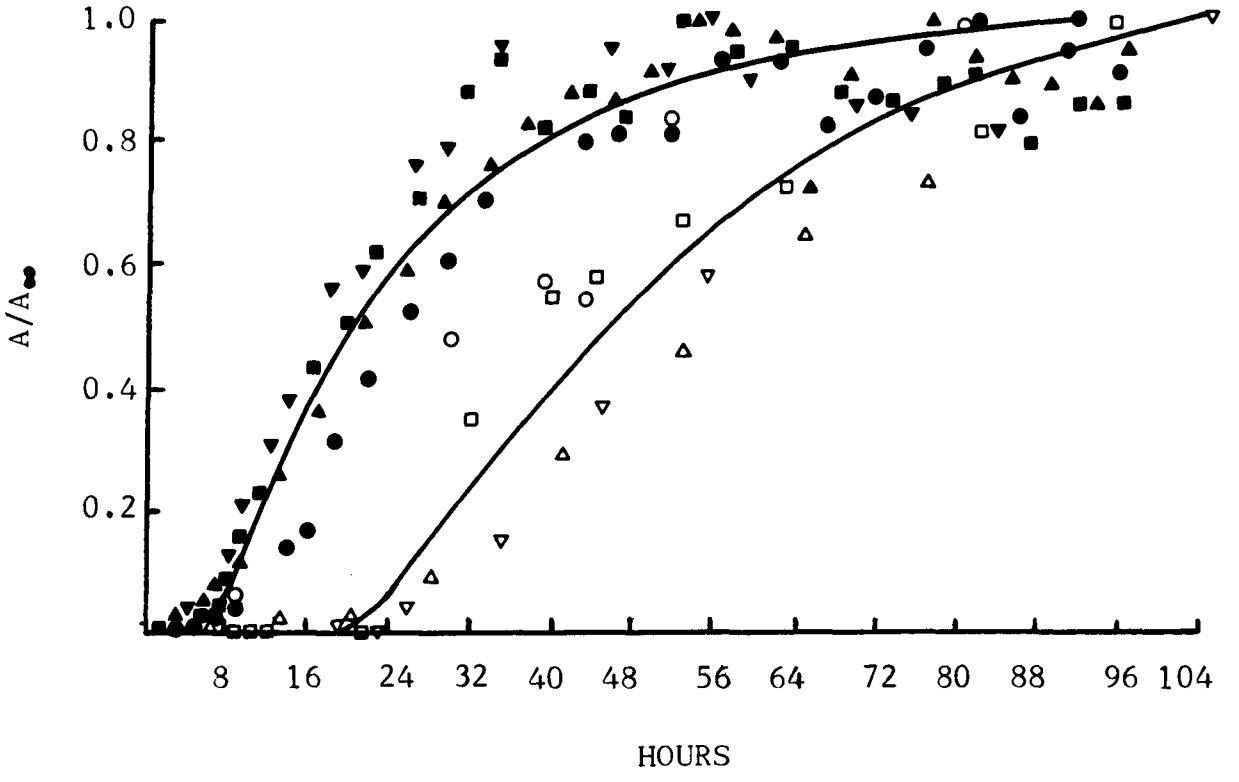
a. Protein synthetic rate ($k_{12} + k_{14}$)

Total alanine flux in protein (excluding AFGP) was obtained as described for leucine. The average polypeptide chain was estimated to contain 56 alanine residues. The resultant total flux, 0.24 μmole/gm/hr, was divided by the free alanine concentration (Table 7) to obtain $(k_{12} + k_{14}) = 0.12\ hr^{-1}$. The pool of alanine in plasma protein is 64 μmole/gm liver based on an 8% alanine content in protein by weight. This is multiplied by the turnover rate constant ($1.38 \times 10^{-3}\ hr^{-1}$) and divided by liver free alanine concentration to obtain $k_{12} = 0.044\ hr^{-1}$. Thus, k_{14} is $0.076\ hr^{-1}$.

FIGURE 6

Incorporation of ^{14}C-alanine into plasma protein (closed symbols) and antifreeze glycoproteins (open symbols) in D. mawsoni at -1.5°. Four different individuals are represented by the various symbols. Theoretical curves based on the kinetic analysis are shown.

FIGURE 6



b. Synthetic rate for AFGP (k_{15})

If the AFGP is synthesized from the same alanine pool as other plasma proteins, the ratio of final steady state incorporation levels may be used to obtain relative synthetic rates. The data of Table 6 indicate that incorporation into AFGP is one-fourth that into plasma protein, thus, $k_{15}/k_{12} = 0.25$. From the result above for k_{12}, one obtains $k_{15} = 0.011\ hr^{-1}$.

c. Other rate constants

It was assumed that no degradation of AFGP occurs at the site of synthesis, thus, $k_{51} = 0$. Liver protein degradation (k_{41}) was varied to obtain agreement with the alanine compartment data (Table 6). As expected, it was similar to that derived in the leucine analysis. The secretion constants, k_{23} and τ_1 for plasma protein, and k_{56} and τ_2 for AFGP, were adjusted in order to fit the time course for secretion, as shown in Figure 6. The absolute lag times δ_1 and δ_2 were obtained by transposing the theoretical curves along the time axis.

Table 8 summarizes the final alanine kinetic constants. Those for plasma protein synthesis and secretion are consistent with the leucine results, as expected. The constants for AFGP secretion are significantly different, however, and reflect the slow labelling pattern that is apparent in the experimental data. This has been analyzed

in terms of a prolonged migration time (14 hr) and a slower secretory rate (k_{56}). Residence time has been maintained at the same value as for plasma protein. However, a longer time could be accommodated if k_{56} were increased. Determination of a unique set of secretory constants would require additional data, e.g., on intracellular concentrations of AFGP.

d. Inhibition of protein synthesis

Injection of cycloheximide 2 hr before administration of radioactive alanine in T. bernacchii caused an 85% reduction in incorporation into liver and plasma protein (see Table 9). In cycloheximide-treated fish, incorporation into AFGP was 0.015% of dose, or 1/4 that into plasma protein, as in control D. mawsoni (Table 6). Thus, the effect of the inhibitor on AFGP synthesis was comparable to its effect on liver protein synthesis, as expected if the AFGP polypeptide undergoes normal messenger-directed synthesis on ribosomes. The possibility of an alternate enzymatic assembly of the repeating polypeptide thus appears to be excluded. The experiment, however, does not establish that liver is the site of AFGP synthesis, since other cells would also be affected by the inhibitor.

TABLE 9

Inhibition of protein synthesis by cycloheximide.

	Percent of Dose		
	Liver		
	Total Acid-soluble	Protein	Plasma Protein
Control	1.6 ± 0.8 (6)	1.5 ± 0.7	0.40 ± 0.15
Cycloheximide-treated	1.9 ± 0.4 (4)	0.24 ± 0.10	0.06 ± 0.03

Data represent percent of injected ^{14}C-alanine recovered in various compartments in Trematomus bernacchii 85 hr after venous injection. Results include SD and number of animals. Average body weight was 198 ± 58 gm; average liver weight was 4.5 ± 0.5 gm.

V. DISCUSSION

A. A New Kinetic Model for Liver Protein Synthesis and Secretion

A new model has been presented in this thesis (see Section II. M and N) to evaluate incorporation data related to liver protein synthesis and secretion in vivo. The scheme is based upon the existence of three basic types of reaction: first, a set of monomolecular reactions describing synthesis and degradation of protein in liver as in previous work (Haschemeyer, 1973); second, a simple concentration-dependent term for the secretory flux including an absolute lag time for vectorial migration; and third, a chemical reactor approximation to describe a process of continuous collection and release of material from some type of vesicles, either of the endoplasmic reticulum or the Golgi apparatus. These reactions are intended to approximate as closely as possible the processes described by electron microscopic studies of secretory tissues (Section I. E). In addition, a major objective was to establish the simplest possible kinetic scheme which could account for the observed kinetics of plasma protein labelling following a pulse injection of radioactive amino acids. The results obtained in the present experimental studies are adequately accommodated by the model. This is particularly evident for the low tempera-

ture data in toadfish and in the Antarctic cod. This model provides a framework in which to evaluate liver protein metabolism in relation to a variety of external factors, including temperature and other environmental parameters, hormones, and conditions of stress such as hemorrhage (see Appendix IV.).

Certain limitations of the model also provide a clue to future areas for research. A major problem is the determination of an intrahepatic protein degradation constant which provides a suitable average over a vast array of proteins which turn over with different half-lives. This will be discussed in relation to the toadfish results in the following section, but it can already be concluded that k_{41} representing the degradative pathway for labelled proteins must be time-dependent in a pulse-label experiment. This is the reason that attempts to measure total intrahepatic protein half-life by isotopic techniques, including the double-label method (Schimke, 1973), have yielded results which are apparently incompatible with known synthetic fluxes in liver (Haschemeyer, 1976). This problem could be solved by developing a theoretical equation for $k_{41}(t)$ representing a reasonable spectrum of protein half-lives. A second area of ambiguity concerns the relative rates of concentration-dependent flux (k_{23}) and movement through collecting vesicles ($\uparrow$). These two constants can be

varied together over at least a two-fold range, provided that the ratio is constant. For example, an increase in residence time, which would slow the secretion process, can be offset by an increase in k_{23}. Data on intrahepatic levels of proteins undergoing secretion would serve to differentiate these processes and yield a unique set of constants. These data could also answer the question whether proteins destined for secretion are ever degraded or directed elsewhere within the tissue of origin, as suggested by Rothman (1975). If an excess capacity for plasma protein synthesis exists, it could account for increased plasma protein secretion in pathological states, such as in recovery from hypoalbuminemia (Morgan and Peters, 1971).

B. Direct Temperature Effects on Plasma Protein Synthesis and Secretion

The data obtained here in winter toadfish (acclimated to 7^{o}) provide new insight into the temperature-dependency of liver protein synthesis and secretion *in vivo*. Liver protein synthesis in summer fish can be broken down into three temperature ranges: at higher temperatures (18^{o} to 30^{o}) Q_{10} is about 2.5; at lower temperatures Q_{10} increases to about 5, both for total protein synthesis and for polypeptide elongation rate; below 7^{o} incorporation falls to extremely low levels in most individuals (Mathews, 1977).

Winter fish, which are for the most part dormant, show evidence of temperature acclimation (elevation of protein synthetic rate) when measured at higher temperatures but not at low temperatures. Levels of a critical enzyme, polypeptide elongation factor 1, are elevated in winter fish as in summer cold-acclimated fish (Nielsen et al., 1977); nonetheless, activity in these fish is extremely depressed at the low temperatures.

Elongation rate data in winter fish indicate a Q_{10} in the 9^{o} to 19^{o} range of about 4.3. This temperature dependency was used in generating the synthetic rate constants for the present analysis. A major question is whether the temperature dependency for the other rate constants will be the same. If not, the system will be out of balance or must be assumed to operate with a different means of regulation. Examination of the present results indicates that plasma protein synthesis makes up the same proportion of total synthesis at both low and high temperatures; thus, Q_{10} for synthesis of these proteins is the same as for total liver synthesis. The temperature dependency of the secretion process yields a value of $Q_{10} = 4.0$ for residence time and $Q_{10} = 2.1$ for k_{23}. Since the two processes are not independent in this analysis, the average ($Q_{10} = 3.0$) should be considered a more reliable estimate of the temperature dependency for secretion. This result compares quite closely with a

determination in cooled rabbits and in turtles where secretion times varied with a Q_{10} of 3.2 (Green and Anker, 1955).

The degradative rate constant k_{41} proved to be the most difficult to determine from the available data. One problem is the fact that the final compartment values at the two temperatures were not obtained at times that could be considered kinetically comparable. The synthetic rate data indicate an 8-fold difference in rates between 7^{o} and 20^{o}, thus the comparable termination points for the experiment would be 1 day at 20^{o} and 8 days at 7^{o}. The fact that the 7^{o} data were obtained after only 2 days may account in part for the high value of k_{41} obtained. Previous analyses in toadfish made at 6 hr after injection also yielded a higher result for the degradative flux (Haschemeyer, 1973). Nevertheless, a reasonable fit to the 7^{o} data was obtained when a value of k_{41} was used which had been calculated from the 20^{o} data with $Q_{10} = 4.3$. This indicates another source of difficulty in analyzing the endogenous liver protein labelling pattern, i.e., that the results are not very sensitive to rather large changes in the degradative rate constant.

In summary, a reasonable picture of plasma protein secretion in toadfish has emerged, which is consistent with protein synthesis studies in winter toadfish and with results in other organisms subjected to direct temperature changes. The relatively large Q_{10}'s found in the toadfish

in this temperature range, when compared with the Antarctic species, indicate a lack of adaptation to winter temperatures. This conclusion is supported by the observed general dormancy of toadfish in winter.

C. Protein Synthesis and Cold Adaptation in the Antarctic Fishes

The techniques developed in this thesis were applied to the study of Antarctic fishes with several objectives in mind: first, to obtain kinetic parameters for liver protein synthesis *in vivo* at -1.5^{o} for comparison with rat and toadfish data; second, to attempt an evaluation of cold adaptation in terms of protein metabolism; and finally, to gather information on the synthesis of the unusual antifreeze glycoprotein responsible for freezing resistance in these fishes.

1. Plasma Protein Secretion at -1.5^{o}

The process of protein synthesis and secretion by liver in *D. mawsoni* has been broken down in this analysis into the components of synthesis (polypeptide chain assembly time = 17 min); vectorial concentration-independent migration (minimum transit time = 2 hr); residence in collecting vesicles, which may include both endoplasmic reticulum and Golgi locations (6 hr); and a concentration-dependent flux characterized by $k_{23} = 0.2\ hr^{-1}$ ($t_{\frac{1}{2}} = 3.5$ hr).

The amounts of protein associated with the various compartments can be directly calculated from these values and from the total plasma protein synthetic flux (0.075 mg gm^{-1} hr^{-1}). Thus, at any time 0.15 mg gm^{-1} is undergoing vectorial migration; 0.45 mg gm^{-1} occupies collection sites (A_3^c); and from the steady state equation $dA_2/dt = 0$, compartment A_2 contains 0.4 mg gm^{-1}. The total is 1.2% of liver protein and is consistent with estimates of intrahepatic plasma protein in rat (Edwards et al., 1976).

Although no comparable kinetic analysis has been carried out for secretion in rat, two of the experimental quantities may be directly compared. In rat the minimum transit time (time elapsed before appearance of radioactive albumin in circulation after an intravenous pulse label) is estimated at 16 min. Average transit time (time required to reach half of the final steady state level) is 35 min (Peters et al., 1971). In the present study, minimum transit time is δ or 2 hr. Average transit time is δ plus the half-time for accumulation (Table 6) or 18 hr in the alanine labelling study. An Arrhenius plot of these values yields a temperature coefficient Q_{10} (between rat and Antarctic fish) of 2.4 for both the minimum and average transit times of secretion. This is striking in that it is significantly below values obtained when temperature is varied directly, as in the winter toadfish studies reported here, previous work in toadfish (Q_{10} about 5) and in the early study by Green and Anker (1955) in rabbits and turtles (Q_{10} = 3.2).

Thus, the Antarctic fish may be considered to have accomplished a degree of adaptation in maintaining a level of function consistent with a moderate Q_{10}. A similar conclusion may be drawn from consideration of total protein synthetic rate below.

2. Total Liver Protein Synthesis at -1.5°

The data from I. hansoni and D. mawsoni used to obtain the rate constants k_{12} and k_{14} yield a value of 5 mg gm^{-1} day^{-1} for total liver protein synthesis. In D. mawsoni, plasma proteins account for 40% of synthesis, well above the range of 20-25% observed in rat (Peters and Peters, 1972), toadfish (Table 1), and I. bernacchii (Table 9). The remaining synthesis of intrahepatic proteins represents a fractional replacement rate of 4% per day, a result consistent with values of 70% in rat at 37° (Peters and Peters, 1972; Haschemeyer, 1976; Garlick et al., 1975) and 17% in toadfish at 20° (Haschemeyer, 1969b), based on a Q_{10} of 2.5 for protein synthesis. As in the case of the secretion parameters, however, direct temperature dependency is much steeper within an individual species: e.g., Q_{10}'s are as high as 8, as discussed in Section I. D. Although the results for the Antarctic species are not indicative of a high level of cold adaptation when compared with respiration data (Holeton, 1974), one may conclude that these species

have at least far extended the range over which a moderate Q_{10} holds.

3. AFGP Synthesis and Secretion

This study has provided the first direct information on the synthesis of a freezing-point depressing protein. Hew and Yip (1976) have suggested that a similar anti-freeze protein occurring in winter flounder is synthesized in liver on the basis of experiments with oocytes injected with flounder liver mRNA. The identification of the product of synthesis in the oocyte, however, was not complete. The possibility that AFGP is synthesized by liver formed the basis of the kinetic analysis of the data of the present study. Because of the high concentration of AFGP in plasma (equal to the sum of all other plasma proteins combined) it is conceivable that AFGP might account for a significant proportion of total liver protein synthesis. The experimental results, however, did not give a completely clear picture of the synthesis of this protein. The appearance of labelled AFGP was inhibited by the standard protein synthesis inhibitor, cycloheximide, suggesting that the normal protein synthetic machinery is responsible for its production. In other respects, however, AFGP synthesis is markedly different from that of the liver-synthesized plasma proteins. Radioactive AFGP could not be identified in the blood until nearly

20 hr after injection, or about 18 hr after the first appearance of radioactive acid-insoluble plasma protein. Average transit time was estimated at about 40 hr. The results of kinetic analysis yielded a migration time of 14 hr; a collection period of 6 hr, similar to that for plasma protein; and a secretion rate constant of 0.05 hr^{-1}. Although variation in secretion times has been reported for different proteins in rat liver (Morgan and Peters, 1971), the difference between the AFGP and the other plasma proteins is so great as to suggest a different secretion mechanism. The high carbohydrate content of the AFGP may also be a factor in the slow secretion process.

The total synthetic rate for the (Ala-Ala-Thr) polypeptide of the AFGP (from k_{15}), assuming that liver is the source of this protein, amounts to 65 $\mu g \ gm^{-1} \ day^{-1}$, or about 1% of liver protein synthesis. This result is rather low in relation to the size of the pool of circulating AFGP (10 mg AFGP polypeptide/ml plasma). At this rate of replacement, circulating AFGP would have a half-life of 23 weeks. A value of 3 weeks has been estimated from the loss of labelled AFGP in the circulation (DeVries, 1974). The discrepancy may be explained if synthesis occurs at a site other than the liver. Because the technique of hepatic portal vein injection produces a very high uptake of radioactivity by liver, other tissues receive less radioactive amino acid, and

their proteins will be poorly labelled. This would account for the low ratio of ^{14}C-alanine incorporation into AFGP relative to the liver-synthesized plasma proteins. In that case, however, AFGP synthesis must differ from that reported for the antifreeze protein in winter flounder (Hew and Yip, 1976). Examination of protein synthetic activity of other tissues in Antarctic species is needed to resolve this question.

APPENDIX I.

Computer Programs

VES90

```
10    dimension yai(1000),ya2(1000),ya3(1000),ya4(1000)
20    dimension yae(1000),yac(1000),xt(1000)
30    real k1,k2,k3,k6,k7,k9,k10,k11,xtau
40    yai(1)=1.0
50    ya2(1)=0.0
51    yac(1)=0.0
52    ya3(1)=0.0
53    ya4(1)=0.0
54    xt(1)=0.0
55    dt=0.125
57    n=728
58    write (6,512)
60    read (5,*)k2,k6,k10,k11,xtau
70    k7=k2*(8.7*k6-1.0)
80    a2=0.103/k2
100   write (6, 515)
110   write (6,*)k7,k9
250   101 do 400 i=1,n
260   yae(1)=0.0
290   di=dt*(k7*ya2(i)+k11*ya4(i)-(k6+k10)*yai(i))
300   d2=dt*(k6*yai(i)-(k7+k2)*ya2(i))
320   d4=dt*(k10*yai(i)-k11*ya4(i))
330   yai(i+1)=yai(i)+di
340   ya4(i+1)=ya4(i)+d4
350   ya2(i+1)=ya2(i)+d2
355   xt(i+1)=xt(i)+dt
356   sum=0.0
360   102 do 365 j=1,i
363   dsum=dt*k2*ya2(j)*exp((xt(j)-xt(i+1))/xtau)
364   sum=sum+dsum
365  365 continue
366   dc=dt*k2*ya2(i)-(1.0/xtau)*sum*dt
```

(VES90, continued)

```
367   yac(i+1)=yac(i)+dc
368   d3=dt*(1.0/xtau)*sum
369   ya3(i+1)=ya3(i)+d3
400   400 continue
529   write (6,513)
530   write (6,514) (yac(k),yae(k),yai(k),ya2(k),ya3(k),ya4(k),%
531   xt(k),k=1,n,120)
587   512 format(t2,'k2,k6,k10,k11,xtau')
590   513 format('0',t2,'ac',t12,'ae',t22,'ai',t32,'a2',t42,%
591   'a3',t52,'a4',t62,'time'//)
592   514 format ('1',t2,f5.3,t12,f5.3,t22,f5.3,t32,f5.3,t42,%
593   f5.3,t52,f5.3,t62,f6.2)
594   515 format(t2,'values of k7 and k9 are')
600   999 stop
610   end
```

DEGV90

```
10    dimension yai(1000),ya2(1000),ya3(1000),ya4(1000)
20    dimension yae(1000),yac(1000),xt(1000)
30    real k1,k2,k3,k6,k7,k9,k10,k11,k13,xtau
40    yai(1)=1.0
50    ya2(1)=0.0
51    yac(1)=0.0
52    ya3(1)=0.0
53    ya4(1)=0.0
54    xt(1)=0.0
55    dt=0.125
57    n=728
58    write (6,512)
60    read (5,*)k2,k6,k10,k11,k13,xtau
70    k7=0
80    a2=0.103/k2
100   write (6,515)
110   write (6,*)k7,k9
250   101 do 400 i=1,n
260   yae(1)=0.0
290   di=dt*(k7*ya2(i)+k11*ya4(i)+k13*ya3(i)-(k6+k10)*yai(i))
300   d2=dt*(k6*yai(i)-(k7+k2)*ya2(i))
320   d4=dt*(k10*yai(i)-k11*ya4(i))
330   yai(i+1)=yai(i)+di
340   ya4(i+1)=ya4(i)+d4
350   ya2(i+1)=ya2(i)+d2
355   xt(i+1)=xt(i)+dt
356   sum=0.0
360   102 do 365 j=1,i
363   dsum=dt*k2*ya2(j)*exp((xt(j)-xt(i+1))/xtau)
364   sum=sum+dsum
365   365 continue
366   dc=dt*k2*ya2(i)-(1.0/xtau)*sum*dt
```

(DEGV90, continued)

```
367   yac(i+1)=yac(i)+dc
368   d3=dt*(1.0/xtau)*sum-k13*ya3(i)*dt
369   ya3(i+1)=ya3(i)+d3
400   400 continue
529   write (6,513)
530   write (6,514) (yac(k),yae(k),yai(k),ya2(k),ya3(k),ya4(k),%
531   xt(k),k=1,n,20)
587   512 format(t2,'k2,k6,k10,k11,k13,xtau')
590   513 format('0',t2.'ac',t12,'ae',t22,'ai',t32,'a2',%
591   t42.'a3',t52,'a4',t62,'time'//)
592   514 format ('1',t2,f5.3,t12,f5.3,t22,f5.3,t32,f5.3,%
593   t42,f5.3,t52,f5.3,t62,f6.2)
594   515 format(t2,'values of k7 and k9 are')
600   999 stop
610   end
```

ALAV90

```
10    dimension yai(1000),ya2(1000),ya3(1000),ya4(1000)
20    dimension yae(1000),yac(1000),xt(1000)
30    dimension yaf(1000),yafc(1000),ya6(1000)
31    real k2,k6,k7,k10,k11,xtau,k12,k13,k14,xtauf
40    yai(1)=1.0
50    ya2(1)=0.0
60    yac(1)=0.0
70    ya3(1)=0.0
80    ya4(1)=0.0
90    yaf(1)=0.0
100   yafc(1)=0.0
110   ya6(1)=0.0
120   xt(1)=0.0
130   dt=0.125
140   n=728
150   write (6,512)
160   read (5,*) k2,k6,k7,k10,k11,xtau,k12,k13,k14,xtauf
180   a2=0.088/k2
190   af=0.255/k14
220   101 do 400 i=1,n
230   yae(1)=0.0
240   di=dt*(k7*ya2(i)+k11*ya4(i)+k13*yaf(i)-(k6+k10+k12)*yai(i))
250   d2=dt*(k6*yai(i)-(k7+k2)*ya2(i))
260   d4=dt*(k10*yai(i)-k11*ya4(i))
270   df=dt*(k12*yai(i)-(k13+k14)*yaf(i))
280   yai(i+1)=yai(i)+di
290   ya2(i+1)=ya2(i)+d2
300   ya4(i+1)=yai(i)+d4
310   yaf(i+1)=yaf(i)+df
320   xt(i+1)=xt(i)+dt
330   sum=0.0
340   sumaf=0.0
350   102 do 365 j=1,i
```

(ALAV90, continued)

```
360   dsum=dt*k2*ya2(j)*exp((xt(j)-xt(i+1))/xtau)
370   dsumaf=dt*k14*yaf(j)*exp((xt(j)-xt(i+1))/xtauf)
380   sum=sum+dsum
390   sumaf=sumaf+dsumaf
400   400 continue
410   dc=dt*k2*ya2(i)-(1.0/xtau)*sum*dt
420   dfc=dt*k14*yaf(i)-(1.0/xtauf)*sumaf*dt
430   yac(i+1)=yac(i)+dc
440   yafc(i+1)=yafc(i)+dfc
450   d3=dt*(1.0/xtau)*sum
460   d6=dt*(1.0/xtauf)*sumaf
470   ya3(i+1)=ya3(i)+d3
480   ya6(i+1)=ya6(i)+d6
490   400 continue
500   write (6,513)
510   write (6,514)(yac(k),yae(k),yai(k),ya2(k),ya3(k),ya4(k),%
520   xt(k),k=1,n,20)
530   512 format (t2,'k2,k6,k7,10,k11,xtau,k12,k13,k14,xtauf')
540   513 format ('0',t2,'ac',t12,'ae',t22,'ai',t32,'a2',%
550   t42,'a3',t52,'a4',t62,'time'//)
560   514 format ('1',t2,f5.3,t12,f5.3,t22,f5.3,t32,f5.3,%
570   t42,f5.3,t52,f5.3,t62,f6.2)
590   write (6,516)
600   516 format ('0',t22,'af',t32,'afc',t42,'a6',t52,'time'//)
610   write (6,517) (yaf(k),yafc(k),ya6(k),xt(k),k=1,n,20)
620   517 format ('1',t22,f5.3,t32,f5.3,t42,f5.3,t52,f6.2)
630   999 stop
640   end
```

APPENDIX II.

A Study of the Distribution of Radioisotope in Toadfish Liver Immediately Following ^{14}C-leucine Injection at 20°

The experiments described in this thesis depend upon the introduction of radioisotopes into liver via the hepatic portal vein. The following experiments were done to determine whether the isotope is distributed evenly throughout the liver or whether there is some preferential distribution. A solution containing both ^{14}C-leucine and ^{3}H-mannitol was injected into the hepatic portal vein of toadfish; the fish were of both sexes and weighed 200-300 gms, and they were acclimated to 20°. Two minutes after injection, the liver was excised and placed on aluminium foil over ice. The liver was then cut into sections, as described below, and each piece was weighed, homogenized, and analyzed for free radioactivity and incorporation into protein, as described in Materials and Methods.

In most toadfish, the liver is composed of three sections. There is a large lobe to the right of the fish's ventral midline, a small lobe about on the midline, and another small lobe to the left of the midline. The liver is anchored at the anterior end. In this experiment, the liver lobes were first separated, and each lobe was cut into at least two pieces. All cuts were made roughly perpendicular to the longitudinal axis of the lobe.

Although there was variation from animal to animal in the results of these experiments, it was clear that radioactive distribution following the 2 min pulse label was not homogenous. Table A1 presents data from an example in this series. ^{3}H-mannitol, used as a marker for extracellular space, showed highest concentration in the distal section of the largest liver lobe (lobe A). Distribution was more uniform in the two smaller lobes. The distribution of ^{14}C-leucine as free radioactivity and in protein was similar.

Table A2 presents distribution data for ^{14}C-leucine given at a 15 mM dose. ^{3}H-mannitol was also given in this experiment at the same dose as given in the tracer experiments. Under these conditions, the distribution of ^{14}C-leucine in both free radioactivity and protein-bound form became more even, although there was a somewhat higher concentration toward the distal end of the larger liver lobes. In this experiment, distribution of the ^{3}H-mannitol space marker was also relatively even throughout the liver.

Table A3 summarizes the recovery of radioactivity among the various lobes. Recoveries ranged as high as 72% of the injected dose. Distribution among the lobes varied widely, and it is difficult to draw any general conclusion. Observation of the circulatory system showed a large branch of the hepatic portal vein going directly to the tip of the largest lobe. No large circulatory

vessels were obvious in either of the two smaller lobes.

In the experiments described in this thesis, however, the uneven distribution of radioactive tracer within the liver should not be a significant factor affecting the results. All liver homogenates from toadfish were made from the entire liver, and the results presented for any given experiment therefore represent the average over the entire liver for each parameter analyzed. In the experiments described for the Antarctic cod, the liver was too large for quick homogenization of the entire organ. In order to obtain a representative sample, each liver was thoroughly minced, after which a portion was taken for homogenization.

TABLE A1

Distribution of radioactivity in liver sections following a pulse injection of ^{3}H-mannitol and ^{14}C-leucine at tracer levels.

Piece	^{3}H (μCi/gm)	^{14}C in protein (μCi/gm)	^{14}C free (μCi/gm)	Total ^{14}C (μCi/gm)
A-1	.089	.070	.012	.082
A-2	.073	.071	.009	.081
A-3	.040	.027	.004	.032
B-1	.039	.016	.002	.017
B-2	.034	.016	.002	.018
C-1	.022	.006	.0006	.007
C-2	.040	.024	.003	.027

In this table, the letter for each piece corresponds to a lobe of the liver: A represents the largest lobe, B is the smaller central lobe, and C represents the small lobe to the left of the ventral midline. The number for each piece designates the position of that piece when the lobe was intact. The subscript 1 indicates pieces taken from the unattached tip (distal portion) of each lobe. Numbers increase for pieces taken toward the end proximal to the point of attachment.

TABLE A2

Distribution of radioactivity in liver sections following a pulse injection of ^{3}H-mannitol at tracer level and 15 mM ^{14}C-leucine.

Piece	^{3}H (μCi/gm)	^{14}C in protein (μCi/gm)	^{14}C free (μCi/gm)	Total ^{14}C (μCi/gm)
A-1	.054	.025	.013	.038
A-2	.057	.030	.019	.049
A-3	.041	.018	.008	.026
B-1	.056	.032	.021	.054
B-2	.043	.021	.011	.031
C-1	.032	.016	.009	.025
C-2	.050	.027	.016	.043

In this table, the letter for each piece corresponds to a lobe of the liver: A represents the largest lobe, B is the smaller central lobe, and C represents the small lobe to the left of the ventral midline. The number for each piece designates the position of that piece when the lobe was intact. The subscript 1 indicates pieces taken from the unattached tip (distal portion) of each lobe. Numbers increase for pieces taken toward the end proximal to the point of attachment.

TABLE A3

^{14}C-leucine distribution in lobes of toadfish liver (as percent of dose).

Expt.	Injection	Time after Injection	Lobe A	Lobe B	Lobe C	Total
D-1	tracer	2 min	26.6	9.48	9.14	45.2
D-3 #1	tracer	2 min	13.8	36.0	21.8	71.6
#2	tracer	2 min	14.6	22.3	3.86	40.8
D-2	tracer	4 min	10.2	9.70	18.6	38.5
D-3	15 mM	2 min	12.7	7.58	3.41	23.7
D-4	15 mM	2 min	8.55	5.29	5.26	19.1
Average weight of lobe (gm)			4.7 (± 2.2)	2.5 (± 2.2)	1.8 (± 1.3)	9.0 (± 5.3)
Lobe as fraction of total liver weight			0.52	0.28	0.20	1.0

APPENDIX III.

Carbohydrate Labelling Experiments

A. Glucosamine-labelling of Plasma Proteins and Liver Proteins at 7° in Toadfish

Previous studies which have monitored the incorporation of glucosamine into liver proteins have indicated that the attachment of core carbohydrates to glycoproteins begins while the nascent polypeptide chain is still attached to the membrane-bound ribosome (Lawford and Schachter, 1966). Sialic acid, a derivative of glucosamine which is found usually in the terminal position in the carbohydrate portion of glycoproteins, is attached to the glycoprotein after it has left the ribosome and while it is resident in the smooth endoplasmic reticulum. Another study has shown that glucosamine is added to the polypeptide chain while it is associated with tRNA on the ribosome (Molnar and Sy, 1967).

In the course of the present work on toadfish at 7° a series of experiments was performed in which ^{14}C-glucosamine was injected into the hepatic portal vein. A time course of appearance of label in completed plasma protein was determined, as described in Materials and Methods, as well as the amount of label in liver protein and the amount of label occurring as liver free radioactivity at the termination of the experiment.

The appearance of ^{14}C-glucosamine in plasma protein over time at 7^{o} in the toadfish is shown in Figure A1. The time course for appearance of label is sigmoidal and is similar to the time course of appearance of ^{3}H-leucine label in plasma protein at the same temperature. Absolute lag time was 5.0 hr, the same as that for leucine, and the $t_{\frac{1}{2}}$ for accumulation was 13.1 hr, again not significantly different from the $t_{\frac{1}{2}}$ for leucine at this temperature.

Table A4 presents data on the amount of label found in the various compartments at the termination of the experiment at 48 hrs. These data clearly indicate that the incorporation of glucosamine into plasma proteins is favored over glucosamine incorporation into liver proteins. The ratio of label found in plasma protein alone to the total label found in both plasma protein and liver protein is 52%. The comparable ratio for leucine incorporation is only 20%.

The results of this study are consistent with the previous reports on ^{14}C-glucosamine incorporation into protein. The similar lag time and $t_{\frac{1}{2}}$ of accumulation for glucosamine and leucine labelling indicate that the two isotopes are being incorporated at very nearly the same time in the synthesis-secretion process. This is consistent with the addition of glucosamine to the nascent polypeptide chain while associated with the ribosome.

FIGURE A1

Time course of appearance of radioactivity in completed plasma protein in toadfish following hepatic portal vein injection of ^{14}C-glucosamine. Experimental temperature is 7^{o}.

FIGURE A1

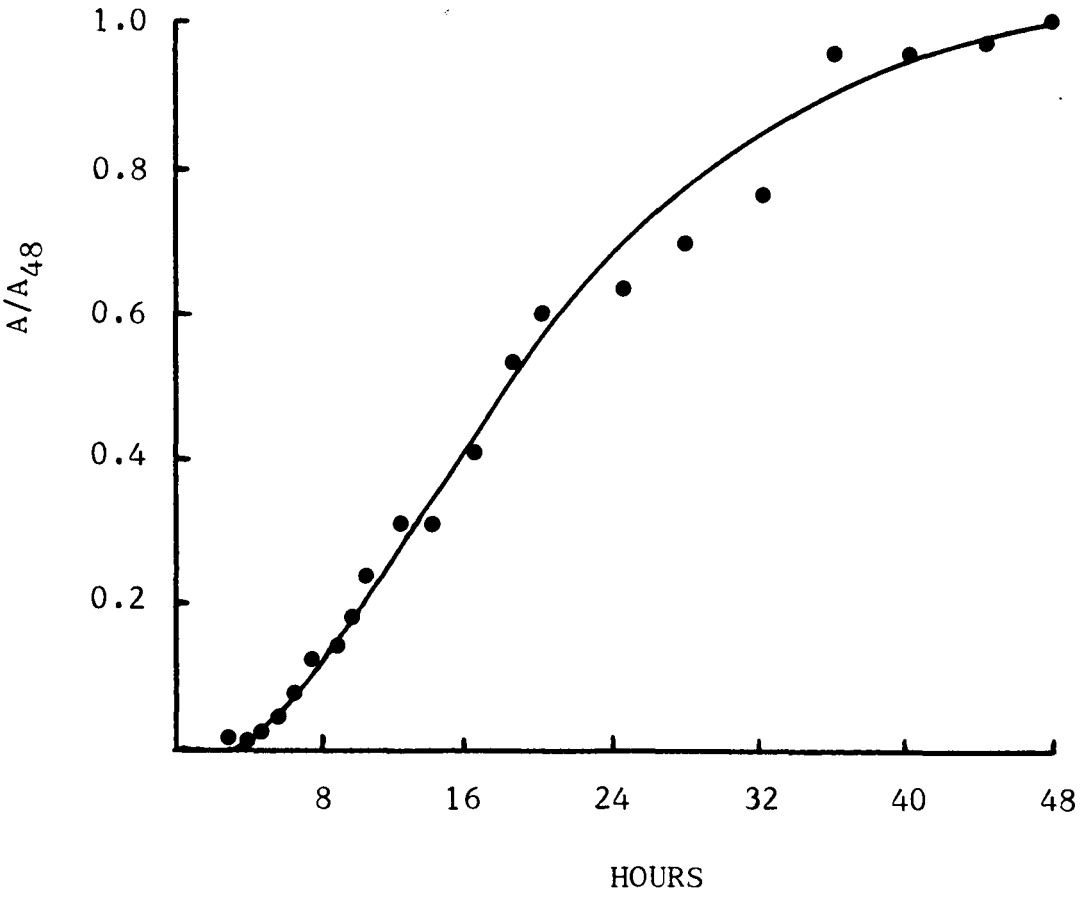


TABLE A4

Recovery of ^{14}C-glucosamine in various compartments at 48 hr in toadfish acclimated to 7°. Also included are estimated time constants for secretion. Number of animals = 13.

A_3 Lag	$t_{1/2}$	$A_2 + A_4$	A_3	A_1	Fraction of Dose Recovered	P/L+P
5.0 hr	13.1 hr	0.44 ± 0.07	0.47 ± 0.07	0.08 ± 0.03	0.185 ± 0.04	0.52

B. Galactose and N-acetylgalactosamine Labelling in Antarctic Cod

Two experiments on sugar labelling were also performed in the Antarctic cod. The time dependency of labelling was somewhat erratic in the case of galactose (Figure A2). N-acetylgalactosamine showed a continuous slow accumulation in both total plasma protein and in AFGP. The distribution of label at the end of the experiments is given in Table A5. The incorporation into AFGP is very low, in spite of a high carbohydrate content (60% by weight). Because sufficient animals and isotope were not available to repeat these experiments, it is hazardous to draw any conclusion at this point. However, it is conceivable that the low recovery of label in the AFGP may be the result of synthesis at a site other than liver.

FIGURE A2

Time course of appearance of radioactive plasma protein, excluding AFGP, (closed symbols) and AFGP alone (open symbols) in Antarctic cod following hepatic portal vein injection of ^{3}H-galactose. Experimental temperature is -1.5^{o}.

FIGURE A2

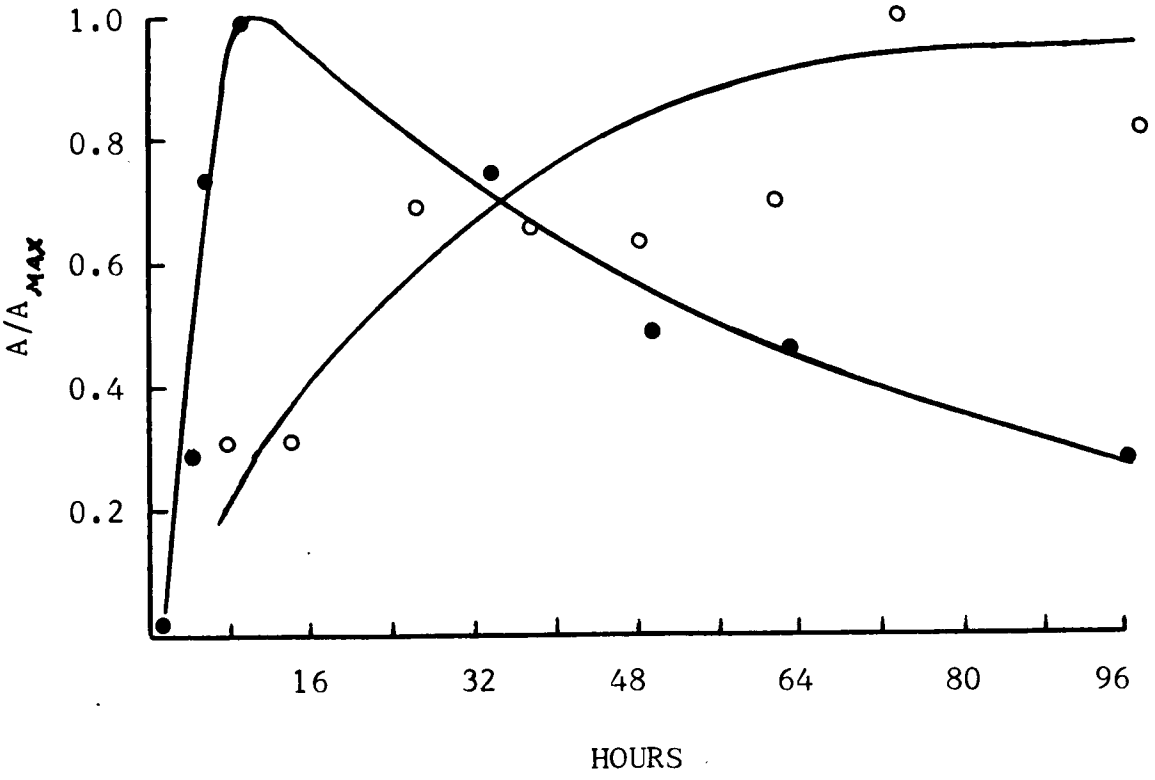


FIGURE A3

Time course of appearance of radioactive plasma protein, excluding AFGP, (closed symbols) and AFGP (open symbols) in Antarctic cod following hepatic portal vein injection of ^{3}H-N-acetylgalactosamine. Experimental temperature is -1.5^{o}.

FIGURE A3

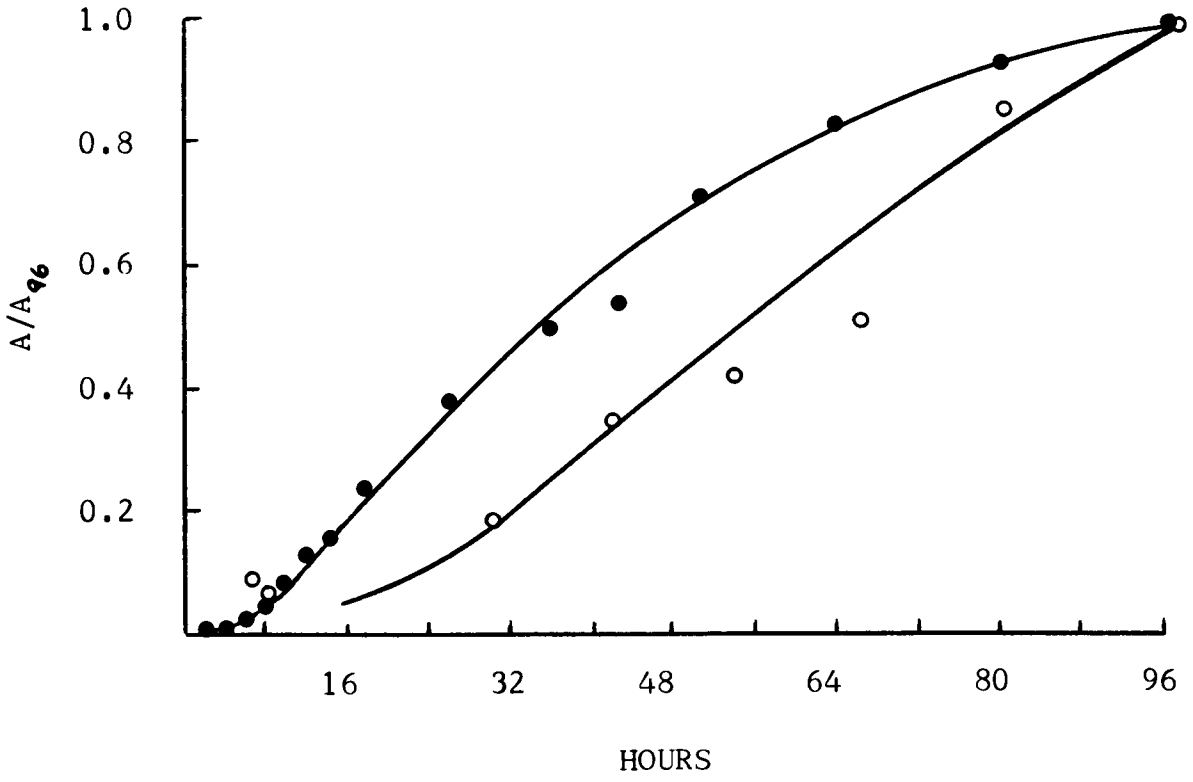


TABLE A5

Recovery of ^{3}H-galactose and ^{3}H-N-acetylgalactosamine at 96 hr in Antarctic cod at -1.5°. A single animal is represented in each experiment.

Radioisotope	$A_2 + A_4$	A_3	A_1	AFGP	Fraction of Dose Recovered
^{3}H-galactose	0.14	0.33	0.50	0.03	0.034
^{3}H-N-acetylgalactosamine	0.19	0.67	0.13	0.005	0.27

APPENDIX IV.

Hemorrhage Experiments at 20° in Toadfish

In the course of determining the time course of labelling of plasma proteins in the experiments described in this thesis, serial blood samples were taken from each fish over time. This meant that each fish was subjected to some degree of blood loss. Reports in the literature indicate that there are substantial changes in liver as a result of hemorrhagic hypertension. Malt et al. (1969) have reported that, in dogs subjected to a 15% hemorrhage, the synthesis of albumin is increased 75% within 1 hr of hemorrhage. Miller et al. (1974) found reduced surface oxygen tension and reduced ATP in liver of hemorrhaged rats.

To examine the effect of hemorrhage in toadfish, experiments were performed in order to measure the appearance of ^{14}C-leucine plasma protein over time in control fish and in fish subjected to a substantial hemorrhage. All experimental animals in this study were toadfish acclimated and maintained at 20°. Blood was withdrawn from arteries of the gill arches up to 50% of total blood volume, determined as 2% to total body weight. For two fish, the blood was centrifuged and the cells were resuspended in an equal volume of isotonic saline or isotonic Dextran 75 for reinjection into the fish. Injection of ^{14}C-leucine took

place 30 min after the hemorrhage; the standard hepatic portal vein injection procedure was used (see Materials and Methods). Serial blood samples were taken, and the liver was analyzed at the conclusion of the experiment (t = 6 hr).

Figure A4 illustrates the results of these experiments. For the non-hemorrhaged control fish, the absolute lag time for appearance of radioisotope in plasma protein was 1.1 hr, while that for the 50% hemorrhaged fish was 1.3 hr. $t_{\frac{1}{2}}$ for accumulation in control fish was 1.1 hr; $t_{\frac{1}{2}}$ for accumulation in the hemorrhaged fish was 2.0 hr. Table A6 presents the recovery of radioactivity in the various compartments at the termination of the experiment.

Figure A4 shows a clearly slower rate of labelling of plasma protein in the hemorrhaged fish. The experimental time was not sufficient for plateau to be reached in either group; however, it appears that controls will plateau well before the hemorrhaged fish. It is uncertain whether the latter would eventually reach the same P/L+P ratio as the controls. It seems doubtful that a significant increase in this ratio, as suggested by the studies in dogs, will occur. In general, the hemorrhaged fish show a severely depressed secretion process. The reason for this is unknown.

The results, however, should not be a serious source of error in the experiments described in this thesis. In

FIGURE A4

Time course of appearance of radioactive plasma protein in control toadfish (closed symbols) and 50% hemorrhaged toadfish (open symbols) following hepatic portal vein injection of ^{14}C-leucine. Experimental temperature is 20^{o}.

FIGURE A4

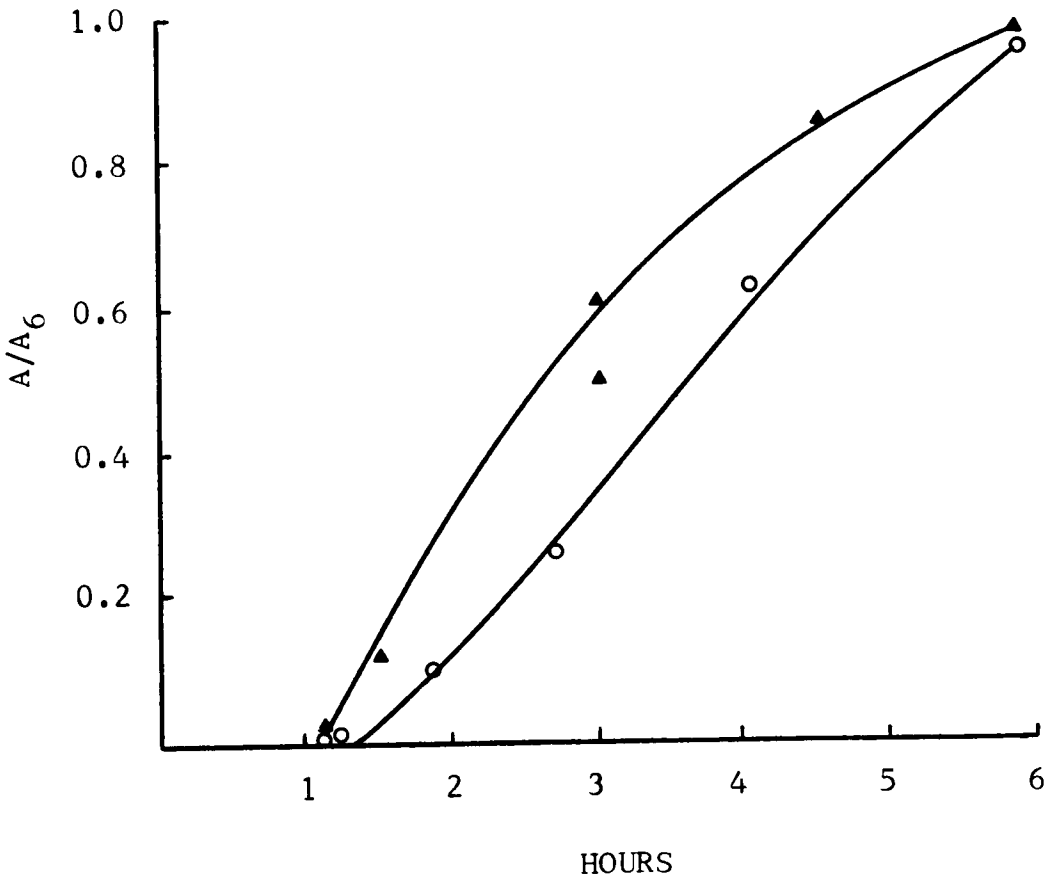


TABLE A6

Recovery of radioactivity in various compartments in control fish and 50% hemorrhaged fish after ^{14}C-leucine injection at 20^{o} in toadfish. Data for controls represents 3 animals; data for hemorrhaged fish represents 2 animals.

	$A_2 + A_4$	A_1	A_3	P/L+P
Controls	0.67	0.10	0.17	0.18
Hemorrhaged	0.61	0.29	0.10	0.13

all of the experiments presented earlier, no fish lost more than 10-15% of its total blood volume as a result of the serial blood sampling.

Several experiments designed to measure the polypeptide chain assembly time in these hemorrhaged fish at various times after blood removal were also performed. Fish were bled of approximately 50% of total blood volume, and this loss was immediately replaced with either an equal volume of isotonic saline or isotonic Dextran 75 containing the withdrawn blood cells. The normal value for polypeptide chain assembly time at 20^{o} is 5.5 min (Nielsen et al., 1977). Values obtained in the hemorrhaged fish were 5.8 min one hour after bleeding and 4.6 min 48 hr after bleeding. These experiments indicate that there is no significant alteration in the transit time of ribosomes over the messenger RNA in response to hemorrhage.

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