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Seasonal Changes in Uptake of L-Phenylalanine by the Intestine of Winter Flounder, *Pseudopleuronectes americanus*

by

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Abstract

Seasonal changes in body paremeters, intestinal structure and L-phenylalanine uptake were examined in winter flounder, *Pseudopleuronectes americanus*. Winter flounder from Passamaquoddy Bay, New Brunswick, ceased feeding in late October and resumed feeding in early May. Condition factor and the slope of the regression line of the length-weight relationship declined during the non-feeding period. Of the 14% decline in condition factor, 71% of it could be attributed to starvation. Muscle and liver lipid levels declined, while muscle water content increased an average 2.4% during the non-feeding period. Intestinal mucosal mass and surface area also declined, but the decreasing proximodistal gradient of these variebles was maintained. The decline in mass was not as great as that observed in starving freshwater rainbow trout, nor was there any loss of intestinal length. The continuing osmoregulation in the winter flounder is postulated to ameliorate the effects of starvation on these intestinal parameters. Surface area was 25% less in September than in May, and 50% less in January than in May.

Intestinal folding was reduced in the non-feeding period, but no obvious ultrastructural change in the enterocytes was observed. The number of goblet cells appeared to decline in winter. The number and length of microvilli on each cell appeared to remain constant. Leucine aminopeptidase activity and protein concentration were decreased only in the hindgut by the winter fast.

The intestine was subdivided into caeca, foregut, midgut and hindgut, and uptake of L-phenylalanine in those sections was measured *in vivo* (as disappearance from the perfusate) and *in vitro* on emusculated mucosal sheets. Starvation and the resultant loss of mucosa was the major influence on phenylalanine uptake, resulting in a decline in the non-feeding period. Phenylalanine uptake could be resolved into a saturable, sodium-dependent component and a non-saturable, linear, sodium-independent component (apparent diffusion). The apparent diffusion constant, K_d, decreased in January more than would have been expected due to the effects of temperature alone. Temperature sensitivity was reduced in January except in midgut. The decreased apparent diffusion constant suggests changes in membrane composition occurred. J_{max} also decreased in winter. The apparent K_t was an unreliable indicator of seasonal change because of influences of the unstirred layer. *In vitro*, J_{max} declined more than expected due to temperature effects alone, but, when compared to *in vivo* J_{max}, was higher than expected in foregut and hindgut and the same in caeca and midgut. These results indicate that apical saturable uptake is reduced in winter, while net flux is increased. This may be due to low plasma phenylalanine levels at this time, or to decreased backflux at the apical membrane due to membrane alterations.

Phenylalanine uptake per cm^2 was similar in all sections of the intestine, but tended to be highest in the foregut in summer. Pyloric caeca did not show preferential uptake. Total uptake was generally highest in the foregut because of its greater surface area. At least two types of carriers for L-phenylalanine appear to exist in the caeca, foregut and hindgut, but there is only one in the midgut. Phenylalanine shares a common transporter with taurine in all sections except the midgut.

Resumé

Les changements saisonniers, la structure intestinale et l'absorption de la L-phenylalanine furent examinés chez la plie rouge, *Pseudopleuronectes americanus*. La plie rouge de Passamaquoddy Bay du Nouveau Brunswick cesse de se nourrir à la fin d'octobre et recommence au début de mai. Le coefficient d'engraissement et la pente de la régression lineaire (longueur-poids) diminue durant la période d'hivernation. Le coefficent d'engraissement diminue de 14% et 71% de ce décroissement est dû à la diète absolue. Le contenu en lipides du muscle et du foie diminue alors que la concentration d'eau dans le muscle augmente à un taux de 2.4% durant le jeûne d'hiver. Le poids et la surface de la muqueuse intestinale décroissent, mais le gradient proximo-distal qui découle de ces deux variables n'est pas aboli. Cépendant la diminution de la masse n'était pas aussi grande que celle observée chez la truite arc-en-ciel d'eau douce et il n'y avait pas de perte de longueur intestinale. Le besoin continuel pour la régulation osmotique de la plie rouge ameliorerait les effets de l'inanition sur la masse mucosale, et le maintien du gradient proximo-distal. La surface était de 25% de moins en septembre qu'en mai et 50% de moins en janvier qu'en mai.

Les crêtes intestinales étaient réduites durant la periode de jeûne, mais aucun changement dans les entérocytes ne fut observé. Le nombre de cellules à mucus semble diminuer l'hiver alors que le nombre de microvillosités pour

chaque cellule semble rester constant. L'activité de la leucine aminopeptidase et la concentration de la protéine ne diminuent pas au jeûne d'hiver excepte dans l'intestin distal.

On reconnait quatre sections d'intestin : soit les sections caecales, proximales, moyennes et distales. L'accroissement de la captation de la L-phenylalanine dans ces tissus était mesuré in vivo (comme disparition du perfusat) et in vitro par la gaine mucosale du muscle enlevé. La perte de la muqueuse, secondaire à l'inanition, semble être la cause majeure de la diminution de la captation de phenylalanine. La captation de phenylalanine comprend en fait une composante sodium dépendante qui suit la cinétique de Michaelis-Menten, et une composante linéaire, non-saturable et indépendente des ions sodium.

La constante de diffusion K_d décroit plus en janvier, ce qui ne peut être expliqué uniquement par les effets de la température. La sensibilité à la température était réduite en janvier excepté dans la partie moyenne de l'intestin. La diminution apparente de la constante indique un changement dans la composition des membranes. J_{max} décroit aussi en hiver. K_t n'était pas consideré comme indicateur des changements saisonniers dû à les effets de la couche non-agitê. In vitro, J_{max} décroit plus que la normale dû à l'effet de la température, mais devient plus élevé que la normale dans l'intestin proximal et distal, mais reste identique dans les caeca et l'intestin distal, quand la mesure se fait in vivo. Ces résultats indiquent que l'absorpton saturable apicale est réduite en hiver, pendant que le flux net augmente. Ceci peut etre dû au niveau tres bas de la phenylalanine à cette période de l'année , ou à la diminution du reflux au niveau de la membrane apicale secondaire aux modifications de cette membrane.

L'absorption par cm^2 de phenylalanine qui est similaire dans toutes les parties de l'intestin, tend a s'élever dans l'intestin proximal au cours de l'été; les caeca pyloriques ne montrent pas d'absorption préférentielle. L'absorption totale était generalement plus élevée dans l'instestin proximal parce que la surface est plus grande. Au moins deux voies de transport pour la L-phenylalanine semblent exister dan les caeca, l'intestin proximal et l'intestin distal. La phenylalanine partage une voie commune avec la taurine dans toutes les sections étudiées excepté dans l'intestin moyen.

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Chapter 1

Introduction

Proteins represent an important dietary component, providing nitrogen, essential amino acids and calories. Fish require relatively high levels of dietary protein (National Academy of Sciences, 1973). The ability of the intestine of fish to handle proteins has received increasing attention from scientists in recent years. The processing of protein by the fish digestive tract does not differ greatly from the process in mammals (Fänge and Grove, 1979) and involves enzymatic and mechanical mechanisms.

Kenyon, in 1925, reported that fish possess trypsin-like and chymotrypsin-like enzymes similar to those synthesized by the pancreas of mammals. Many fish also produce a pepsin-like enzyme (Fänge and Grove, 1979), although the Cyprinidae and Cyprinodontidae are stomachless and produce neither pepsin nor acid. Unlike mammals, those fish which do have stomachs synthesize pepsin and acid in the same cell. Pancreatic carboxypeptidases A and B have also been measured in several species of fish, although in some species only one or the other has been detected (Fänge and Grove, 1979).

Mechanical breakdown is often minimal in fish. Acid denaturation, peptic hydrolysis and some mechanical breakdown occur in the stomach. In fish without stomachs, there is often a compensatory increase in intestinal length which increases the exposure time of the digesta to the pancreatic enzymes (Fänge and Grove, 1979). Acid is neutralized upon passage of the chyme into the intestine, where the pancreatic enzymes begin their hydrolytic activities. The result of the hydrolysis by the pancreatic proteases is a mixture of peptides and free amino acids that must be absorbed across the intestine. Oligopeptides in mammals apparently undergo hydrolysis by brush border membrane enzymes (Adibi and Kim, 1981; Semeriva et al., 1982), and the resulting tri- and dipeptides are transported intact across the membrane (Adibi and Kim, 1981), as are the amino acids, by various pathways. Few studies on peptide uptake in fish have been done, but peptide hydrolases have been identified in the intestine of brown trout, *Salmo trutta* (Ash, 1979) and rainbow trout, *Salmo gairdneri* (Ash, 1980), and uptake of intact glycylglycine has been observed in the rainbow trout (Bogé et al., 1981a).

In addition to the above methods of uptake, which are similar to those which occur in other vertebrates, many fish are able to absorb protein macromolecules by pinocytosis. Pinocytotic uptake of protein macromolecules is common in larval and juvenile teleosts (Watanabe, 1981; 1982; 1984), and is thought to be related to the late development of complete digestive function. However, such uptake has also been observed in adult fish, including the stomachless tench (*Tinca tinca*) (Noaillac-Depeyre and Gas, 1976) and grasscarp (*Ctenopharyngodon idella*) (Stroband and van der Veen, 1981), and also in the stomach-possessing catfish, *Ictalurus punctatus* (Krementz and Chapman, 1974), perch, *Perca fluviatilis* (Noaillac-Depeyre and Gas, 1979) and rainbow trout, *Salmo gairdneri* (Ezeasor and Stokoe, 1981). Only the posterior portion of the intestine is involved in such transport in these fish, and the physiological importance of macromolecular protein uptake is uncertain (Stroband and van der Veen, 1981).

Fish which possess stomachs often have a number of blind sacs projecting from the intestine just posterior to the pyloric sphincter of the stomach. These pyloric caeca may number from one to more than one thousand, depending on the species (Fänge and Grove, 1979). Early studies (Greene, 1913) suggested that the pyloric caeca functioned largely in the absorption of lipid, and microscopic surveys supported this finding (Ezeasor and Stokoe, 1981). Beamish (1972), however, found that the ratio of lipid to protein in the pyloric caeca of largemouth bass (*Micropterus salmoides*) was higher than that in the feed, suggesting the preferential absorption of protein. Few uptake studies have been done. Collie (1985) found that proline uptake was 4 times greater in pyloric caeca than in the foregut of coho salmon (*Oncorhynchus kisutch*), supporting a protein handling function for these structures.

The cells of the pancreas, which produce the major enzymes involved in intestinal digestion, and the enterocytes or absorbing cells of the intestine are similar in appearance to those of mammals (Fänge and Grove, 1979). They must, however, perform their functions under variable conditions of temperature in many fish. In addition, the intestine has an added importance for osmoregulation in marine fish (Lahlou, 1983). While salt uptake does not appear to interfere with nutrient handling (Dabrowski et al., 1986), few studies have addressed the perturbing effects of temperature on specific digestive functions.

The activity of pepsin from cold acclimated brook trout (*Salvelinus fontinalis*) was higher than that from warm acclimated trout when measured at the same temperature (Owen and Wiggs, 1971). Smit (1967) found that gastric acid secretion was thermally compensated between 20 and 30°C in brown bullheads (*Ictalurus nebulosis*). Neither trypsin nor chymotrypsin, however, demonstrated thermal compensation in rainbow trout (*Salmo gairdneri*) (McLeese, 1982). The affinity of trypsin for its substrate was found to be independent of temperature in trout and in bluefin tuna, *Thunnus thynnus* (McLeese,1982), and in several other species of fish and in tadpoles (Hofer et al., 1975). Hofer and his colleagues (1975) were unable to demonstrate temperature independence of trypsin affinity from adult frogs or reptiles, and suggested that this was an adaptation to aquatic life.

Few studies on environmental perturbations of uptake have been reported. Before such effects can be understood, the nature of the systems which are involved in transport must be elucidated. Transport systems in mammalian and avian cells other than those of the intestine and renal tubule are reasonably well understood. Less is known of the nature of the transport systems in the intestine and kidney, and still less about their nature in these organs in fish. A summary of current knowledge follows.

1.1 Amino Acid Transport

The development of techniques including the measurement of unidirectional fluxes in emusculated sheets in flux chambers, microelectrode transmembrane electrical measurements and flux measurements in membrane vesicles, have distinguished a number of mechanisms of amino acid uptake. Amino acids are a broad group of solutes with differing properties. As might be expected, they are handled by cells in a variety of ways. A summary of known amino acid transport systems for cells other than those of the intestine and renal tubule is presented in Table 1. They include systems which transport the cationic or basic amino acids (e.g. lysine, arginine), the anionic or acidic amino acids (e.g. glutamate, aspartate), and the zwitterionic or neutral amino acids (e.g. phenylalanine, glycine, alanine, leucine).

Many of these systems are affected by pH, because in most cases, it is the charge state of the amino acid which characterizes the transporting system. In some cases, however, the charge can be modified by the presence or absence of sodium ions and, to some extent, lithium ions. The ASC system will transport arginine and lysine independently of sodium, while system y^+, which generally transports cationic amino acids in a sodium-independent fashion, will also transport several

Table 1. Summary of amino acid transporting systems in cells other than enterocytes and renal tubule cells (after Christensen, 1984).

Type of Amino Acid	System Name	Sodium Dep.*	Characteristics
Cationic e.g. Lysine	y^+ formerly Ly^+	No	ubiquitous
Anionic e.g. Glutamate	X_{AG}^-	Yes	Wide occurrence, reacts similarly with aspartate and glutamate.
	X_A^-	Yes	Carries aspartate but excludes glutamate: where characterized, e.g. hepatocytes and erythrocytes, is identical to ASC.
	X_G^-	No	Carries glutamate and analogs, but excludes aspartate and analogs: occurs in endogenous neuronal vesicles.
	X_C^-	No	Carries glutamate and cystine, possibly avariant of X_G^-.
Zwitterionic e.g.leucine	Gly	Yes	Widespread. Carries glycine and sarcosine. Tolerates N-methyl or other group on α-amino N.
	A	Yes	Ubiquitous. Carries most zwitterionic amino acids. Tolerates N-methyl or other group on α-amino N.

*Dependence

Table 1 : continued

Type of Amino Acid	System Name	Sodium Dep.*	Characteristics
	ASC	Yes	Ubiquitous. May carry prolines, but excludes N-methyl amino acids.
	N	Yes	Glutamate, asparagine and histidine. Characterized only in hepatocytes and hepatoma cells so far.
	β	Yes	β-alanine, taurine, 4-aminobutyric acid.
	L	No	Ubiquitous. Prefers amino acids with branched or apolar side-chains. Active in exchange of amino acids. May be heterogeneous.
	L_1	No	Low K_t component which develops on extended incubation of hepatocyte monolayers.
	T		Prefers benzenoid amino acids. Occurs in human red blood cells.

*Dependence

neutral amino acids in a sodium-dependent fashion. The sodium ion in this latter case presumably acts as the charge group of the arginine or lysine normally transported by the y^+ system (Christensen, 1984).

Carrier heterogeneity, or variants of a carrier system, has been observed within a cell type, particularly for the L-system. It tends to be prevalent in the cells of the intestine and the renal tubule, where carrier systems of broad and differing characteristics are abundant. Christensen (1984) suggests that the renal tubule and the intestinal brush border have transport systems which differ from those of most other plasma membranes.

Difficulties in defining the transport systems of intestine arise for several reasons. The tissue is heterogeneous, consisting of endocrine cells, goblet cells, gland cells and enterocytes (the absorbing cells) in differing stages of maturity. In rabbit ileum, autoradiographic evidence indicates that the uptake of amino acids occurs only in the upper third of the villus (King et al., 1981). Thus most techniques used at present include a large number of non-transporting cells. Cell asymmetry is an additional difficulty. The enterocyte consists of a brush border, or luminal surface, and a basolateral surface, both of which face strikingly different milieux. It might therefore be expected that transport systems on the two surfaces are distinct, and differences have been found (Stevens et al., 1982; Christensen, 1984; Lash and Jones, 1984).

The current theory for energy dependent uptake of amino acids at the brush border, the sodium gradient hypothesis, takes into account this asymmetry (Munck, 1981). This hypothesis states that sodium concentrations in the enterocyte are maintained low relative to the ambient levels through the expenditure of metabolic energy by the electrogenic Na^+,K^+-ATPase located at the basolateral membrane. The cytoplasm is, therefore, electrically negative with respect to the luminal fluid. Inward movements of sodium following its electro-chemical gradient are coupled through a carrier to inward movement of amino acid. High intracellular concentration of the amino acid results in its net outward movement across a basolateral membrane carrier. The sodium gradient hypothesis for coupled amino acid transport has been confirmed in many studies (e.g., Rose and Schultz, 1971; Sigrist-Nelson et al., 1975), and is generally accepted.

The intestinal transport of anionic amino acids could not be demonstrated using steady state techniques because of transamination of these solutes (Munck, 1981). However, in unidirectional flux studies, Schultz and his coworkers (1970) found anionic amino acid transport in rabbit ileum to be saturable, sodium-dependent, and competitively inhibited by other anionic amino acids. Lerner's review (1984) also describes an anionic amino acid transporter in the domestic fowl *Gallus domesticus.*

Transport of cationic and neutral amino acids is less well defined. A system similar to y^+ appears to be present in rabbit ileum (King et al., 1981), humans (Bannai,1984) and the domestic fowl (Lerner, 1984). This system has been examined in humans with a genetic transport disease called cystinuria, in which high levels of cystine and basic amino acids are excreted in the urine. These studies suggested that cystine in the intestine shares a single transport system with the basic amino acids lysine, arginine and ornithine, and that its uptake is saturable and sodium-dependent. Thus, this system resembles the y^+ system with Na^+ ions providing the charge necessary for the transport of cystine, which is largely neutral at luminal pH. Although Lerner (1984) lists the y^+ system as being present in the domestic fowl, he also reports that cystine shares a single saturable system with L-leucine and the basic amino acids, but that its transport may be sodium-independent. White (1985) states that although system y^+ is sodium-independent in most other cells, there is evidence in the intestine that there may be both sodium-dependent and -independent components to basic amino acid trasnsport. It is not yet known if these two systems represent a single system or two independent systems. Of interest, however, are findings in brush border membrane vesicles from rats fed a low protein diet which show only the sodium-independent lysine transport system, while brush border membrane vesicles from rats fed a high protein diet show both sodium-dependent and -independent systems (White, 1985).

The early uptake studies on the transport of neutral amino acids were hampered by the assumption that a single carrier system existed. Matthews and Laster (1965) performed competition studies in hamster intestine and concluded that alanine, glycine and leucine shared a common transporter. Sigrist-Nelson and her colleagues (1975) showed that uptake of L-alanine was inhibited by the addition of glycine, threonine, serine, valine, phenylalanine, leucine and methionine in the presence of a sodium gradient (interior negative) in membrane vesicles. However, Baker and George (1971) suggested from competition studies in rat intestine that two neutral carriers existed.

Atkins and Gardner, in 1977, demonstrated that most published data for absorption kinetics best fit a model which included a saturable component described by Michaelis-Menten kinetics and a linear non-saturable component. The idea of two transport systems began to gain support. Sepulveda and Smith (1978) suggested that two transporters, one that was sodium-dependent and inhibited by serine, and one that was less sodium dependent, uninhibited by serine and showed a preference for hydrophobic amino acids, occurred in rabbit ileal mucosa. This same laboratory (Paterson et al., 1979) calculated uptake of serine, methionine and alanine as a single carrier mediated entry mechanism, and as a mediated entry mechanism plus a linear diffusional component. They found that the two component model fit best, but they were doubtful that the linear component was diffusional because a portion of it could be inhibited in the presence of a second amino acid.

Total inhibition was impossible because the levels of the competitor required were too high. They suggested that both components were carrier mediated, the first with high affinity and low capacity (saturable) and the second with low affinity and high capacity (linear). In a subsequent study (1980), these authors found that only the linear component remained when uptake was examined in the absence of sodium (Paterson et al., 1980). A sodium-independent carrier mediated mechanism susceptible to competitive inhibition (the second mechanism, above) and a passive diffusive component may therefore exist.

A third entry mechanism for neutral amino acids was reported (King et al., 1981) when it was demonstrated that alanine in rabbit ileum shared a high affinity, sodium-independent lysine transporting pathway distinct from the basic amino acid system which resembled y^+. Munck (1981) lists two neutral amino acid carriers for the intestine, one which transports proline, hydroxyproline, N-methylated glycines, sarcosine, β-alanine, γ-aminobutyric acid, D-alanine and taurine, and one which is sodium-dependent and transports L-amino acids, proline and hydroxyproline. Munck states, however, that a second saturable system which prefers lipophilic amino acids has been demonstrated, but its resemblance to the L-system has yet to be established.

Stevens and his colleagues (1982) suggested the presence of five neutral amino acid carriers in rabbit jejunal brush border membrane vesicles. Two systems were sodium-independent: one which seemed to resemble the L-system in that it showed a preference for lipophilic amino acids; and one which resembled y^+, with lysine uptake inhibited by several neutral amino acids. Three systems were sodium-dependent: one which transported neutral L-amino acids but did not carry β-alanine or N-methyl aminoisobutyric acid (MeAIB); one which favoured phenylalanine and methionine; and one which favoured imino acids and MeAIB. They found that none of these systems resembled A or ASC, and that β-alanine was not taken up by a carrier mediated mechanism. Whether differences between these findings and those of previous workers, based mainly on studies on rabbit ileum, reflect differences in methodology or differences in position along the intestine is open to question. Differences in regional uptake of neutral amino acids have been demonstrated in rat (Baker and George, 1971), dog (Robinson et al., 1980), domestic fowl (Levin et al., 1983) and hamster (Navab et al., 1984). In the latter study, Navab et al. (1984) reported two possible mediated and one non-mediated component of L-histidine uptake. In agreement with the findings of Stevens et al. (1982), β-alanine was not transported by a carrier mediated mechanism.

Lerner (1984) described two sodium-dependent transporters for neutral amino acids, one which transports L-leucine and other neutral amino acids and a second which transports β-alanine, proline and related amino and imino acids in the domestic fowl. Lerner also mentioned that in the absence of sodium, some transport of leucine, methionine, glycine and proline still occurred. Thus, there is a sodium-independent uptake of these amino acids.

Stevens et al. (1982) found that, in addition to the carrier mediated mechanisms of entry, there was a significant diffusional component. At 5 mM phenylalanine and 23°C, this passive diffusion accounted for 25% of the uptake in rabbit jejunum. In rat ileum, Bikhazi et al. (1985) described sodium-dependent transporters for alanine and phenylalanine, and added that 15 to 20% of these amino acids was passively translocated.

Systems which transport amino acids in mammalian and avian intestine therefore include a carrier for anionic amino acids, at least one carrier for cationic amino acids, and possibly two, with differing sodium requirements, and several carriers for neutral amino acids, one of which is sodium-independent, and possibly three which are sodium-dependent. Diffusional uptake may represent a significant proportion of uptake.

Transport at the basolateral membrane appears to be more similar to that of other cells. Mircheff et al. (1980) reported that in rat intestinal basolateral membrane vesicles, a transporter that resembled the classic A system and one that resembled the classic ASC system seem to be present (Table 1). In addition, a sodium-independent system that resembles the L system was also found. They suggested that the A-like and ASC-like systems would be important in the transport of amino acids from the blood to the cell when luminal amino acids are absent, while the L-like system probably was responsible for efflux of neutral amino acids during absorption.

There are still many questions to be answered, especially with respect to neutral amino acid transport. However, there appear to be saturable, sodium-dependent and -independent transporters of broad and overlapping specificity, and sodium-independent passive uptake (the linear component of the equation of uptake) which may or may not be carrier mediated. Many of the observed differences may be species differences, technical differences or positional differences.

1.2 Intestinal Studies in Fish

Studies on fish have concentrated less on the carrier specificities than on factors influencing uptake, particularly ions and temperature. Using everted sacs, proline and glycine uptake was found to be concentrative against a gradient in puffer (*Spheroides maculatus*), sea robin (*Prionotus carolinus*), scup (*Stenotomus chrysops*) and toadfish (*Opsanus tau*) (Wilson, 1957). Concentrative uptake has been reported in many fish by many authors: tyrosine in winter flounder, *Pseudopleuronectes americanus* (Huang et al., 1965), killifish, *Fundulus heteroclitus* (Huang and Rout, 1967), and catfish, *Ictalurus punctatus* (Chen and Huang, 1972); threonine, alanine, serine, histidine, valine, methionine, phenylalanine and leucine in the goldfish, *Carassius* *auratus* (Mepham and Smith, 1966a); L-leucine in scup, *Stenotomus versicolor* and catfish, *Ictalurus nebulosus* (Neff and Musacchia, 1967); glycine in white grunt, *Haemulon plumieri* (Smith, 1969); L-leucine in rainbow trout, *Salmo gairdneri* (Ingham and Arme, 1977); glycine in rainbow trout (Bogé et al., 1977a); phenylalanine in tench, *Tinca tinca* (Cartier et al., 1979); L-lysine in rainbow trout (Hokazono et al., 1979); 2-amino isobutyric acid in *Dicentrarchus labrax*, *Boops salpa*, *Anguilla anguilla* and *Mugil cephalus* (Bogé et al., 1982); proline in coho salmon (Collie, 1985); and taurine in winter flounder (King et al., 1986). Most of these studies also demonstrated sodium-dependence and a requirement for metabolic energy of at least a part of the uptake. It is therefore apparent that in fish intestines, the sodium gradient hypothesis for active amino acid uptake applies.

Uptake of neutral amino acids in the presence of sodium in goldfish was divisible into two groups: phenylalanine, valine, methionine and leucine, which were concentrated to more than two times the levels achieved under similar conditions in hamster intestine; and threonine, alanine, serine and histidine, which were concentrated only to 1.2 times the level in hamster intestine (Mepham and Smith, 1966a). These authors suggested that the difference between the two might reflect species differences, and be related to the ability of the goldfish to adapt to different temperatures. In a second study, Mepham and Smith (1966b) found that valine and threonine uptake behaved differently after temperature acclimatization. Threonine was not transported at all at temperatures below the acclimatization temperature, while valine was. These data raise the possibility that two neutral amino acid carrier systems are present. This study also demonstrated that while increased incubation temperature increased amino acid uptake, decreased acclimation temperature also resulted in higher values when compared to uptake in warm acclimated fish at the same temperature. A constant relationship between transmural potential difference, and uptake measured by the change in potential difference in the presence of the solute was demonstrated by Mepham and Smith (1966b). Therefore, they avoided the difficulty encountered in standardizing uptake to such things as mucosal weight, which may change with temperature acclimation. Smith (1970) found that the decline in amino acid uptake at lower assay temperatures consisted of two components. One was associated with a concomitant decline in water and sodium uptake, and was general for all eighteen amino acids examined. The second was more specific, with only certain amino acids affected. Those not affected, termed by Smith non-adaptors, were methionine, tyrosine, phenylalanine, leucine and histidine. Smith felt that an explanation which invoked two carriers to account for the difference between adaptors and non-adaptors was not satisfactory. He suggested that the lipophilicity of the non-adaptors might provide a clue, and that changes in the fatty acid composition of the membrane with temperature adaptation probably affected transport. Indeed, the time course of changes in membrane lipid and amino acid transport were found to be similar in goldfish (Smith and Kemp, 1971). However, these authors noted that influx of alanine and phenylalanine was not affected by adaptation, but net flux of alanine was, suggesting that the change in transport may be due to changes at the basolateral membrane.

Kitchin and Morris (1971) found that the uptake of valine, an adaptor according to the classification of Smith, increased with decreasing temperature in goldfish, due largely to an increase in carrier affinity for valine. However, uptake of methionine decreased at decreased acclimation temperature, while affinity was independent of acclimation temperature, suggesting that the number of carriers had decreased. Competition studies indicated that valine and methionine shared a common carrier, but the authors suggested that methionine may be transported by a second carrier. More work is required before the effects of temperature on such carrier systems can be resolved.

The above systems were measured in the presence of sodium, and are probably sodium-dependent. Sodium independent systems operate in goldfish (Kitchin and Morris, 1971), catfish, *Ictalurus punctatus* (Chen and Huang, 1972) and rainbow trout (Ingham and Arme, 1977), and are probably of general occurrence in fish. Whether these systems are diffusive or carrier mediated remains to be established, but Miller and Kinter (1975) demonstrated that only the non-meditated portion of uptake remains in the absence of sodium in goldfish and killifish.

Ferraris and Ahearn (1984) noted that the apparent diffusional component of amino acid uptake is generally higher in marine compared to freshwater fishes. Significant backflux into the intestinal lumen from the cell therefore prevents tissue (T) to medium (M) ratios of greater than one. Their statement that T/M ratios for amino acids are below one is based on data for two fish species only, and may not be a general phenomenon. However, Smith (1969) did note backflux of glycine in *Haemulon plumieri* when no mucosal glycine was present. Collie (1985) also found that serosal to mucosal unidirectional fluxes of proline were higher in saltwater compared to freshwater coho salmon, while mucosal to serosal fluxes were lower, resulting in lower net flux in the saltwater fish. In winter flounder, the intestinal transmural electrical potential difference (TEP) has been found to be serosa negative, unlike all mammals and most freshwater fish examined to date (Ferraris and Ahearn, 1984). Serosal negative TEP has been found in almost every marine fish species examined so far, as have low electrical resistances. The latter is a measure of the "leakiness" of the tissue, and indicates that the junctional complexes and paracellular pathways offer little resistance to the flow of ions (Ferraris and Ahearn, 1984).

High apparent diffusional components, lower net transmural flux of solutes on adaptation to seawater, serosa negative transmural potential difference and lower electrical resistances in marine fish all point to important structural differences both in the membrane composition of the enterocytes (Ackman, 1967; Ferraris and Ahearn, 1984) and also in the tight junctions. It is also apparent that characteristics for amino acid uptake may differ in marine fish. Recent work on brush border membrane vesicles from several marine fish has demonstrated a requirement for Cl^- ions for the uptake of glycine (Bogé and Rigal, 1981; Bogé et al., 1983; Bogé et al., 1985); Cl^- apparently increases the accessibility of the carrier site to sodium.

These differences in membrane structure, in the demands made on the intestine and in the requirements of the transporters in marine fish suggests that the intestine may respond in a different manner to such stressors as temperature when compared to freshwater fish. The nature of this response is examined in the present study on seasonal changes in uptake of phenylalanine by the intestine of winter flounder (*Pseudopleuronectes americanus*).

1.3 The Winter Flounder

Winter flounder is a common marine flatfish which ceases feeding in late fall and resumes again in spring, at least in the northern part of its range (Kennedy and Steele, 1971; Fletcher et al., 1981). The gonads of mature flounders begin to develop in September in preparation for spring spawning (Burton and Fletcher, 1983). In fall, levels of antifreeze peptides rise (Fourney et al., 1984). Thus, flounders undergo a natural period of starvation at a time when they face considerable metabolic energy demands.

Concomitantly, however, they are faced with reduced demands for metabolic energy because they become inactive, often burying themselves in the sediments (Fletcher et al., 1981). Winter temperatures probably further reduce metabolic rate. Energy requirements will reflect a balance of these factors plus maintenance requirements, and the extent of metabolic reserve depletion will be determined by that balance. The intestine may be expected to respond to these changing energy demands. In spite of these major changes, no studies on seasonal changes in intestinal function have been performed on the winter flounder. In a study of glucose and leucine uptake in the intestine of winter flounder, Thompson and Kleinzeller (1985) noted that leucine uptake was much greater than glucose uptake, but that neither was detectable in intestines taken from fish caught in December. They speculated, however, that the electrical measurement of uptake in these fish might not be sensitive enough to detect low levels of uptake. Indeed, Shears and Fletcher (1983) reported that zinc uptake by the intestine of winter flounder was reduced, but not abolished, in the winter. Seasonal changes in sodium flux rates and electrical properties of the intestine of the earthworm, *Lumbricus terrestris*, have been reported (Cornell, 1984). Csaky and Gallucci (1977) found a decline in 3-O-methylglucose and L-phenylalanine uptake as well as a decline in Na^+,K^+-ATPase activity in the winter in non-feeding *Rana pipiens*. Na^+,K^+-ATPase activity also decreased in winter in *Rana esculenta*, with a second decrease in July to September. A third species, *Rana catesbeiana*, showed no seasonal variations in intestinal active transport under the same conditions. These results in other species suggest that intestinal transport may be sensitive to seasonal changes, but that the nature of the response differs with species. Csaky and Gallucci attributed these differences to evolutionary adaptation.

1.4 Objectives

This study was initiated in order to test the hypothesis that the intestine of the winter flounder undergoes considerable functional and structural change throughout the year in response to the changing energy requirements of the fish, and especially from the feeding period (summer) to the non-feeding period (winter). It is predicted that diffusional uptake of amino acids will continue through the winter non-feeding period, and the presence of ion gradients necessary for the uptake of salts for osmoregulation, the major function of the intestine in winter, will allow continued saturable uptake. It is also predicted that the intestine of the winter flounder does not behave as a single unit, but is differentiated into sections which will take up phenylalanine differently, and respond differently with respect to season. Finally, this study was undertaken to establish whether the pyloric caeca of the winter flounder are involved primarily in protein handling.

These questions were addressed by:

1. An assessment of body composition of the winter flounder throughout the year to estimate the degree of depletion of metabolic reserves. If protein uptake provides calories, nitrogen and essential amino acids, it would be predicted that the uptake of an essential amino acid would be the highest at the onset of feeding when replenishment of energy reserves is the most critical.

2. An examination of structural changes in the intestine. Overall uptake will be affected by the surface area of the intestine. Intestinal surface area is enormous due to folding of the mucosa and to the presence of microvilli, finger-like projections of the apical surface of the cell. Changes in either of these structural features could significantly alter uptake by reducing the absolute number of carriers. Starvation is known to cause atrophy of the intestine (Karasov and Diamond, 1983a), while in goldfish, low temperature acclimation results in hypertrophy. The intestine of winter flounder may therefore be expected to atrophy, but the decrease may be ameliorated by low temperatures and a continued requirement for osmoregulation.

3. Measurement of uptake of phenylalanine throughout the year. Phenylalanine is an essential amino acid in fish (National Academy of Sciences, 1973). Its uptake may be more closely regulated than that of a non-essential amino

acid. *In vivo* assessment allows estimation of uptake in the presence of the normally operating controlling factors (hormonal, neural or feedback). *In vivo* uptake estimations by measurement of disappearance gives a measure of net flux. Factors acting at both the basolateral and brush border membrane are predicted to be influencing uptake rate. If compared to *in vitro* estimations of uptake which measure influx and not net flux, then factors acting at the basolateral and brush border may be differentiated. The effect of temperature will be assessed since it is likely to be a major influence on uptake.

4. Assessment of regional functional variations in uptake. Pyloric caeca function can be assessed and compared with the rest of the intestine to ascertain if they are a site for preferential amino acid uptake.

5. Assessment of uptake with respect to nutrients other than amino acids. Such an assessment will ascertain if any changes observed are specific to amino acids, to protein-associated materials or general for nutrient handling in the gut.

6. Delineation of some of the characteristics of the carrier(s). A change in sodium dependency with season could conceivably affect the function of a carrier. Measurements of uptake in the presence and absence of sodium at different times of the year would reveal any such changes.

Observation of regional carrier differences in mammalian intestine (King et al., 1981; Stevens et al., 1982) suggest that such differences may also occur in flounder, and studies of phanylalanine uptake in the presence of competitively inhibiting amino acids may help to elucidate such differences.

This study is divided into three sections. Chapter 3 reports physical changes in the fish, structural changes in the intestine and activities of two brush border membrane enzymes, leucine aminopeptidase and alkaline phosphatase. Chapter 4 reports the results of the *in vivo* studies of phenylalanine uptake. Chapter 5 reports the results of the *in vitro* uptake studies which include the effect of temperature, the effect of the use of a sodium-free uptake medium and the effect of competitive inhibitors on phenylalanine uptake.

Chapter 2

Materials and Methods

2.1 Care of Flounders

Winter flounder were caught by otter trawl in Passamaquoddy Bay and returned to the laboratory where they were placed in large holding tanks of running seawater. The seawater supply was obtained from the bay adjacent to the laboratory, so temperatures reflected the ambient temperature of the Bay, and varied between 3°C in the winter and 15°C in the summer. Salinity varied naturally between 25 and 29.5 °/₀₀. Holding tanks were either indoors, in a room with windows to allow for natural photoperiod, or outdoors.

Fish would take food only in the summer, and they were provided with food every other day. This schedule ensured that the intestine was regularly stimulated by food. Fish to be used for experiments were removed from the tank prior to feeding so that the intestines were not full. Flounders were fed a diet of shrimp caught in Passamaquoddy Bay, which they took readily. Fish were used within two weeks of capture.

In late fall, prior to the cessation of feeding, a large supply of flounders was brought to the holding tanks for the winter experiments. They were offered food as above but by mid-October, 1984 and late-October, 1985 all fish had ceased feeding. In the spring of 1985, fish were offered food beginning in mid-April, but did not begin feeding until early May.

2.2 Light and Electron Microscope Studies

In order to divide the intestine into sections appropriate for uptake studies, the intestines of two small (5.0 and 7.2 cm standard length; standard length is the distance between the tip of the jaw and the caudal peduncle) flounders were serially sectioned. These fish were feeding fish captured in July. Flounders were sacrificed by spinal transection, and the intestine removed. The intestines were rinsed with saline, cut into approximately 1 cm lengths and fixed in Bouin's solution. Sections were then embedded in Paraplast and cut serially in 8 um sections using a microtome. Staining was with hematoxylin and eosin.

Seasonal changes in intestinal structure were examined from pieces of intestine taken from fish at the end of the *in vivo* uptake experiments. Sections were removed just posterior to the perfused intestinal section. The fourth and most posterior of the caeca was cut free of the foregut for sectioning. Intestinal sections were fixed and stained as described for serial sections, but were cut 6 um thick.

For electron microscopy, one fish from October and one from February was sacrificed by spinal transection. Pieces of caeca, foregut, midgut and hindgut were rapidly removed, fixed with Karnovsky fixative for 2 h, washed in 0.1 M sodium cacodylate, post-fixed for 1 h with osmium tetroxide (1%), and then transferred to 0.1 M sodium cacodylate buffer. They were then transported to the University of New

Brunswick in Fredericton, where they were embedded in Epon-Araldite. Sectioning and transmission electron miscroscopy was performed at the University of Ottawa Faculty of Health Sciences, Department of Anatomy.

2.3 Surface Area Measurements

Light microscopic sections from the three experimental periods, January/February, May/June and September/October, were chosen to examine seasonal changes in the degree of intestinal folding. Sections were enlarged 17- or 22-times and traced onto paper from projections of the slides. Internal (mucosal surface, excluding microvilli) and external (outer intestinal circumference) perimeters were drawn for each section. Through the use of computer-assisted morphometry (Department of Anatomy, Faculty of Health Sciences, University of Ottawa), the inner and outer perimeters were measured, and the cross-sectional areas calculated. The ratio of the measured inner perimeter to cross-sectional area of the intestine was then compared for each of the four divisions of the intestine, and for each of the three sampling periods.

2.4 Enzyme Assays

Three fish from October and three fish from February were killed by spinal transection. The intestines were rapidly removed, and separated into the four sections (caeca, foregut, midgut and hindgut). The mucosa of each section was scraped from the musculature using a glass slide, and placed into a tared vial. These were weighed, and nine volumes of flounder Ringer's (Naftalin et al., 1978: 150 mM NaCl, 15 mM $NaHCO_3$, 5 mM $KHCO_3$, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.3 mM NaH_2PO_4 and 1.65 mM Na_2HPO_4) were added. The mucosa was then homogenized for five seconds using a Polytron PC-U homogenizer (Brinkman Instruments).

2.4.1 Alkaline Phosphatase

Homogenates were further diluted 1:3 with flounder Ringer's (final dilution 40 times). Alkaline phosphatase activity was measured by kit (Sigma Chemical Co., St. Louis, Mo.), using the rate of appearance of *p*-nitrophenol from *p*-nitrophenyl-phosphate at 405 nm and 30°C.

2.4.2 Leucine Aminopeptidase

Homogenates were diluted a further 100-times for fish sampled in October (final dilution 1000-times) and a further 10-times for fish sampled in February (final dilution 100-times). Leucine aminopeptidase activity was estimated according to the discontinuous method of Appel (1974). A synthetic substrate, L-leucine-*p*-nitroanilide, was hydrolyzed for 2 h at 8°C to *p*-nitroaniline and leucine, and the *p*-nitroaniline was then diazotized, coupled and converted to an azo dye. The optical density was read at 546 nm on a spectrophotometer (LKB Ultrospec 4050) and compared to a standard curve of various concentrations of *p*-nitroaniline similarly converted to an azo dye.

2.4.3 Protein

Protein was measured by the method of Lowry et al.(1951) using bovine serum albumin as a standard.

2.5 *In vivo* Uptake Studies

2.5.1 Surgical Techniques

In vivo uptake studies were based on the methods of Bogé and his colleagues (1977b). A fish was brought into the laboratory and placed in 4 L of fresh seawater containing 100 mg/L MS222 (tricaine methanesulfonate) for 10 minutes. It was then transferred to a tray where the gills were bathed by a continuous anaesthetic solution of 70 mg/L (Fig. 1). All anaesthetic solutions were buffered to pH 8.4 with Trizma base. Incisions were made on the dorsal side of the flounder to expose the intestine. Two cannulae, an anterior inflow and a posterior outflow, were inserted into a segment of the intestine and tied securely. Fore- and midgut segments averaged 3 to 5 cm in length, while the hindgut was the full length of the intestine posterior to the valve (Fig. 2). The pyloric caeca were too short to cannulate individually in the winter, so an inflow cannula was placed immediately posterior to the pyloric sphincter in the foregut, and an outflow cannula was placed immediately anterior to the last caecum on the foregut. Thus, the three anterior caeca were perfused as a group in both summer and

Figure 1. Schematic diagram of set-up for surgical procedure. A: Anaesthetic inflow. B: Anaesthetic ouflow. C: Site of incision.

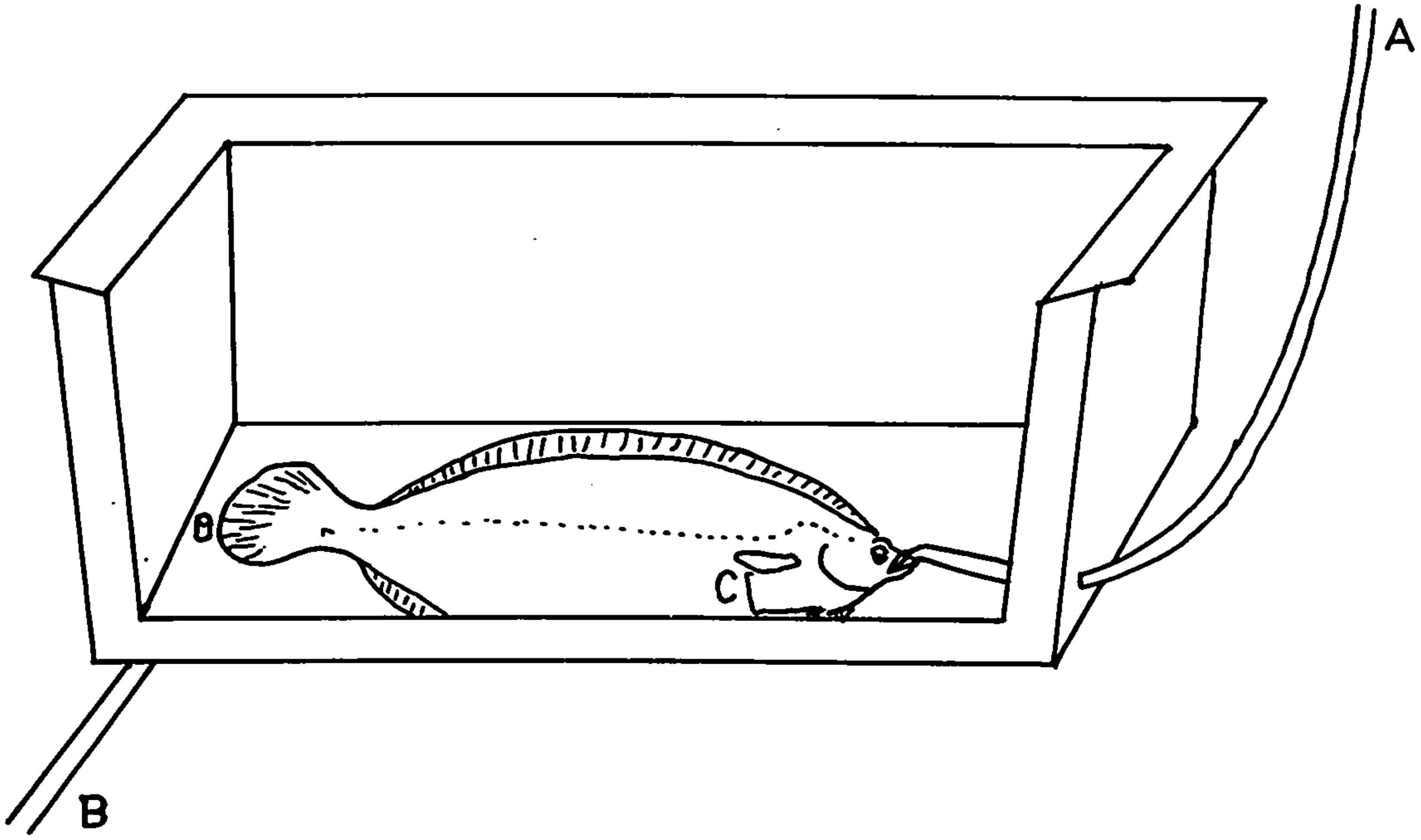
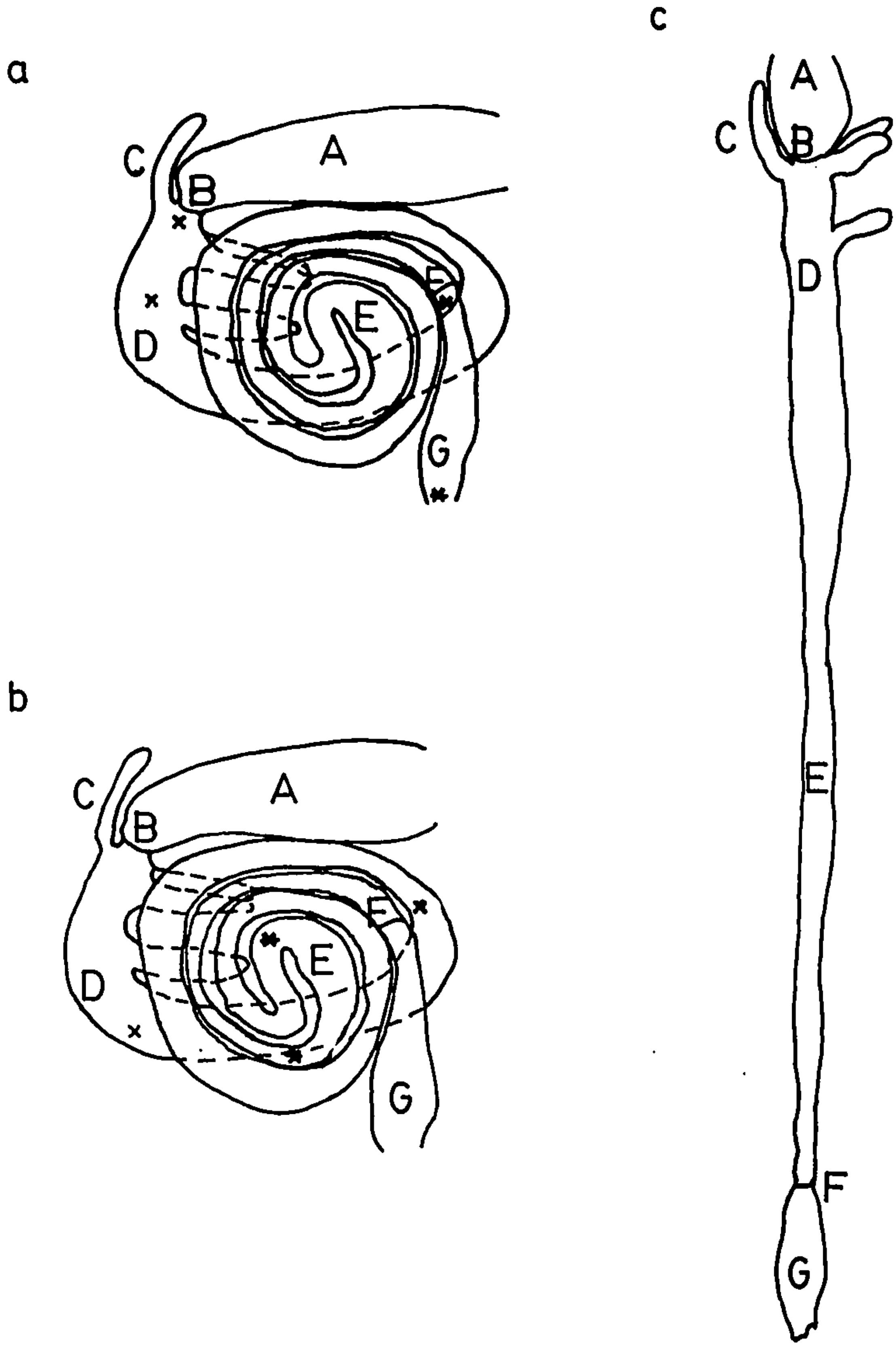

A
B
C
B

Figure 2. Schematic diagrams of the intestine of winter flounder as it appears *in situ* (a and b) from a dorsal incision (head of the fish is to the right), and extended to show the four divisions (c). a: *In situ* intestine marked to show placement of the cannulae which were used to perfuse the caeca (x), and the hindgut (*). b: *In situ* intestine marked to show placement of the cannulae which were used to perfuse the foregut (x) and the midgut (*). c: Intestine as it appears when removed from the fish and straightened out. A: Stomach. B: Pyloric Sphincter. C: Pyloric Caecum (one of four). D: Foregut. E: Midgut. F: Intestinal Valve. G: Hindgut.


a
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winter. Care was taken at the inflow cannula to avoid occluding the major blood vessels which supply the caeca and sphincter when tying the cannula into place. Two segments were prepared for perfusion in each fish, usually the foregut and midgut or the hindgut and caeca.

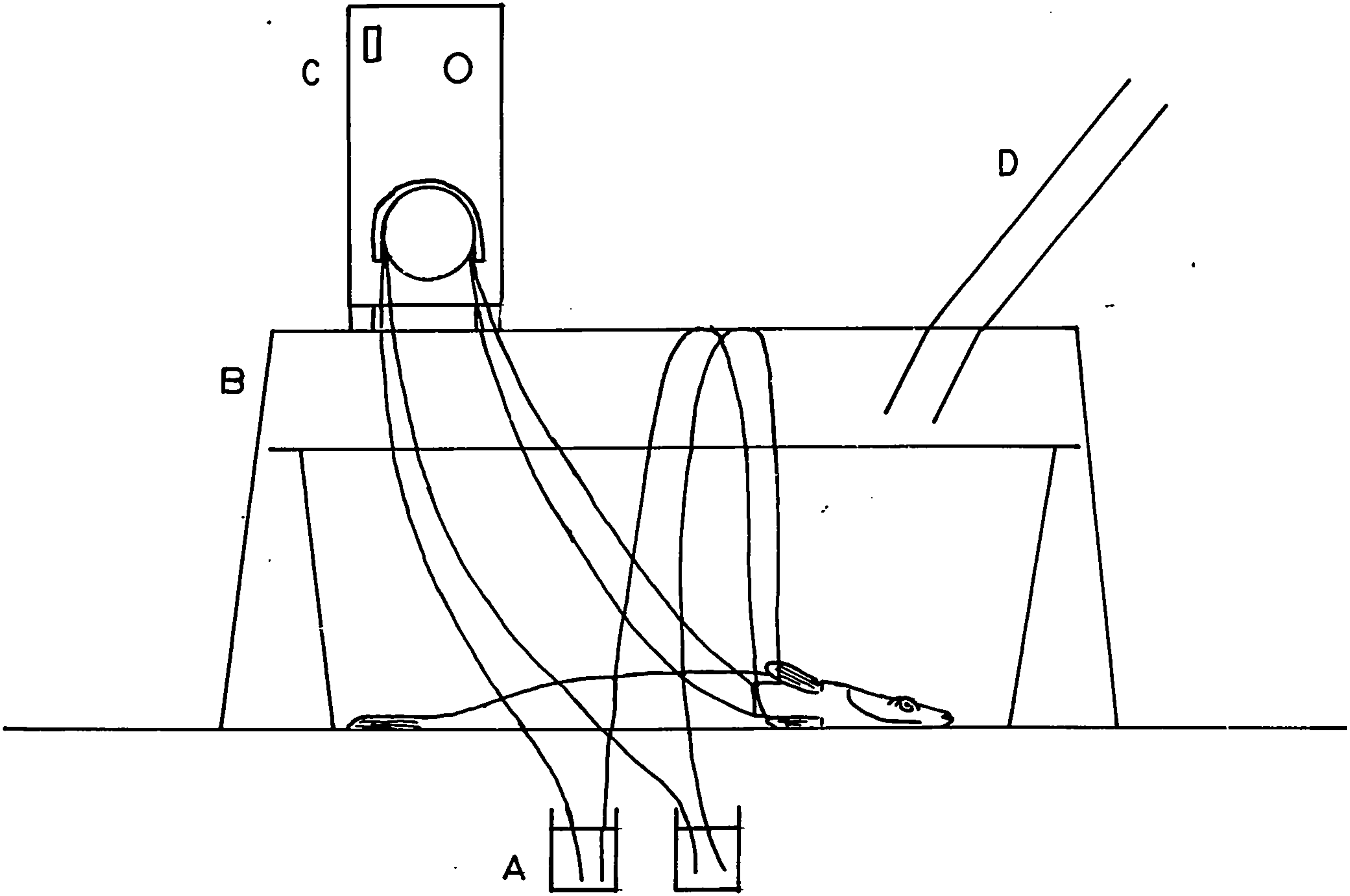
Following insertion of the cannulae, the incision was sutured closed and the cannulae tacked with thread to the dorsal skin a short distance from the incision to prevent movement when the fish recovered. The fish was then transferred to a pan (40 x 27 x 10 cm) supplied with running seawater. Under these conditions, fish recovered from the anaesthetic in no longer than 5 min. A cage was placed over the pan to prevent the fish from jumping out, and to support the peristaltic pump during the perfusion. Ambient water temperature was maintained at all times throughout the procedure. Fish were allowed to recover overnight before perfusion.

2.5.2 Perfusions

Intestinal sections were perfused at a rate of 0.15 ml/min (± 0.03 ml/min) in a closed circuit system (Fig. 3). Perfusates were bubbled for 30 min with 99% O_2/1% CO_2 prior to perfusion, and pH adjusted to 7.3. All perfusates were made up in flounder Ringer's, with a total of 20 mM solute added. Replacement of phenylalanine was achieved through the use of equimolar mannitol.

The experiment began with a 1 h pre-perfusion with 10 mM phenylalanine. The intestinal section was then

Figure 3. Schematic diagram of the set up for *in vivo* perfusion. A: Reservoirs for perfusion solutions for intestinal segments (two segments perfused simultaneously). Each reservoir contained one inflow and one outflow cannula. B: Cage which supports perfusion pump and prevents fish from jumping out of tank. C: Perfusion pump. D: Seawater inflow.


C
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A

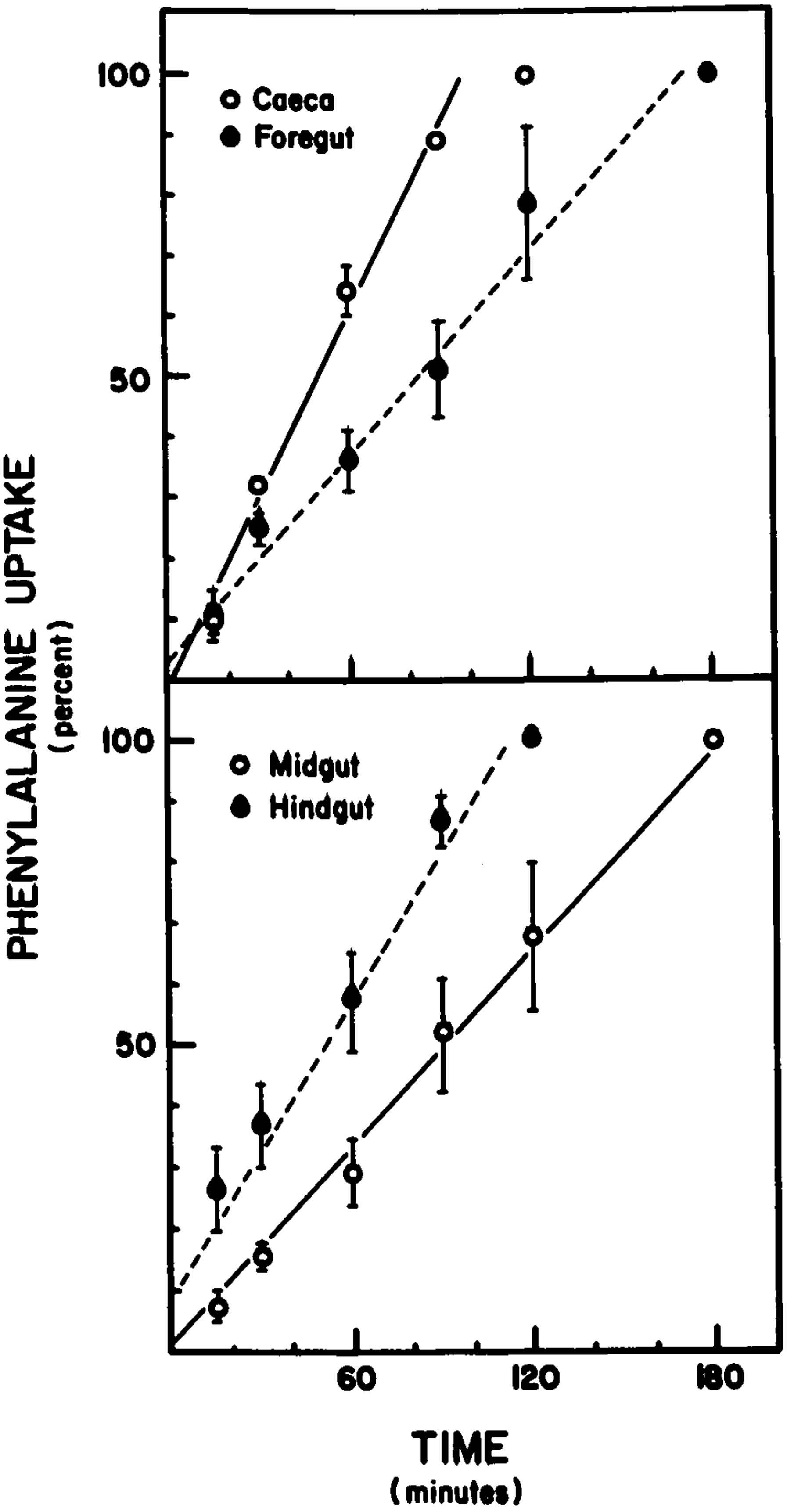
perfused a minimum of 30 min with a Ringer's containing 20 mM mannitol (mannitol Ringer's) to clear it for the next perfusion. The section was perfused with the test solution for 1 h, at which time uptake was linear (Fig. 4). Test solutions were 5 ml of 0.5, 1.0, 2.0, 5.0, 10.0, 15.0 or 20.0 mM phenylalanine. Perfusions on each section were begun with the lowest concentration, with the last perfusion for each fish 20.0 mM. Approximately 0.1 uCi of ^{14}C-phenylalanine was added to each solution, to give a specific activity of approximately 9.5×10^7 dpm/mmol at 0.5mM and 2.5×10^6 dpm/mmol at 20.0 mM.

A 25 ul sample of perfusate was taken from the reservoir at the start and the end of each 1 h perfusion, and added to 8 ml of scintillation cocktail (Scintiverse; Fisher Scientific). Radioactivity was determined using a LKB 1211 Rackbeta liquid scintillation counter with automatic quench correction. The uptake of phenylalanine was then calculated from the difference between the initial and the final values.

Changes in the concentration of the perfusate due to absorption or secretion of water across the intestinal segment was monitored by adding 0.1 g/L phenol red to all perfusion solutions (Winne, 1978). Samples of 50 ul were taken as above, added to 2.5 ml of 0.1 mM NaOH and the optical density read at 558 nm on a spectrophotometer.

Possible diurnal changes in uptake were estimated by five consecutive 1 h perfusions of 5 mM phenylalanine (plus ^{14}C-phenylalanine) in midgut and caeca segments only.

Figure 4. Linearity for *in vivo* uptake of L-phenylalanine in caeca and foregut (upper panel), and midgut and hindgut (lower panel). Perfusion time was a maximum of 180 min. Phenylalanine concentrations were 0.5 mM and 5 mM, and uptake was expressed as a per cent of total uptake (final uptake = 100%). Both concentrations showed the same pattern, and they were therefore combined (Mean ± SE; n = 6 (foregut and caeca), 7 (midgut) or 9 (hindgut)).



PHENYLALANINE UPTAKE
(percent)
100
50
○ Caeca
● Foregut
100
50
○ Midgut
● Hindgut
60
120
180
TIME
(minutes)

The intestinal segments were pre-perfused as already described, and each test perfusion was separated from the others by a mimimum of 30 min perfusion with mannitol-Ringer's.

2.5.3 Final Sampling Procedures

At the end of each *in vivo* experiment, the fish was killed by spinal transection, then weighed and measured to the nearest 0.5 cm. The cannulae were removed, and the perfused segment cut from the intestine. A section posterior to the perfused segment was removed and placed in Bouin's fixative for light microscopy (see 2.2 above).

Intestinal uptake was normalized to nominal surface area and to the length of the segment so that changes due to increased mucosal mass could be distinguished from changes in carrier number or efficiency. The length and outer circumference of the perfused section were measured, then the segment was slit longitudinally, and the thickness of the wall (musculature and mucosa) measured. These measurements were made with a micrometer, and were to the nearest 0.01 mm. The inner circumference was calculated from the wall thickness and the outer circumference, and was multiplied by the segment length to give the surface area of the perfused segment. The mucosa was scraped free of the musculature with a glass slide and placed in a tared pan. It was weighed, then dried overnight to a constant weight in an oven (50°C) for dry weight.

The gonads were removed and weighed. Samples of muscle were taken for estimation of muscle water content, and samples of liver and muscle were frozen for estimation of lipid content. Muscle samples were removed from the dorsal surface just above the lateral line and contained only white muscle fibres. A small muscle sample was weighed, then dried to constant weight in an oven for water content. The difference between wet and dry weight was the water content. Lipid content of the muscle and liver was estimated from thawed samples by the method of Bligh and Dyer (1959), using a chloroform:methanol mixture to extract the lipid from the homogenized tissue.

2.5.4 Measurement of Unstirred Layer Thickness

Estimation of the thickness of the unstirred layer was attempted based on the methods of Read and his colleagues (1974) for *in situ* measurements. Surgery was performed on the flounder as for the uptake experiments. An inflow cannula was prepared which possessed an agar bridge (2% agar in 3 M KCl) containing a Ag/AgCl wire electrode. The bridge was joined at a "T" junction to the inflow cannula and thus was in contact with the perfusate. A second agar bridge with a Ag/AgCl electrode was used as a reference electrode. It was inserted under the skin on the dorsal side of the fish above the lateral line, and tacked into place with thread. The incision for the reference electrode and the open tip of the agar bridge (at the point where the silver wire extended) were sealed with Super Glue to prevent movement of the wire and to seal the electrodes from salt water.

Fish were brought to the recording laboratory the day after surgery and placed in the experimental tank. The electrode wires were connected to a Keithly model 602 electrometer, which was connected to a Grass polygraph recorder. The intestinal segment was perfused with a solution of 154 mM NaCl for 10 min, or until a stable baseline was achieved, then the solution was replaced with one containing 104 mM NaCl and 100 mM mannitol (to maintain osmolarity). The time at which the new solution entered the intestine was noted (time zero) and the time required to reach a new, steady potential difference was recorded. The thickness of the unstirred layer was then calculated according to Diamond (1966):

$$d^2 = t_{1/2} \cdot D/0.38 \qquad (1)$$

where d is thickness of the unstirred layer, $t_{1/2}$ is the half time required to reach the new potential difference, D is the diffusion coefficient of NaCl, and 0.38 is the constant of proportionality of the build-up and decay of solute concentration at the membrane.

The inflowing seawater in the experimental tank was 21°C (ambient temperature = 3°C). A low flow was maintained for fish health, and incoming water was cooled as much as possible by passing the inflow tube through ice, and holding the experimental tank on a bed of ice. Temperatures for these studies ranged from 6 to 12°C and were noted for each recording. The experimental tank was continuously aerated.

The unstirred layer thickness was estimated in midgut and hindgut only, and in non-feeding fish only. In spite of the importance of the unstirred layer to the *in vivo* uptake studies (Karasov and Diamond, 1983a), no further attempts at estimation of its thickness were made. The difficulty in maintaining an acceptable water temperature even in winter and the necessity of transporting the fish between laboratories after surgery resulted in significant stress. These problems were insoluble and were considered too perturbing to be acceptable.

2.6 *In vitro* Uptake Studies

2.6.1 Preparation of Tissues

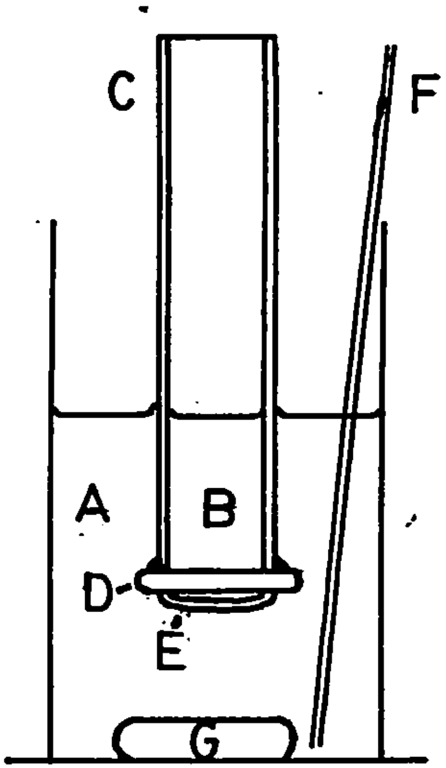
In vitro uptake studies were performed on mucosal sheets. Flounders were brought into the laboratory, and a blood sample was taken from the caudal vein for measurements of plasma phenylalanine levels. The fish was sacrificed by spinal transection, weighed to the nearest 0.1 g and measured for length to the nearest 0.5 cm. The intestine was rapidly removed, slit and rinsed with cold mannitol-Ringer's. It was divided into the four sections, then each was placed in a beaker of cold, oxygenated Ringer's containing 10 mM glucose and 10 mM mannitol. Sections were held in this solution until they could be mounted. Samples of muscle and liver were taken from the fish for water content and lipid content as described above (2.5.3).

The intestinal segments were divided into smaller sections, 1 to 2 cm in length. The sections were slit lengthwise for removal of the musculature. Sections were pinned, mucosal side down, to a smooth board covered with plastic wrap. The tissue was kept wet with mannitol-Ringer's, and the procedure was completed in the cold (8°C). The tissue was stretched with a glass slide, then the musculature was carefully cut with a scalpel blade. The musculature was then gently stripped free of the mucosa with a pair of fine forceps. The stripped mucosa was cut free and mounted, luminal side out, on a hollow glass tube (8 mm in diameter, 1 mm wall thickness). The tissue was held in place by a rubber O-ring (Fig. 5). Petroleum jelly was applied to the face of the O-ring away from the tissue, to prevent leakage. The serosal side (inside the tube) was filled with approximately 200 ul of mannitol-Ringer's, and the tube was placed in an oxygenated solution of mannitol-Ringer's until the uptake experiment began, between 30 and 90 min later.

2.6.2 Uptake Experiments

Uptake of phenylalanine was estimated from 5 ml of solution containing 0.05, 0.1, 0.2, 0.5, 1.0 or 5.0 mM phenylalanine. Osmolarity was maintained by replacement with mannitol, to a concentration of 20 mM solute. Approximately 0.15 uCi of ^{14}C-phenylalanine was added

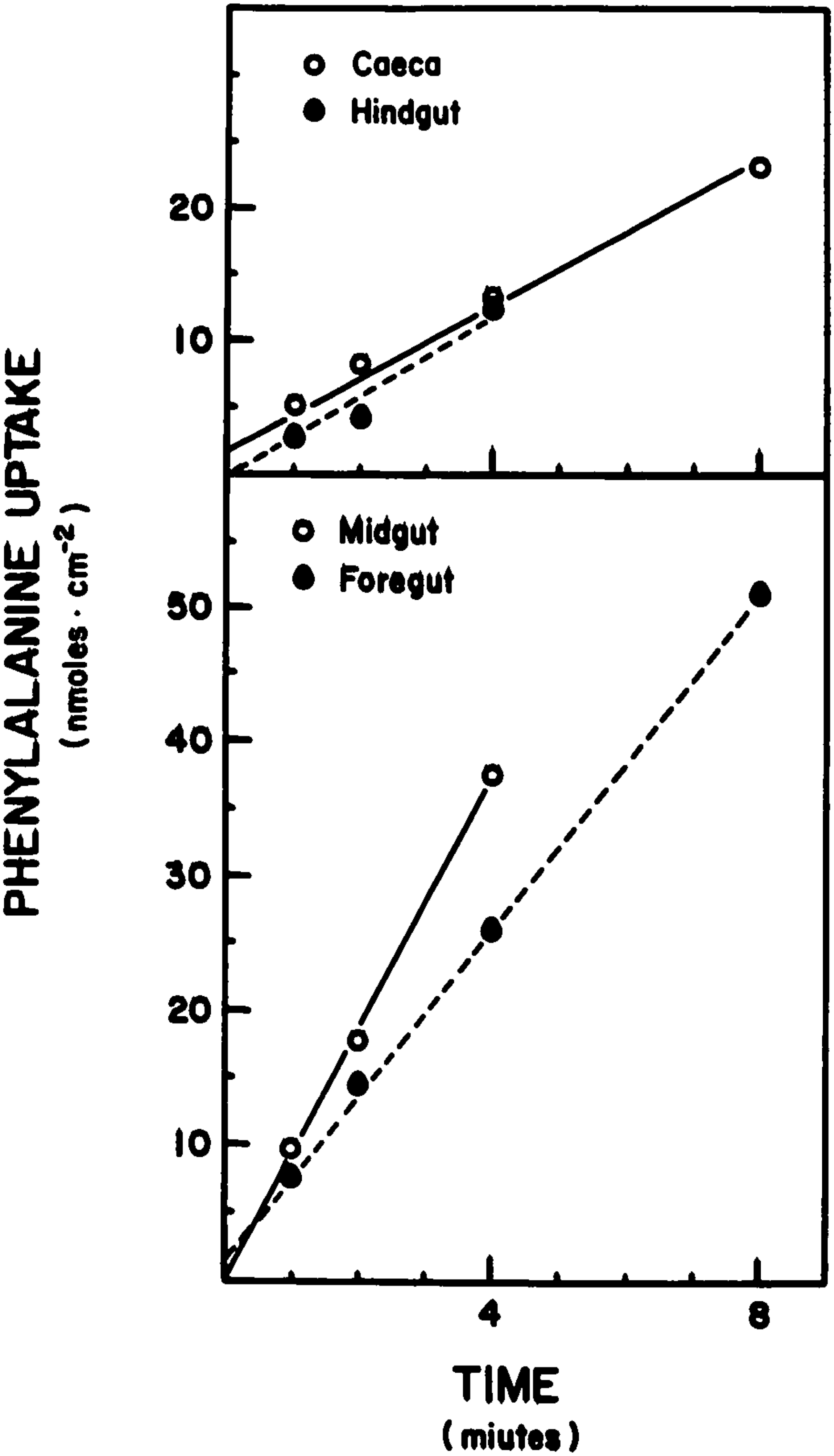
Figure 5. Schematic diagram of the chamber used for the *in vitro* method. A: Mucosal phenylalanine solution. B: Serosal phenylalanine solution. C: Hollow glass tube on which tissue is mounted. D: Rubber O-ring to hold tissue in place, with upper surface coated with petroleum jelly to prevent leaks. E: Tissue. F: Oxygen inlet. G: Magnetic stir bar.


C
F
A
B
D
E
G

to give specific activities of approximately 1.2 x 10^9 dpm/mmol (0.05 mM) to 1.2 x 10^7 dpm/mmol (5 mM). ^{3}H-mannitol was used to correct for adherent water. Negligible amounts of mannitol are taken up by the intestine of the winter flounder (McKibbin and Kleinzeller, 1979). Mannitol is preferred to polyethylene glycol (PEG) because its molecular weight (182 Daltons) is more similar to that of phenylalanine (165 Daltons) than is that of PEG (900 or 4000 Daltons), and it will therefore diffuse at a similar rate. Concentrations of mannitol in the adherent water will more accurately reflect that of phenylalanine in the short incubation times used in this study.

Uptake of L-phenylalanine was found to be linear for at least 4 min (Fig. 6), so an incubation time of 2 min was chosen. Mounted tissues were suspended in 5 mL of a phenylalanine test solution to a depth equal to the serosal bath level to prevent effects of hydrostatic pressure. The solution was stirred with a magnetic stir bar and vigourously bubbled with 99% O_2/1% CO_2 for the duration of the incubation to minimize the effect of the unstirred layer (Wilson and Dietschy, 1974). The tissue was then removed and rinsed for 20 sec in mannitol-Ringer's (Karasov and Diamond, 1983b) to remove adherent water but not absorbed solute. The diameter of the tissue was then measured with a micrometer to the nearest 0.01 mm for area calculations, the tissue was cut free of the tube, and blotted dry for 10 sec with a piece of Whatman No. 1 filter paper. It was then placed in a tared scintillation vial and

Figure 6. Linearity with time of L-phenylalanine uptake measured *in vitro* in caeca and hindgut (upper panel), and foregut and midgut (lower panel). Phenylalanine concentration was 5 mM (Mean ± SE; n = 5).



○ Caeca
● Hindgut
○ Midgut
● Foregut
PHENYLALANINE UPTAKE
(nmoles · cm-2)
20
10
50
40
30
20
10
4
8
TIME
(miutes)

weighed. It was dried to a constant weight at 50°C for dry weight, wetted with one drop of distilled water, then solubilized overnight at 50°C with 0.5 ml of NCS tissue solubilizer (Amersham Corp.). Ten milliliters of Scintillene (Fisher Scientific) was added to the digested tissue, plus sufficient acetic acid to neutralize the medium (15 to 20 ul). Samples were subjected to liquid scintillation counting for ^3H and ^{14}C (dual label programme, LKB Rackbeta). Specific activity of the reservoir was determined by withdrawing 25 ul from the 5 ml solution prior to incubation of tissues. In addition, 25 ul of serosal solution was taken to detect leaks in the mounted tissue. The 25 ul was added to 8 ml of Scintiverse and counted as above. No radioactivity was detected in the serosal solutions of properly mounted tissues after incubation. Tissues which exhibited high serosal counts were not included in the calculations.

2.6.3 Experimental Conditions

In vitro experiments were performed on each of the four intestinal sections in summer (July to September) and winter (January to February). The effect of temperature on phenylalanine uptake was assessed at both seasons, because temperature could not be controlled during the *in vivo* experiments (section 2.6.2) without stressing the fish. Experimental temperatures chosen were 15°C (summer ambient), 8°C, and 3°C (winter ambient). The requirement for sodium was assessed in both seasons, replacing sodium with choline in the Ringer's solution (150 mM choline chloride, 20 mM choline HCO_3, 1.5 mM KCl, 0.3 mM KH_2PO_4, 1.65 mM K_2HPO_4, 1mM $MgSO_4$ and 1 mM $CaCl_2$, pH adjusted to 7.3 after aeration). Experiments were performed as described for the sodium-containing medium. However, Robinson (1970) has indicated that some tissue effects may occur due to choline, so exposure to choline-containing solutions was kept to a minimum. The serosal side was rinsed 3-times with choline-mannitol-Ringer's before the final addition of the 200 ul serosal bath solution. The mucosal side was rinsed with 3 washes of choline-mannitol-Ringers, then immersed for 1 min in choline-mannitol-Ringer's to remove as much sodium as possible. The tissue was then incubated for 2 min in a choline-Ringer's containing the desired phenylalanine concentration and post rinsed in choline-mannitol-Ringer's. Tissues were then treated as already described. Sodium-free uptake rates were measured at 15°C in summer and winter.

In addition to the effects of season, temperature and sodium, the influence of the presence of potentially competing amino acids in the incubating solution was examined. These experiments were performed in November and December, just at the start of the non-feeding period. The incubation temperature was 15°C. The effect of 10 mM solutions of leucine, alanine, histidine, methionine, taurine and arginine on the uptake of 5 mM phenylalanine was measured, as well as the effect of a taurine/leucine mixture at 5 mM each, and of a methionine/leucine mixture at 5 mM each. Uptake of 5 mM phenylalanine plus 15 mM mannitol was measured during the same period as a control.

2.7 Serum Phenylalanine Concentrations

Blood was taken in non-heparinized needles from fish used for the *in vitro* experiment (section 2.6). It was allowed to stand to coagulate in the refrigerator, then the serum was removed and frozen.

Serum samples from each month were thawed just prior to the analysis. Phenylalanine was estimated by the use of phenylalanine ammonia lyase (Pharmacia), according to the method of McKnight et al. (1983). This method measures the rate of production of trans-*p*-coumarate and trans-cinnamate from tyrosine and phenylalanine, respectively. By measuring the production of these products at two wavelengths, 290 nm, where both absorb, and 315 nm, where only the tyrosine product absorbs, the contribution to the change in absorbance due to the production of the phenylalanine product can be calculated, and hence the concentration of phenylalanine by comparison to values obtained from known amounts of phenylalanine.

2.8 Calculations and Statistics

Most statistical comparisons were by t-tests (Steel and Torrie, 1980) and a probability level of 5% was chosen as indicating significance. Statistical values in the tables were computed from values presented in the figures.

For the *in vivo* uptake studies, disintegrations per min were transformed into concentration of phenylalanine using specific activity estimates after correction for volume changes measured with phenol red. The difference between initial and final samples was taken as the amount absorbed.

For the *in vitro* uptake studies, disintegrations per min were transformed into concentration of phenylalanine in the tissue after correction for background levels and adherent water.

2.8.1 Uptake Calculations

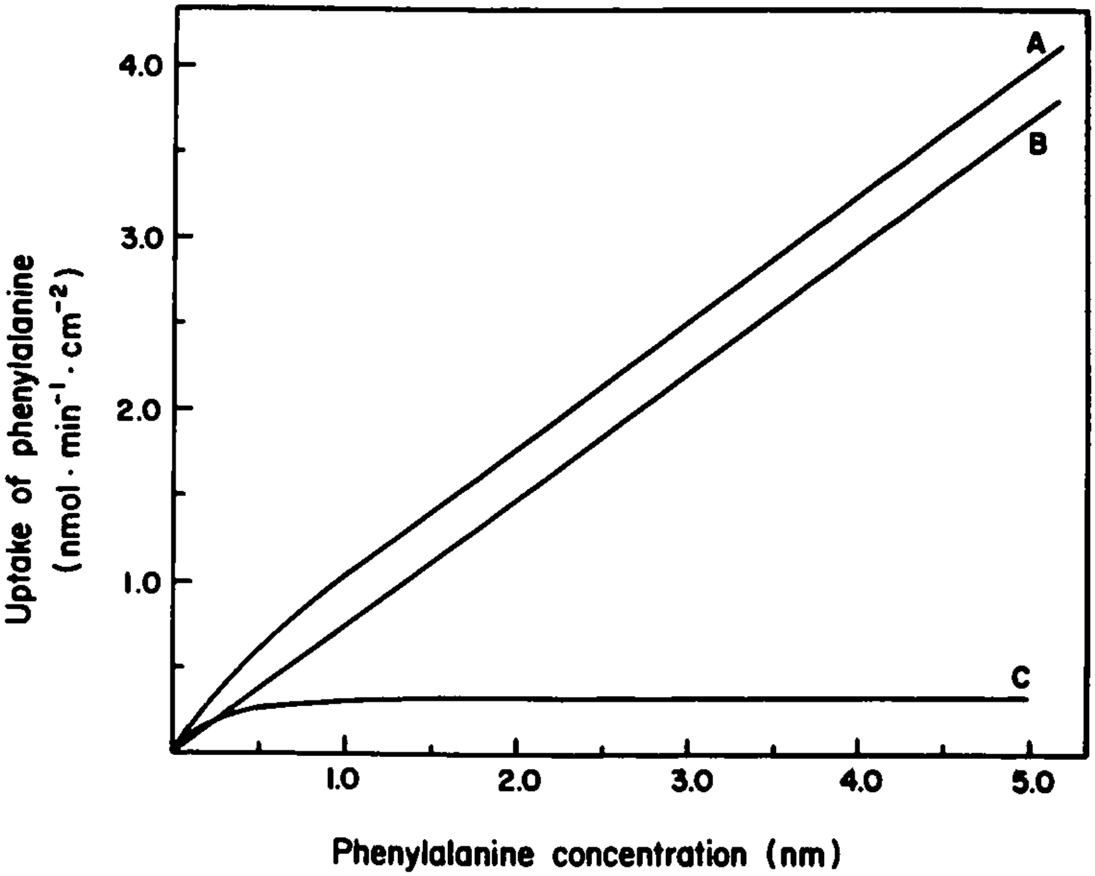
Intestinal uptake has been shown to follow Michaelis-Menten kinetics with an extra term to account for passive uptake (Atkins and Gardner, 1977). The equation which best describes the intestinal uptake of phenylalanine is

$$J = \frac{J_{max} \times [Phe]}{K_t + [Phe]} + K_d \times [Phe] \qquad (2)$$

where J is the rate of uptake, J_{max} is the maximal rate of uptake, K_t is the affinity constant of the carrier, K_d is the diffusion constant, and [Phe] is the concentration of phenylalanine.

The diffusional component can be assessed as a rising straight line at high substrate concentrations (Fig. 7).

Figure 7. A: A typical curve obtained from estimation of uptake rates at different concentrations of L-phenylalanine. This curve was taken from *in vitro* results (Chapter 5) (Winter, midgut, 8°C), but *in vivo* curves appear similar. B: Linear component of the curve (apparent diffusion). C: Saturable component of the curve. A is the sum of B and C.



Uptake of phenylalanine (nmol · min⁻¹ · cm⁻²)
4.0
3.0
2.0
1.0
1.0
2.0
3.0
4.0
5.0
Phenylalanine concentration (nm)
A
B
C

Multiplication of the slope of this line (determined by least squares regression) by the concentration of phenylalanine and the subsequent subtraction of this value from the data leaves the saturable or Michaelis-Menten portion of the uptake.

The saturable component of uptake was characterized by an Eadie-Hofstee transformation. This method is less affected by interference from the unstirred layer than the more commonly used Lineweaver-Burk plot (Winne, 1973; Thomson and Dietschy, 1980a). In addition, the bias inherent in the plot (underestimation of both J_{max} and K_t) can be corrected (Zivin and Wand, 1982). The equation of the resultant line is

$$J = J_{max} - K_t\ (J/[Phe]) \qquad (3)$$

where symbols are as described for equation 2.

Differences in slopes of the calculated lines for the apparent diffusional constant, K_d, were assessed by comparisons of slopes which result in a t value (Steel and Torrie, 1980). Differences in apparent K_t and J_{max} were assessed by a computer program (Department of Fisheries and Oceans, Biological Station, St. Andrews, New Brunswick) which compared slopes and adjusted intercepts from Eadie-Hofstee transformed data to result in values of t.

The apparent K_t measured *in vivo* in January in the midgut and the hindgut was corrected for the effects of the unstirred layer. Corrections were calculated using the equation of Barry and Diamond (1984):

$$K_t = K_t(\mathrm{app}) - \frac{0.5\ J_{max} \cdot d}{D \cdot S_w} \qquad (4)$$

where $K_t(\mathrm{app})$ is the measured K_t, K_t is the corrected K_t, d is the effective thickness of the unstirred layer, D is the diffusion coefficient of the solute and S_w is the surface area of the unstirred layer.

The surface area of the unstirred layer was not measured, but was assumed to be the same as the surface area of the cylinder of the intestine. Since that term is already part of the J_{max} value (expressed against surface area), the S_w term drops out. The diffusion coefficient (D) of phenylalanine at 3°C (the temperature at which the January uptake measurements were performed) was calculated to be 3.3×10^{-6} $cm^2 \cdot s^{-1}$ from values given by Longsworth (1953) for 1°C and 25°C and by Winne (1978) for 38°C.

2.8.2 Length-Weight Relationships

The relationship of length to weight follows the equation

$$W = cL^b \qquad (5)$$

where W is weight, L is length, and c and b are constants.

The length-weight relationship was calculated by least squares regression on the log transformation of the above equation

$$\log W = b \log L + \log C \qquad \text{(Lux, 1969)}$$

Weights were gonad-free weights. Fish were not separated by sex, because male and female flounders show no sexual dimorphism in this relationship (Kennedy and Steele, 1971). Because lengths were estimated to the nearest 0.5 cm, weights for a given length were averaged and the average used to calculate the relationship. Condition factors (100 x weight (in grams)/length^3 (in centimetres)) were calculated from unpooled lengths.

Chapter 3

Seasonal Changes in Fish Condition and Intestinal Structure

3.1 Introduction

Winter flounder undergo a natural fast during the winter (Kennedy and Steele, 1971; Fletcher et al., 1981). Seasonal changes in amino acid uptake may reflect changes in the fish as a whole as well as in the intestine. In this part of the study, I examined certain body parameters of the fish and the degree of their depletion in the winter when feeding was suspended. Length-weight relationships and condition factors were examined throughout the year to establish changes in body shape, as it is a sensitive index of both depletion and reproductive demands (Love, 1970; 1980). Lipid content of muscle and liver was estimated to further assess metabolic reserves. According to Love (1970; 1980), lipid reserves are depleted prior to a loss of muscle protein. He states that non-fatty fish such as the winter flounder use the liver as a lipid reservoir, and thus it is expected that the liver will show the greatest depletion with the cessation of feeding. Muscle water content reflects the utilization of muscle protein during fasting. Condition factor, liver lipid and muscle water are considered by Love and others as adequate indicators of starvation in fish species.

Regional functional differences along the length of the intestine have been documented in rainbow trout (Stokes and Fromm, 1964; Boge et al., 1979; Dabrowski and Dabrowska, 1981; Marcotte and de la Noüe, 1982), goldfish (Mepham and Smith, 1966a), catfish (*Ameirus nebulosus*) and scup (*Stenotomus* *versicolor*) (Musacchia et al., 1961), *Ophicephalus punctatus* and *Heteropneustes fossilis* (Sastry et al., 1977), grasscarp (*Ctenopharyngodon idella*) (Stroband and van der Veen, 1981), carp (*Cyprinus carpio*) (Dabrowski, 1983), surgeonfish (*Acanthurus mata*) and eel (*Gymnothorax undulatus*) (Ferraris and Ahearn, 1983) and coho salmon (Collie, 1985). However, Smith (1969) found no such functional differences in *Haemulon plumieri*, nor did Ingham and Arme (1977) and Hokazano et al. (1979) in the intestines of rainbow trout. Field and his coworkers (1978) found no difference in the transmural potential difference across flounder intestine along its length, and thus no regional differences in ion uptake; however, they did not examine the first and last 4 cm of the intestine. Regional functional and structural assessment of the intestine was considered necessary in view of the conflicting findings in other species and the incompleteness of the assessment in winter flounder. In addition, intestinal morphology was examined to assess any seasonal changes in the mucosal folds and in the enterocytes or absorbing cells.

The intestine of mammals is known to atrophy disproportionately to the rest of the body with starvation (Williamson, 1978). The decline in mucosal mass results largely from the loss of direct stimulation by food (Lipkin, 1981; Karasov and Diamond, 1983a). The length of the intestine decreases during starvation in the rat (Kershaw et al., 1960) and carp (Gas and Noaillac-Depeyre, 1976). In rainbow trout, Bogé and his coworkers (1981b) reported a 69% decrease in intestinal wet weight after eight weeks of starvation, with most of this decline occurring in the mucosa. Gas and Noaillac-Depeyre (1974) observed that epithelial renewal declined in the winter even in feeding carp. Thus, one might predict that the intestine of the winter flounder will undergo considerable atrophy in the winter after the cessation of feeding.

Kitchin and Morris (1971), however, observed intestinal hypertrophy in fed goldfish when acclimated to low temperatures, and this was confirmed by Groot and his colleagues (1983). The low environmental temperatures encountered by the winter flounder throughout the winter may ameliorate the effects of starvation on the intestine. The intestine of marine fish also serves an important osmoregulatory function (Skadhauge and Lotan, 1974; Kirsch and Meister, 1982), and the continuing demands of osmoregulation might further ameliorate the effects of starvation. Indeed, Macleod (1978) found that rainbow trout adapted to full strength seawater showed no decline in intestinal cross-sectional area and a less marked decrease in fold height when starved than fish adapted to half- and quarter-strength seawater. Therefore, it is predicted that although the mucosa may atrophy at the cessation of feeding, the decline will not be as marked as in freshwater fish undergoing a similar period of starvation.

While the amount of mucosa may decline, the changing nutritional status of the fish may affect the functional characteristics of the intestine. The next two parts of this study (Chapters 4 and 5) deal with various aspects of the uptake of one amino acid, phenylalanine. The activities of two enzymes involved in intestinal uptake were also estimated to assess functional characteristics other than those associated with amino acid uptake. Alkaline phosphatase (EC 3.1.3.1), an enzyme implicated in lipid (Linscheer et al., 1971) and ion (Oku and Wasserman, 1978) uptake, was measured to assess the capacity of the membrane to handle uptake of non-protein related nutrients. Leucine aminopeptidase (aminooligopeptidase, EC 3.4.11.2), implicated in the transport of peptides (Adibi and Kim, 1981), was also measured to assess general changes in protein handling capabilities.

3.2 Results

Winter flounder began feeding in late April and ceased feeding in mid-October of 1984, the first year of this study. Feeding resumed in mid-May, and ceased again in mid-November of 1985.

3.2.1 Length-Weight Relationships

The value of the slope of the log transformed length-weight equation is lower in non-feeding than in feeding fish (Table 2). This difference is not significant between feeding fish and the 85/86 group of non-feeding fish, but is significant for the 84/85 data. The slopes for the two non-feeding groups of fish were not significantly different.

The condition factor, calculated with or without gonad weight, of fed fish was significantly higher than that of fish from either non-feeding period (Table 3). Non-feeding fish had condition factors that were not significantly different.

Table 2. Length-weight relationships of flounders sampled in two non-feeding periods and one feeding period. P is the number of points on each line, where each point is composed of from 1 to 8 individual fish at each length. Weights were averages at the particular length because lengths were measured only to the nearest 0.5 cm. (Weight in g; total length in cm; mean ± SE)

Sampling Period	State	Length-Weight Equation	P
Nov '84-May '85	non-feeding	$\log W = 2.54 \log L - 1.23$	14
May '85-Oct '85	feeding	$\log W = 3.17 \log L - 2.14$	16
Nov '85-Feb '86	non-feeding	$\log W = 3.03 \log L - 1.99$	16

Table 3. Condition factors (100 x W/L^3) of feeding and non-feeding fish. Condition factors were calculated using weights including gonads (gonad inclusive) and excluding gonads (gonad free).

Sampling Period	State	Condition Factor ($100g/cm^3$) Gonad Inclusive	Condition Factor ($100g/cm^3$) Gonad Free	n
Nov '84-May '85	non-feeding	1.26	1.18	54
May '85-Oct '85	feeding	1.40	1.36	48
Nov '85-Feb '86	non-feeding	1.31	1.22	44

3.2.2 Lipid Content of Muscle and Liver

Although only a single value from each month was measured for lipid content, there was a trend toward higher lipid content in both muscle and liver in late summer and early fall (Fig. 8). The trend was less apparent in the muscle. The average lipid content of liver for the period from August to December was significantly higher (approximately four times) than for the period from January to July. Lipid content in muscle averaged over the same two periods was not significantly different. The length, weight, sex, gonad weight and gonadosomatic index of the fish used for lipid assessment are presented in Table 4. Fish sampled in November to March showed high gonadosomatic indices. All of these fish would have reproduced in the spring spawning period. The remainder of the fish had low gonadosomatic indices, but the fish from April to June were non-ripe during the spawning period. Thus, the fish used for the three months which show higher muscle lipid content in spring would not have spawned that year.

Observation of the visceral cavities in all the fish used in the course of this study gave no evidence for the presence of discrete fat bodies such as are found in the Salmonids (Weinreb and Bilstad, 1955).

3.2.3 Water Content of Muscle

The flesh of the winter flounder changed in appearance seasonally, becoming greyer and more transparent in the winter compared to the summer. Water content in the white muscle

Figure 8. Lipid content of liver (upper panel) and white muscle (lower panel) of winter flounder by month (one individual per month, same fish for liver and muscle estimations) and as combined averages for August to December and January to July.

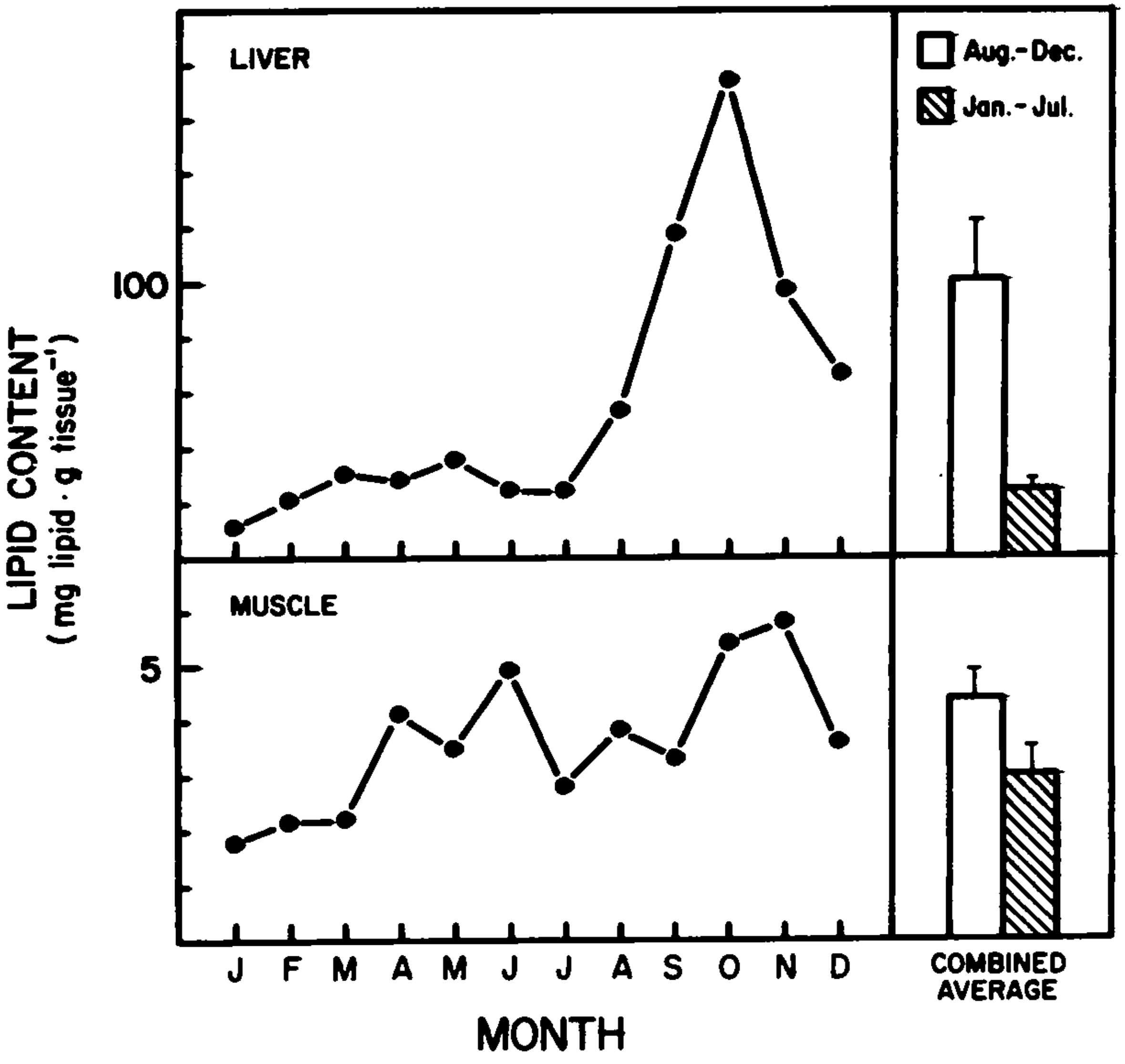

LIPID CONTENT
(mg lipid · g tissue^{-1})
100
5
LIVER
MUSCLE
□ Aug.-Dec.
▨ Jan.-Jul.
J F M A M J J A S O N D
MONTH
COMBINED AVERAGE

Table 4. Characteristics of the flounders used for the analysis of lipid content: weight, standard length (measured from the tip of the jaw to the caudal peduncle), gonad weight, sex and gonadosomatic index. (nr = non-reproductive)

Month	Standard Length (cm)	Weight (g)	Gonad Weight (g)	Sex	Gonado-somatic Index
Jan	29	563.9	73.84	F	0.131
Feb	23	275.5	21.87	M	0.079
Mar	25	407.2	75.96	F	0.187
Apr	23	285.6	nr	M	-
May	26	320.9	2.51	F	0.008
June	28	479.2	4.76	F	0.010
July	26	492.7	6.69	F	0.014
Aug	28	480.9	4.86	F	0.010
Sept	27.5	641.3	9.27	F	0.015
Oct	27	521.4	0.79	M	0.002
Nov	25	529.0	47.0	F	0.089
Dec	28	594.0	41.15	F	0.069

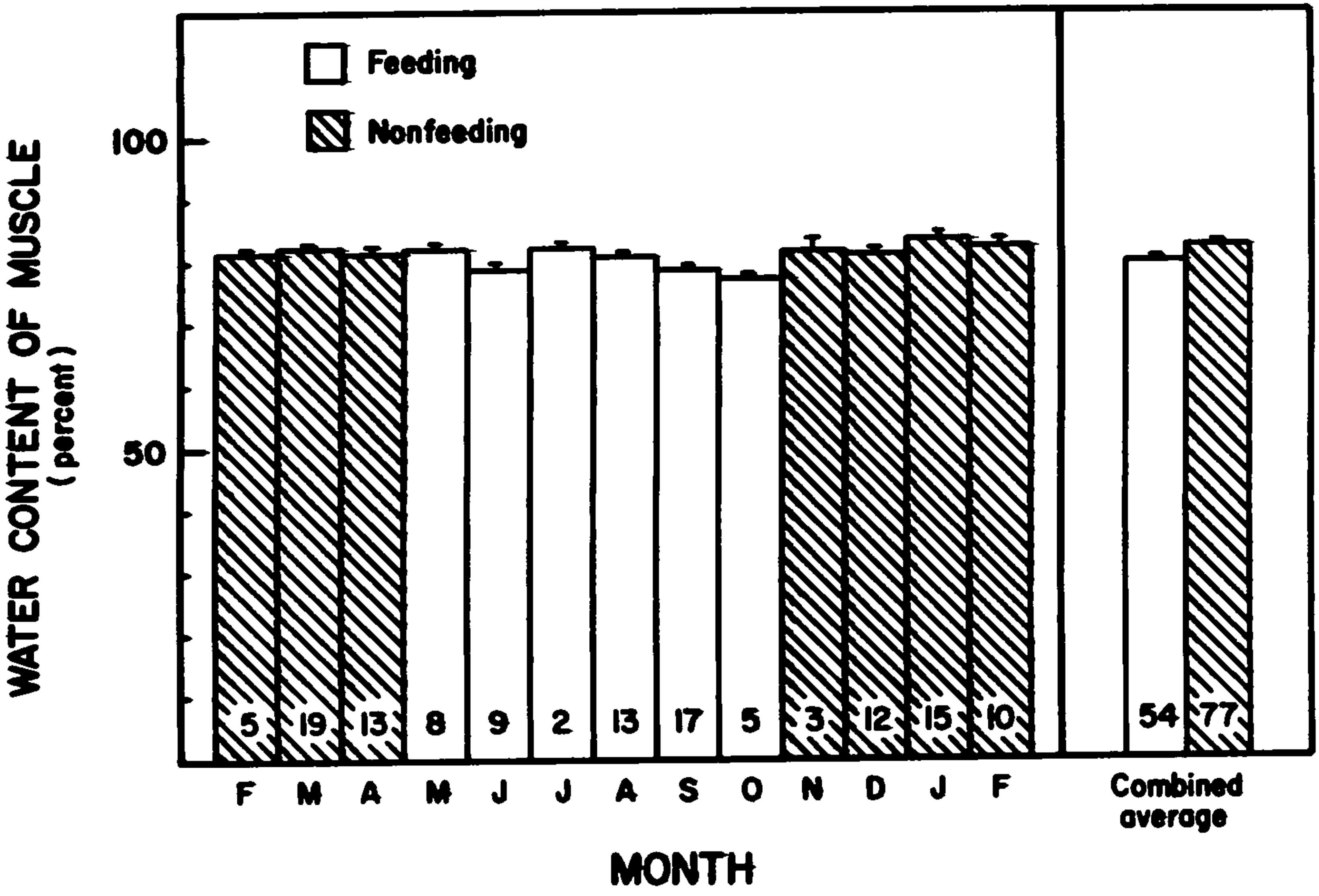
varied monthly from a high of 83.7% in January 1986 to a low of 77.2% in October 1985 (Fig.9), and was generally higher in the non-feeding months. Combined into feeding and non-feeding groups, the percent white muscle water was significantly higher (2.4%) in non-feeding compared to feeding flounders.

3.2.4 Intestinal Morphology

The ratio of intestinal length to standard body length was 1.03 (± 0.19 SE, n = 29) in feeding fish and 0.98 (± 0.18, n = 61) in non-feeding fish. These were not significantly different, and so were pooled, to give a ratio of 0.99 (± 0.18). There were normally four pyloric caeca present at the anterior end of the intestine, although rarely (less than 1%), there were three or five. Three caeca extended from the intestine immediately posterior to the pyloric sphincter (Fig. 2). One extended back along the posterior margin of the stomach, while the other two extended between the turns of the intestine, side by side. The fourth caecum entered the intestine a short distance posterior to the first three, approximately 1 cm back in a 30 cm fish. It also extended between the turns of the intestine. Caeca were approximately the same length in any one fish, averaging 24.51 ± 1.22 mm in the feeding flounder. However, in non-feeding flounder, caeca were significantly shorter, averaging 19.73 ± 1.00 mm.

Externally, the foregut appeared thin walled but with a large diameter. The intestinal diameter decreased posteriorly, and the intestine was thicker walled in the midgut. The midgut was separated from the hindgut by a valve

Figure 9. Water content of the white muscle of winter flounder; average per month, and combined average of feeding and nonfeeding values. Number inside the bar indicates the number of individuals assessed.


Feeding
Nonfeeding
WATER CONTENT OF MUSCLE
(percent)
100
50
5
19
13
8
9
2
13
17
5
3
12
15
10
54
77
F
M
A
M
J
J
A
S
O
N
D
J
F
Combined average
MONTH

which was apparent externally as a pale line. The hindgut, when empty, appeared rugose and thick walled, but was capable of much extension.

All observations concerning the mucosal structure of the winter flounder intestine are qualitative rather than quantitative, both for light and electron microscopic surveys. The only exception is the estimation of intestinal perimeter (section 3.2.6).

3.2.4.1 Light Microscopic Survey

The flounder intestine could be easily subdivided into three distinct sections based on external morphology; the caeca, the foregut and the hindgut. Light microscopic examination, however, revealed a gradual change in the morphology of the foregut, with a thickening of the musculature and a decline in the number of folds, degree of folding and shape of folds. Thus the foregut was subdivided into a foregut and midgut, the foregut being approximately 40% and the midgut approximately 50% of the total intestinal length. The hindgut represented about 10% (Fig. 2). The change in morphology occurred gradually, and therefore subdivisions between foregut and midgut were arbitrary.

The flounder intestine consisted of an outer serosa, a muscularis, a submucosa and a mucosa (Fig. 10). The muscularis was composed of an outer longitudinal and inner circular muscle layer. The muscularis was thin in the caeca and foregut, but thickened posteriorly toward the hindgut. The circular muscle layer was usually thicker than the longitudinal muscle layer. Except in the caeca, there

Figure 10. Light microscopic section (6 um) of caecum. a) summer. b) winter. M = mucosa, SM = submucosa, C = circular muscle layer, L = longitudinal muscle layer. (Hematoxylin and eosin (H+E); 75x)

appeared to be a reduction in the thickness of the circular muscle layer compared to the longitudinal layer with the onset of fasting. The longitudinal layer often took on a more folded appearance in the non-feeding fish, the fore- and midgut becoming more similar to the hindgut.

The submucosa, or the layer between the muscularis and the mucosa, was composed of loose connective tissue and blood vessels. There was no muscularis mucosae separating the submucosa from the mucosa, nor was there a stratum compactum. It is not obviously differentiated into regions. The submucosa appeared less dense in the non-feeding fish.

The mucosa of feeding fish possessed long, largely unbranched folds in both the caeca (Fig. 10a) and the foregut (Fig. 11a), nearly filling the lumen. The degree of folding declined posteriorly toward the midgut, where the folds were more branched and leaf-shaped (Fig. 12a). In the hindgut (Fig. 13a), folds were long and branched. Enterocytes appeared to be similar in all sections of the intestine. They did not appear to differ with position on the fold. Goblet cells were numerous and large throughout the intestine, with possibly a greater number in the hindgut than elsewhere.

During the non-feeding period, all sections of the intestine exhibited a reduction in the height and number of the folds and a change in their shape (Figs. 10b - 13b). The enterocytes themselves were shorter, although the brush border was still clearly visible. The number of goblet cells was much lower in every section in non-feeding fish.

3.2.4.2 Electron Microscopy

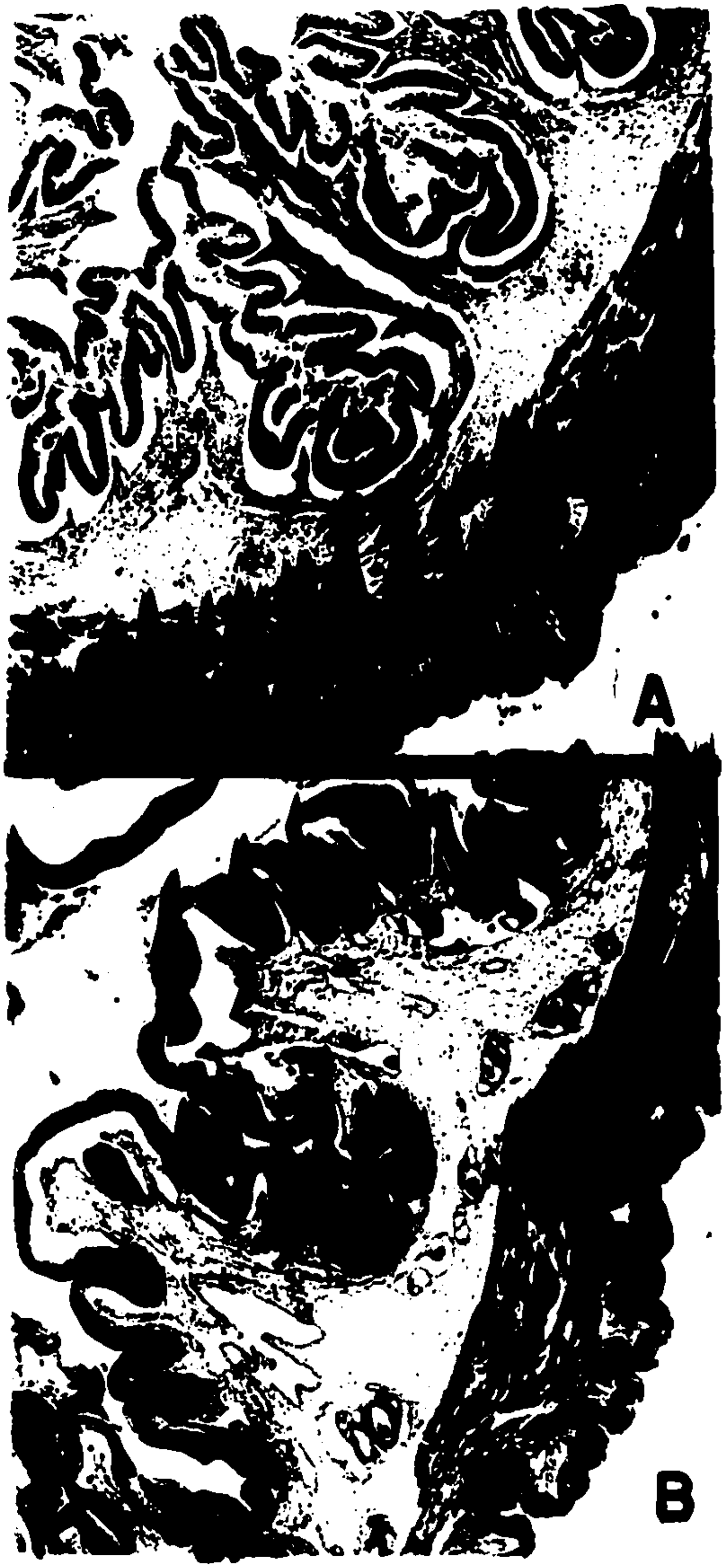
Figure 11. Light microscopic section (6 um) of foregut. a) summer. b) winter. (H+E; 75x)


A
B

Figure 12. Light microscopic section (6 um) of midgut. a) summer. b) winter. (H+E; 75x)


B

Figure 13. Light microscopic section (6 um) of hindgut. a) summer. b) winter. (H+E; 75x)


A
B

Electron microscopic examination of the intestine showed that the enterocytes appeared similar along the length of the intestine and in the feeding and non-feeding periods. Microvilli appeared to be equally numerous in the cells of the caeca, foregut and midgut, but were much more numerous in the hindgut (Fig. 14). No change in the number or length of microvilli was observed in non-feeding fish compared to feeding fish in any section.

3.2.5 Mucosal Weight

Mucosal wet and dry weights were both significantly heavier in feeding fish (May to Oct.) than in non-feeding fish (Nov. to Apr.) in the four sections (Fig. 15). The feeding period could be subdivided into early (May/June) and late (Sept./Oct.) periods because mucosal weights were obtained from fish used for the *in vivo* uptake studies.

All of the intestinal sections exhibited the same mucosal wet weights per centimetre of intestine in the non-feeding period and in the early feeding period. The foregut had a significantly higher wet weight (Table 5, Fig. 15) than the caeca in the late feeding period, while none of the other comparisons showed significant differences. The caecal mucosal dry weights were significantly higher than those of the other sections in the non-feeding period. Mucosal dry weights did not differ between sections in the early feeding period, while the midgut weighed significantly less than the foregut in the later feeding period. The other sections did not differ at that time.

Mucosal wet weights from the early feeding period were

Figure 14. Electron micrograph of the brush borders of enterocytes. A: Hindgut (summer) B: Foregut (summer) C: Foregut (winter) D: Midgut (summer) E: Midgut (winter). (10000x)

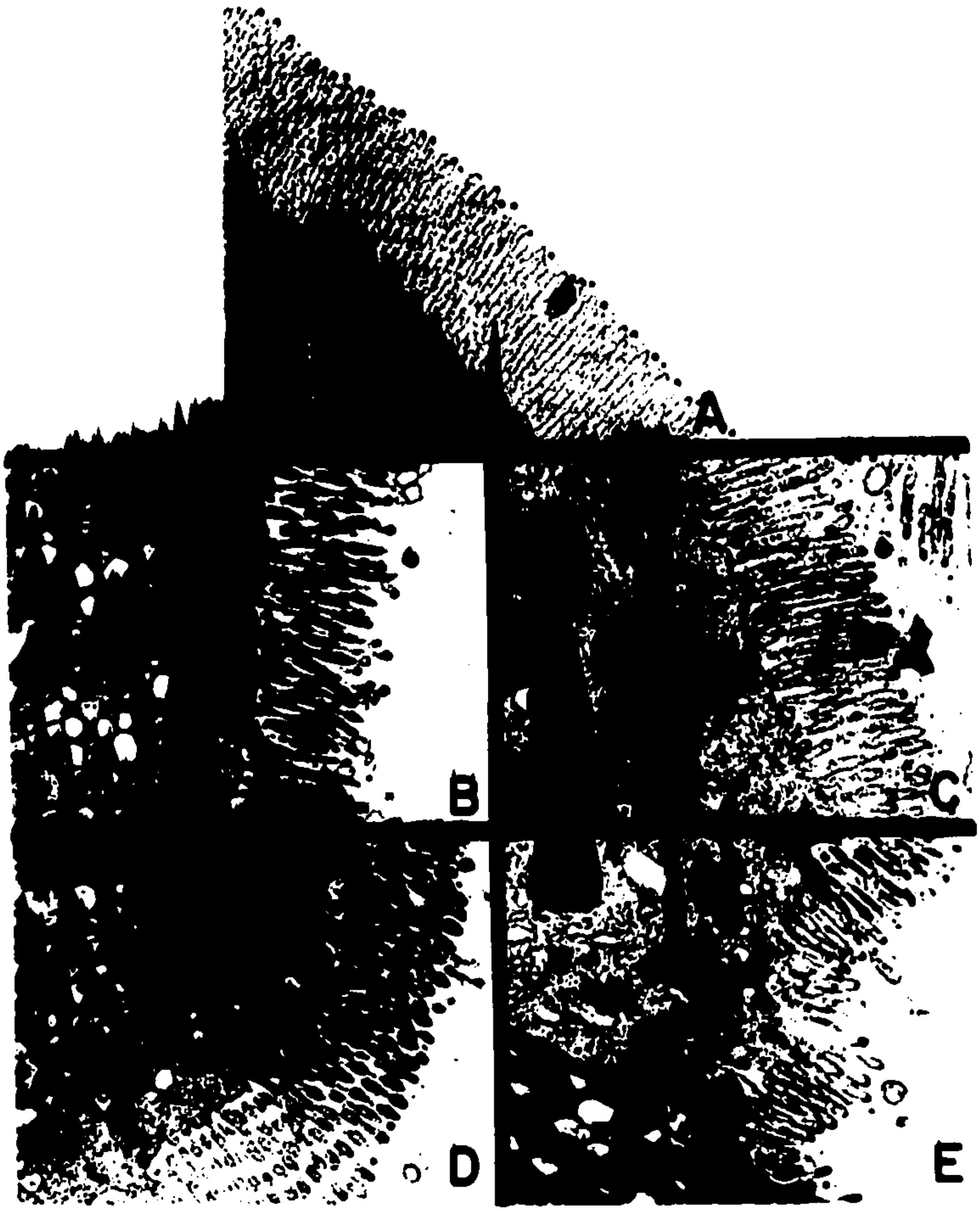
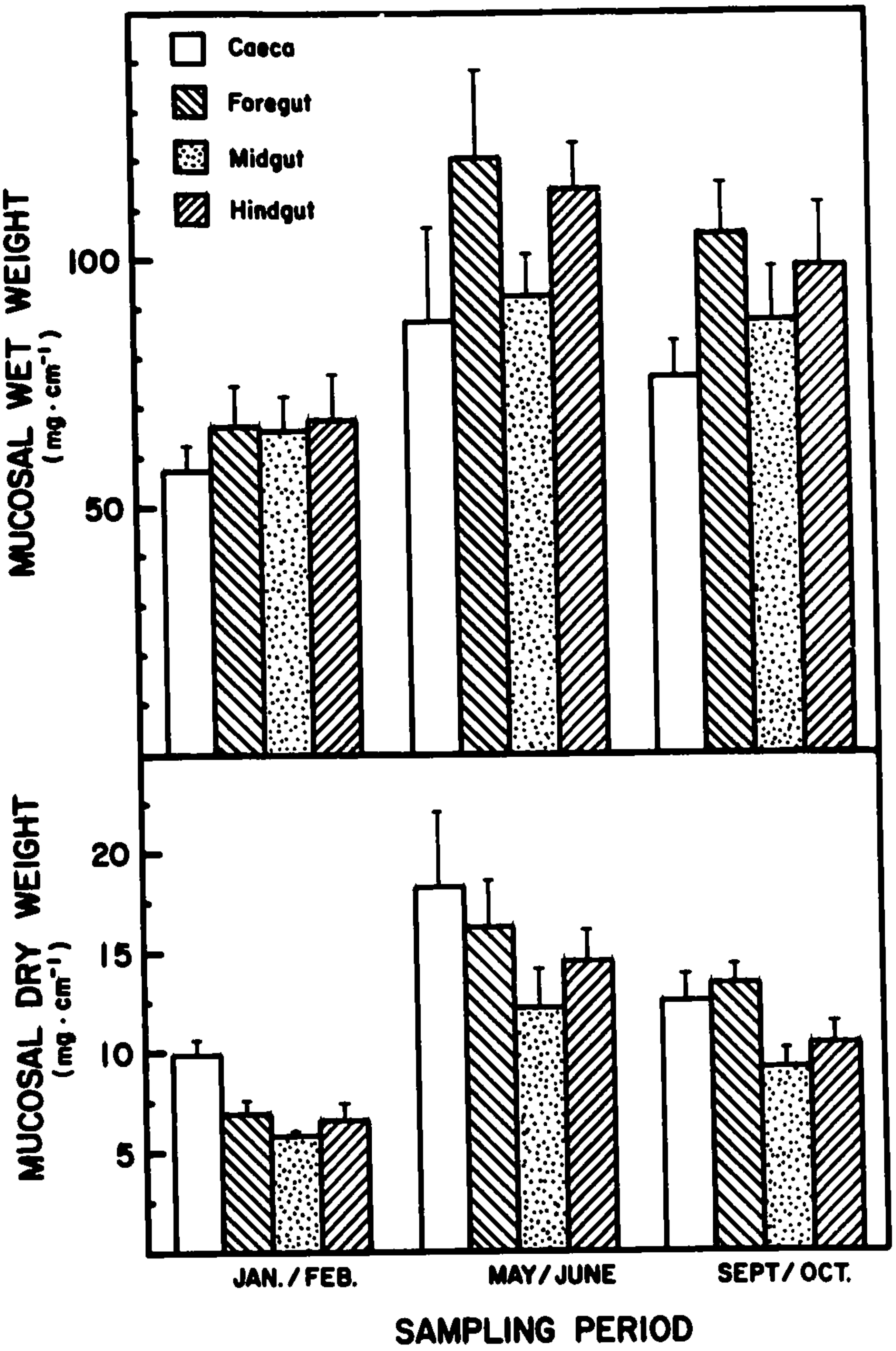

A
B
C
D
E

Figure 15. Seasonal changes in mucosal wet weights (upper panel) and dry weights (lower panel) per cm length of intestine for each section of the intestine (n varied from 5 to 16).



Caeca
Foregut
Midgut
Hindgut
MUCOSAL WET WEIGHT
(mg · cm⁻¹)
100
50
MUCOSAL DRY WEIGHT
(mg · cm⁻¹)
20
15
10
5
JAN. / FEB.
MAY / JUNE
SEPT / OCT.
SAMPLING PERIOD

Table 5. Values of t (degrees of freedom (df) = not less than 14) for t-tests performed to compare mucosal wet and dry weights of fish from non-feeding (Jan), early feeding (May) and late feeding (Sept) periods. Asterisks indicate significance at the 5% level. Data from Figure 15.

		Caeca		Foregut	
		Jan	May	Jan	May
WET	May	2.34*	-	3.18*	-
WEIGHT	Sept	2.35*	0.73	3.05*	0.76
DRY	May	3.62*	-	3.86*	-
WEIGHT	Sept	1.90	1.87	5.97*	0.92

		Midgut		Hindgut	
		Jan	May	Jan	May
WET	May	2.65*	-	8.60*	-
WEIGHT	Sept	2.16*	0.37	2.05	1.03
DRY	May	4.11*	-	6.07*	-
WEIGHT	Sept	3.59*	1.43	3.77*	2.39*

always significantly higher than those from the non-feeding period (Table 5, Fig. 15), while those for the late feeding period were higher in the caeca, foregut and midgut, but not different in the hindgut. In no section did the early and late feeding periods differ from each other in mucosal wet weight, although the weights of the late period were generally lower. Mucosal dry weights were always significantly higher in the early feeding period compared to the non-feeding period (Fig. 15). The dry weights from the late feeding period were not different from those of the non-feeding period in the caeca, but differed significantly in the other sections. Only in the hindgut was the mucosal dry weight significantly lower in the late feeding period than in the early feeding period. The difference between the two summer periods was not significant in the other sections, although the weight was lower later in the summer.

3.2.6 Mucosal Perimeter

The inner perimeter of the mucosal surface is presented as a function of cross-sectional area to correct for differences due to the size of the intestine (Fig. 16). The caeca and foregut had the largest perimeters, and did not differ significantly from each other regardless of the season (Table 6). The midgut had the next largest perimeter, followed by the hindgut.

Mucosal perimeter was significantly smaller in all sections in the non-feeding period compared to the early feeding period (May/June) (Table 7). Similarly, the perimeter was significantly smaller in the caeca, foregut and midgut in

Figure 16. Changes in mucosal perimeter with season. Perimeters were measured from 6 um sections for each part of the intestine standardized to the cross-sectional area of that section (mean ± SE; n varied from 4 to 8).

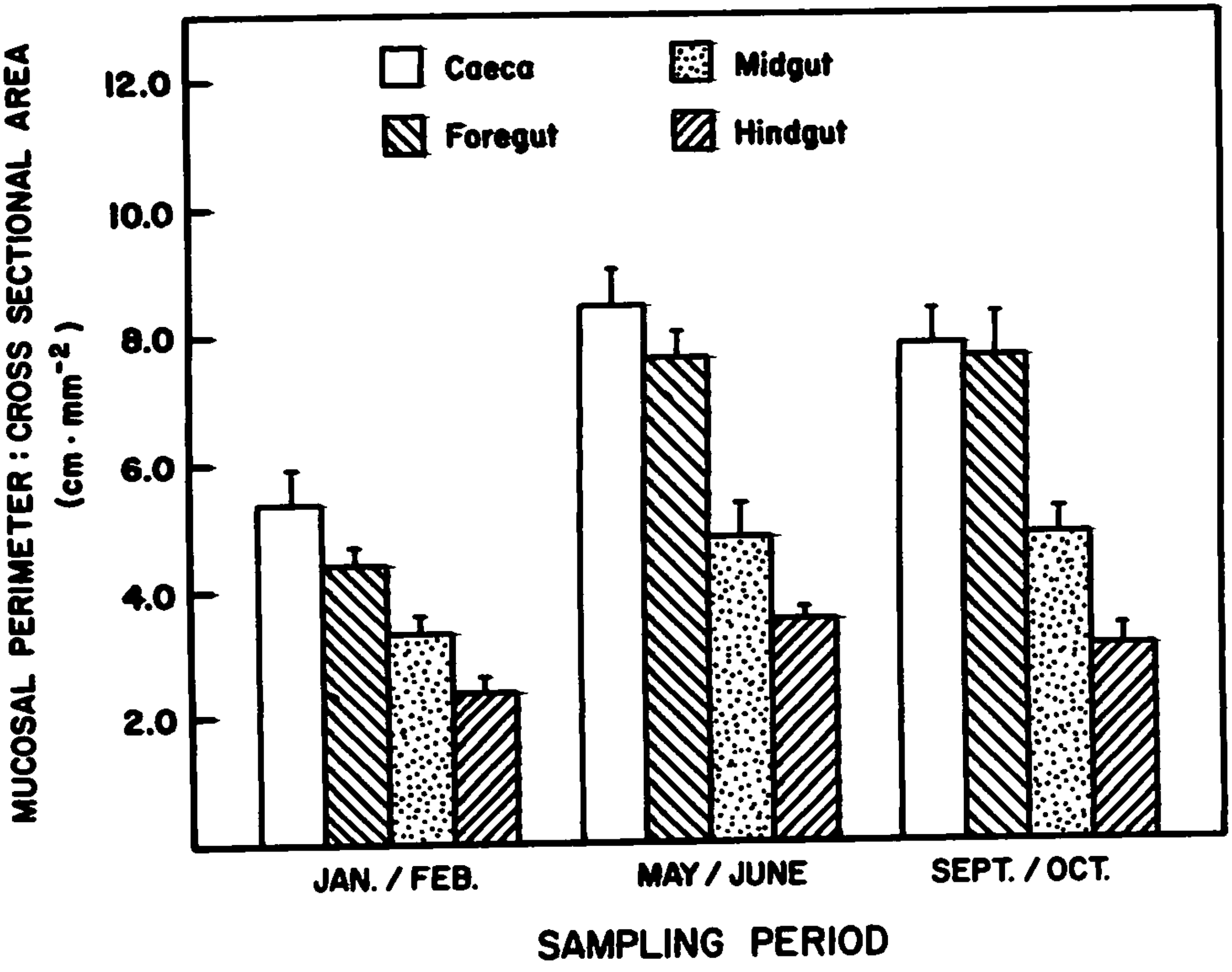

Caeca
Foregut
Midgut
Hindgut
MUCOSAL PERIMETER : CROSS SECTIONAL AREA
(cm · mm^{-2})
12.0
10.0
8.0
6.0
4.0
2.0
JAN. / FEB.
MAY / JUNE
SEPT. / OCT.
SAMPLING PERIOD

Table 6. Values of t (df = not less than 7) for t-tests comparing the mucosal perimeter (standardized to cross-sectional area) from different sections of the intestine. (C = caeca, F = foregut, M = midgut, H = hindgut). Asterisks indicate significance at the 5% level. Data from Figure 16.

	January			May			September		
	F	M	H	F	M	H	F	M	H
C	1.71	3.35*	4.55*	1.30	4.97*	9.47*	0.24	4.44*	6.94*
F	-	2.87*	5.19*	-	4.65*	8.67*	-	3.81*	6.70*
M	-	-	2.62*	-	-	2.46*	-	-	3.34*

Table 7. Values of t (df = not less than 7) for t-tests comparing mucosal perimeters (standardized to cross-sectional area) from non-feeding (Jan), early feeding (May) and late feeding (Sept) periods. Asterisks indicate significance at the 5% level. Data from Figure 16.

	Caeca		Foregut		Midgut		Hindgut	
	Jan	May	Jan	May	Jan	May	Jan	May
May	4.02*	-	7.10*	-	2.65*	-	3.68*	-
Sept	3.21*	0.80	3.23*	0.03	2.92*	0.02	1.77	1.23

the non-feeding period compared to the late feeding period. However, the perimeter of the hindgut for these two periods was the same. There was no difference between the two feeding periods.

3.2.7 Enzyme Activities

3.2.7.1 Alkaline Phosphatase

Alkaline phosphatase activities from feeding flounders, expressed in units of mucosal wet weight, were highest in the hindgut, but showed a proximodistal gradient from caeca to midgut (Fig. 17). The same trend was observed in non-feeding fish, except that caeca and hindgut activities were similar. Declines in alkaline phosphatase activities from feeding compared to non-feeding flounders were 85% in the caeca, 87% in the foregut, 78% in the midgut and 88% in the hindgut.

Expressed in units of protein concentration, alkaline phosphatase activity in feeding fish was highest in the caeca and declined down the intestine to the hindgut (Fig. 18). The same was true for non-feeding fish. Declines in activity from feeding fish compared to non-feeding fish were 90% in the caeca, 85% in the foregut, 83% in the midgut and 77% in the hindgut.

Total activity, or the relative contribution of each section to alkaline phosphatase activity, could be estimated because activity in the whole mucosa from each section was measured. Total activity showed the same trend as activity expressed per unit protein (Fig. 19), with a proximodistal gradient down the intestine.

Figure 17. Activities of alkaline phosphatase and leucine aminopeptidase from the mucosa of winter flounder in winter (non-feeding) and in summer (feeding). Activities are in terms of units per mg tissue (mean ± SE; n = 3).

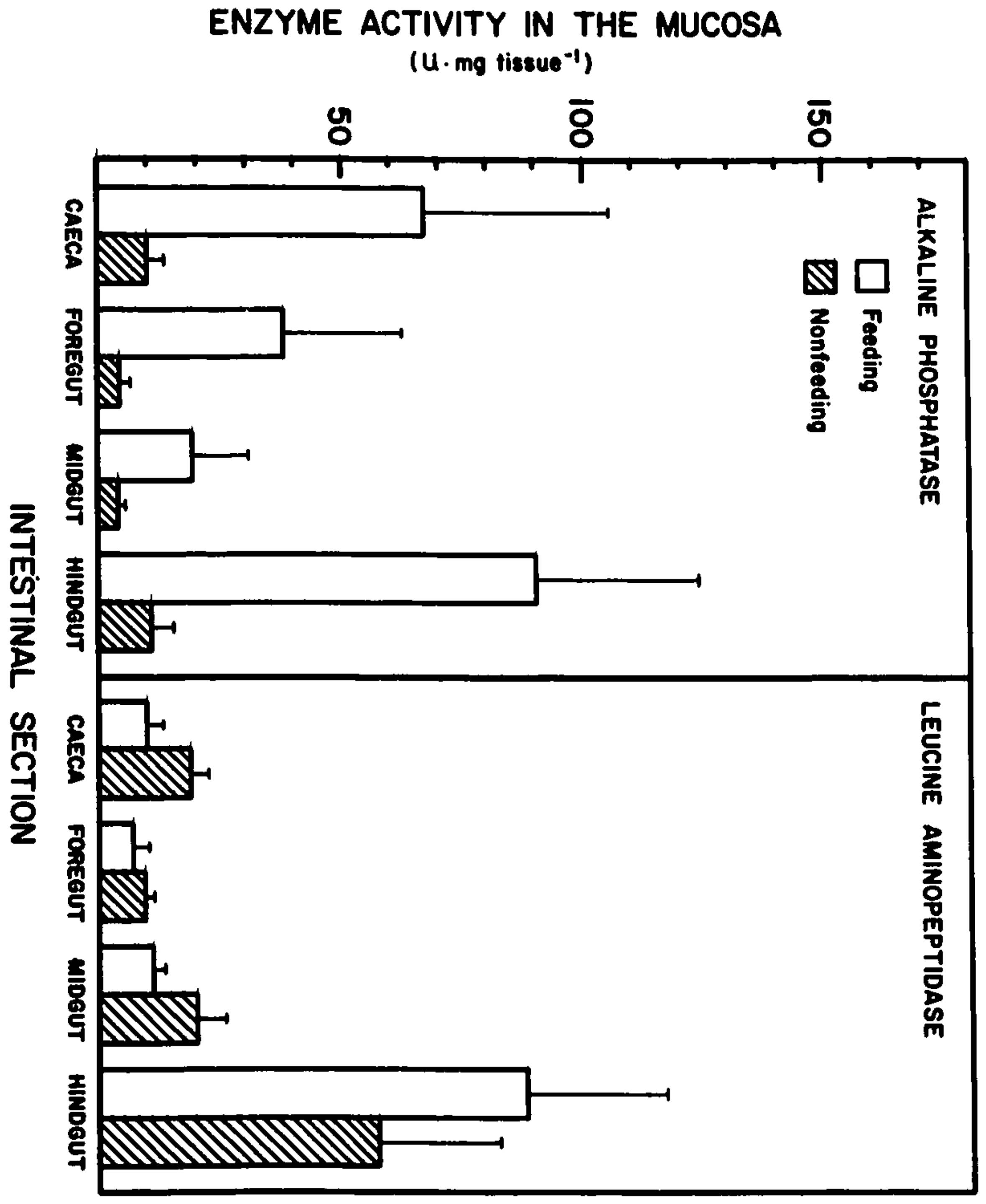

ENZYME ACTIVITY IN THE MUCOSA
(U·mg tissue⁻¹)
50
100
150
ALKALINE PHOSPHATASE
LEUCINE AMINOPEPTIDASE
Feeding
Nonfeeding
CAECA
FOREGUT
MIDGUT
HINDGUT
CAECA
FOREGUT
MIDGUT
HINDGUT
INTESTINAL SECTION

Figure 18. Activities of alkaline phosphatase and leucine aminopeptidase from the mucosa of winter flounder in winter (non-feeding) and in summer (feeding). Activities are in terms of milliunits per mg protein (mean ± SE; n = 3).

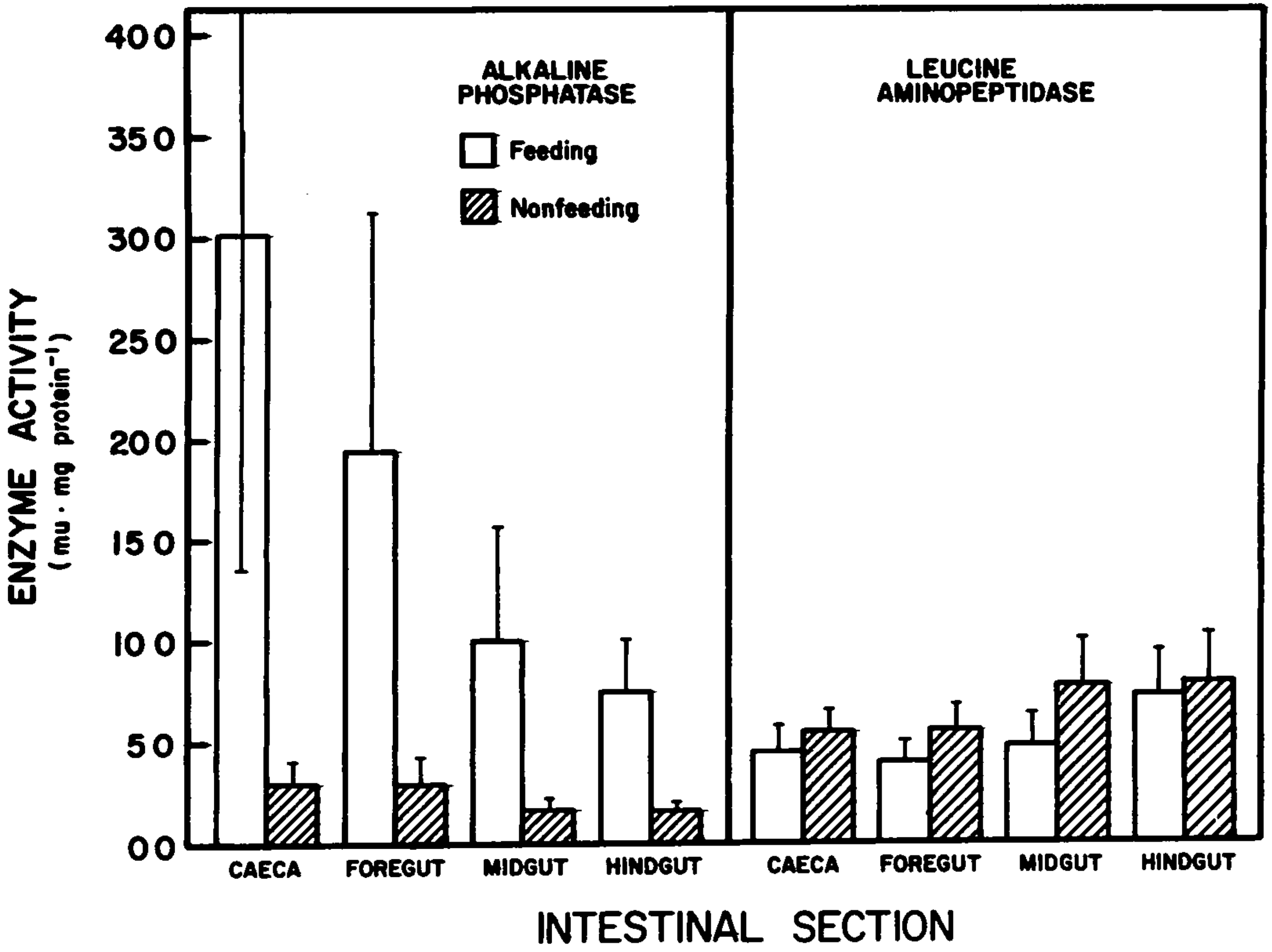
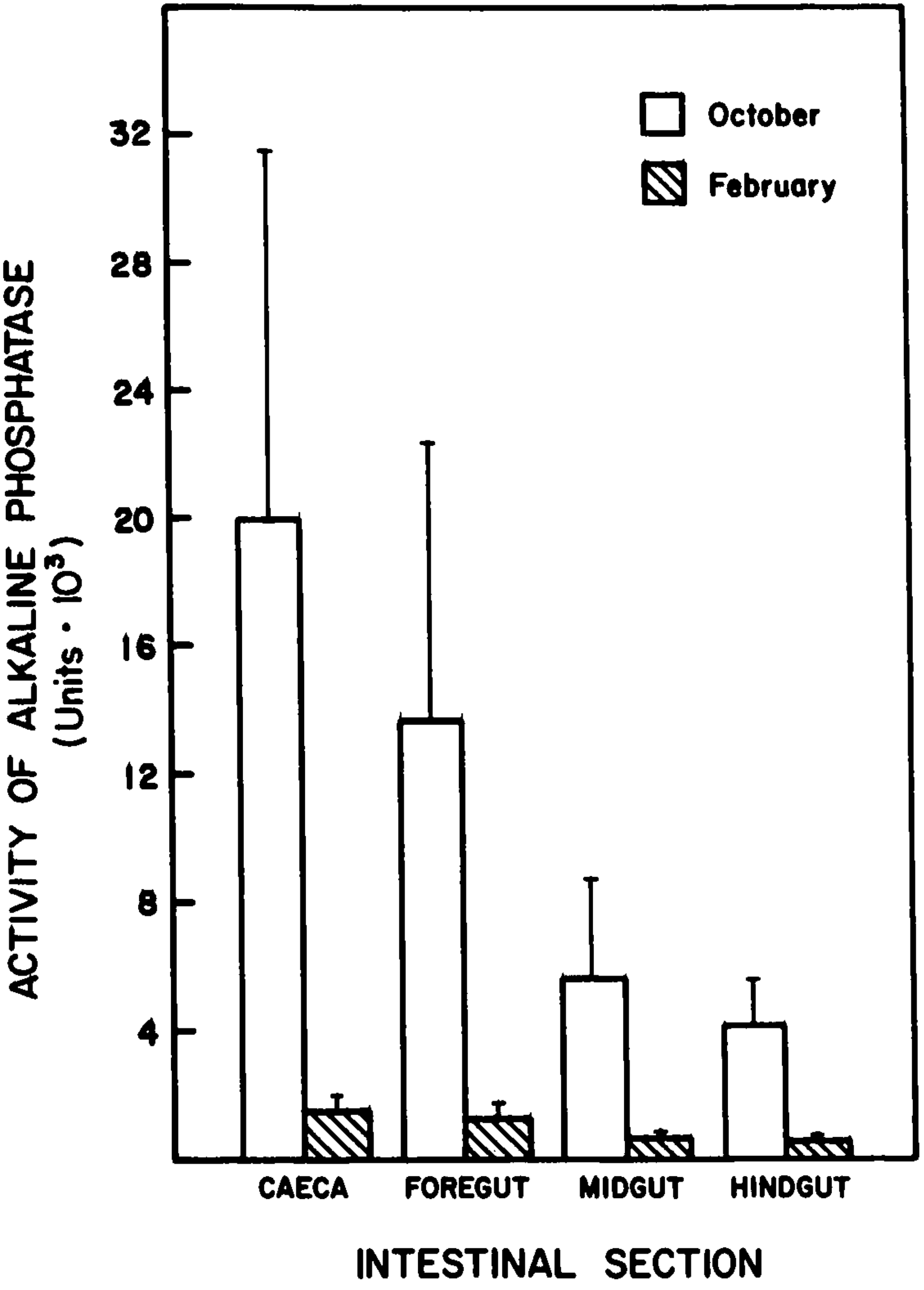
ALKALINE PHOSPHATASE
LEUCINE AMINOPEPTIDASE
□ Feeding
▨ Nonfeeding
ENZYME ACTIVITY (mu · mg protein⁻¹)
400
350
300
250
200
150
100
50
00
CAECA
FOREGUT
MIDGUT
HINDGUT
CAECA
FOREGUT
MIDGUT
HINDGUT
INTESTINAL SECTION

Figure 19. Seasonal change in total activity of alkaline phosphatase in the mucosa from each section of flounder intestine. Total activity is in terms of units (mean ± SE; n = 3).



October
February
ACTIVITY OF ALKALINE PHOSPHATASE
(Units · 10³)
32
28
24
20
16
12
8
4
CAECA
FOREGUT
MIDGUT
HINDGUT
INTESTINAL SECTION

3.2.7.2 Leucine Aminopeptidase

Leucine aminopeptidase activity showed a very different pattern from that of alkaline phosphatase. Expressed in units of wet weight, LAP activity was low and similar in caeca, foregut and midgut, with either no change or a slight increase from feeding to non-feeding flounders (Fig. 17). The hindgut exhibited relatively high activities, which declined when the fish did not feed.

LAP activities, expressed in units per protein concentration, were similar in all sections, and did not change when the fish stopped feeding (Fig. 18). Total activity (Fig. 20) was similar in caeca, foregut and midgut, but was highest in feeding fish in the hindgut. Only the hindgut showed a decline in total activity when feeding ceased.

3.2.7.3 Protein Concentration

Protein concentration per gram tissue (Fig. 21) was highest in the hindgut. Protein concentration increased by 61% from summer to winter in the caeca, stayed the same in the foregut and midgut, and declined by 60% in the hindgut.

3.2.8 Total Mucosal Surface Area

Total mucosal surface area was calculated for a 30 cm (standard length) flounder. The ratio of intestinal length to standard length was 0.99, and therefore gut length would be 29.7 cm. Based on estimates, the foregut represented 40% of the total length, the midgut 50% and the hindgut 10%. Therefore, the foregut would be 11.88 cm, the midgut would be

Figure 20. Seasonal change in total activity of leucine aminopeptidase in the mucosa from each section of flounder intestine. Total activity is in terms of units (mean ± SE; n = 3).

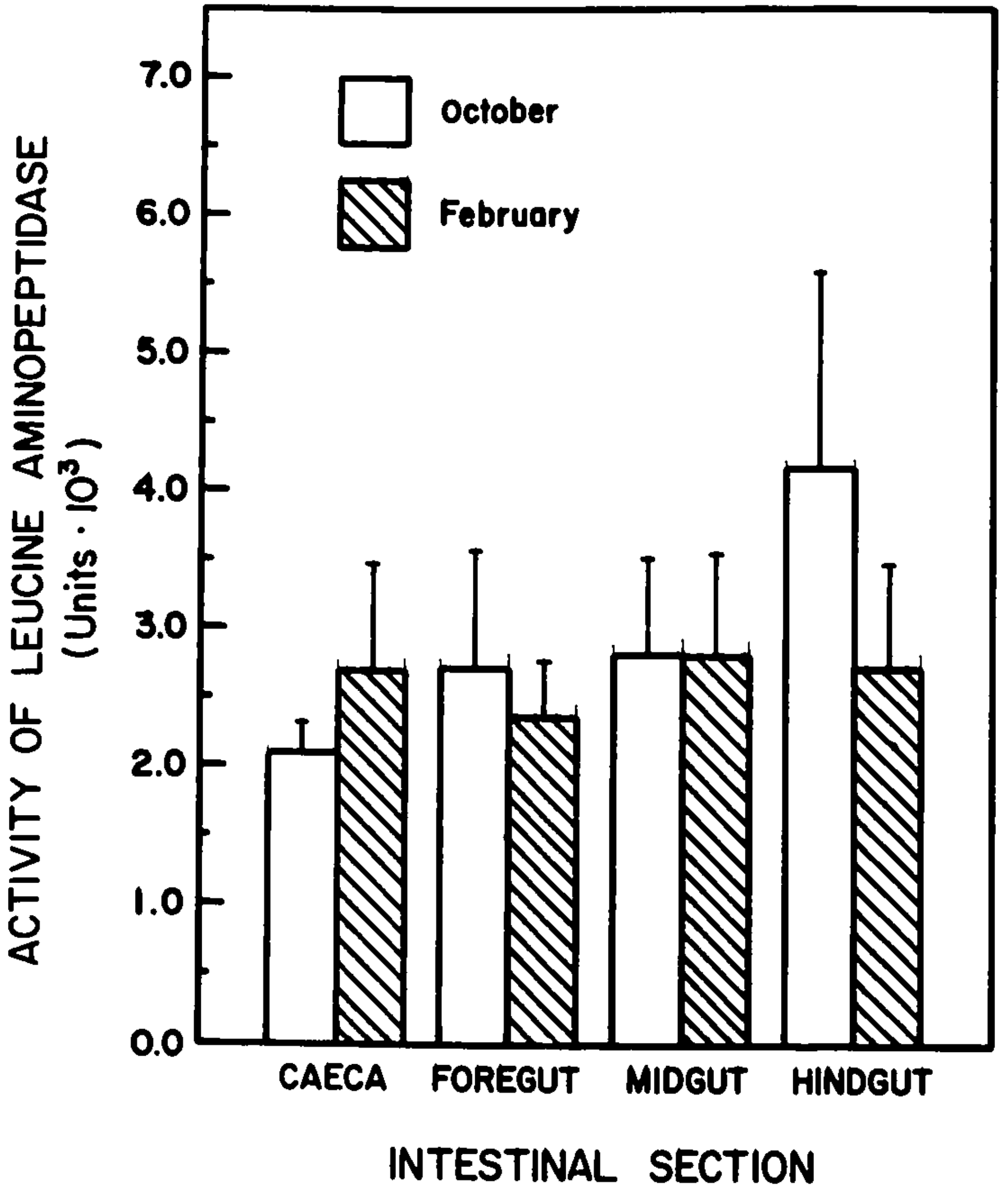
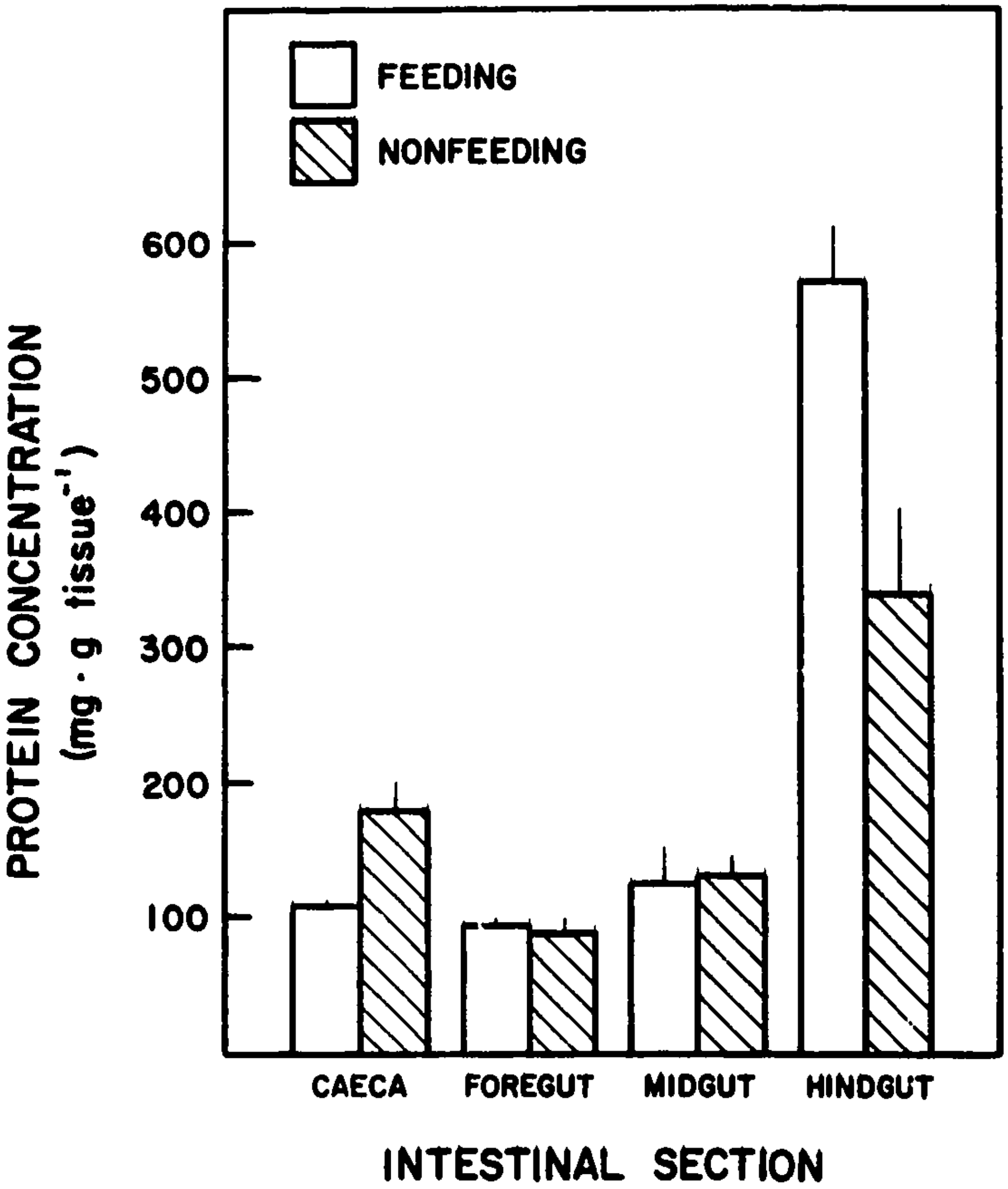

ACTIVITY OF LEUCINE AMINOPEPTIDASE (Units · 10^3)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
October
February
CAECA
FOREGUT
MIDGUT
HINDGUT
INTESTINAL SECTION

Figure 21. Seasonal change in concentration of protein of the mucosa in each section of the flounder intestine (mean ± SE; n = 3).


FEEDING
NONFEEDING
PROTEIN CONCENTRATION
(mg · g tissue^{-1})
600
500
400
300
200
100
CAECA
FOREGUT
MIDGUT
HINDGUT
INTESTINAL SECTION

14.85 cm and the hindgut would be 2.97 cm long. Mucosal perimeters, not corrected for cross-sectional area, and caecal lengths are given in Table 8. Multiplication of the perimeter by the length of the section (and multiplied by four for the caeca) gave the mucosal area for each section of the intestine, and these were summed for total area (Table 8). Caeca accounted for the same amount of surface area in January and September as did the midgut. However, in May, the surface area of the caeca was much greater. The foregut always showed the largest surface area, and the hindgut the least. Except in the hindgut, surface areas were largest in May, declined in September, and were smallest in January. Hindgut surface areas were highest in May, but the same in January and September. Total intestinal surface area was highest in May, declined by 25% by September, and declined a further 25% by January.

3.3 Discussion

Winter flounder from Passamaquoddy Bay exhibit a cycle of summer feeding, commencing about May, and winter non-feeding, commencing about November. During the feeding period, they will readily ingest almost any food presented to them, while they refuse all foods offered in the non-feeding period. Food refusal in winter can be active, when food is presented persistently, in that they will push it away forcibly by a stream of water from the mouth. Kennedy and Steele (1971) noted that, based on stomach contents, feeding declined gradually before it ceased in winter flounder caught off Newfoundland. A gradual reduction in feeding prior to the

Table 8. Seasonal changes in total surface area of the intestinal mucosa. Mucosal perimeters (mean ± SE(n), not standardized to cross-sectional area), average caecal lengths (cm) and calculated mucosal area for the three sampling periods, January, May and September. Mucosal areas were calculated for a 30 cm (standard length) flounder.

Perimeter (cm)

Section	January	May	September
Caeca	6.40 ± 0.27(5)	9.21 ± 3.26(4)	6.70 ± 0.76(8)
Foregut	6.08 ± 0.50(7)	11.96 ± 0.98(7)	8.98 ± 0.75(5)
Midgut	3.28 ± 0.34(5)	4.59 ± 0.40(5)	4.26 ± 0.66(6)
Hindgut	3.77 ± 0.31(4)	5.77 ± 0.52(5)	3.78 ± 0.38(5)
Length of Caeca	1.70 ± 0.11(7)	2.66 ± 0.15(6)	2.29 ± 0.17(8)

Mucosal Area (cm²)

	January	May	September
Caeca	43.5	98.1	61.3
Foregut	72.2	142.1	106.6
Midgut	48.6	68.2	63.3
Hindgut	11.2	17.1	11.2
Total Area	175.5	325.5	242.4

cessation of feeding was not observed in the present study, but no effort was made to measure amount consumed or stomach contents.

The muscle of winter flounder from Passamaquoddy Bay also exhibited annual changes. The flesh appeared opaque and dense and was firm to the touch in summer, while in winter, it became grey and translucent, with a watery feel. Pearcy (1961) noted a watery consistency of the flesh of winter flounders caught in winter from the Mystic River Estuary of Connecticut, although Umminger and Mahoney (1972), studying flounders caught off New Jersey, did not. This may reflect differences in populations, in feeding habits or in temperature regimes in the geographical location of the flounders. Templeman and Andrews (1956) noted a high percentage of American plaice (*Hippoglossoides platessoides*) with high muscle water content ("jellied") when catches were from areas of low bottom temperature (-1 to 0°C). Because only the large, reproductive plaice were found in this condition, it probably reflects the inability of these fish to replenish body reserves at these temperatures after reproduction.

The gonads in mature flounders were thin and watery in June and July after spawning, but began to develop again by September or October. By the cessation of feeding, they were generally enlarged, causing an obvious bulging of the dorsal side of the fish beneath the lateral line. Throughout the winter, the gonads of mature flounders protruded into the visceral cavity to such an extent that the intestine was pushed well forward. The intestine itself appeared less opaque and more thin-walled throughout the winter, and was often distended with fluid. Parasites were increasingly rare as the non-feeding period progressed, until by mid-December to January, they were absent. In the summer, gut parasites were usually present, and occasionally in large numbers. A digenean was common amongst the folds of the mucosa in both the caeca and foregut. Cestodes and, occasionally, nematodes seemed to parasitize the entire intestine, although they were more frequent anteriorly. Cestodes were most abundant in the late summer, sometimes causing distention of the intestine by their numbers.

The cessation of feeding may result in two advantages to the winter flounder. First, it allows the gonads to develop into the visceral cavity without interference from large quantities of food distending the intestine. This may enhance fecundity. Secondly, it permits recovery from parasitic infestation. Whether these are causative or merely coincident is open to speculation.

Winter flounders become emaciated in winter, the thickness of the musculature decreasing. Quantitation of this was attempted by examining both the length-weight relationships and condition factors of the fish. The slope of the length-weight relationship declined in winter, particularly in the first year of the study (84/85) (Table 2), possibly due to the fact that fish were sampled later in the non-feeding period in the first group. Thus, larger fish were more affected by the cessation of feeding than smaller fish.

Values of the slope of the length-weight relationship found for other populations of winter flounder are similar to those of the present study. Slope values ranged from 2.9 to

3.2 for flounders caught off New England (Lux, 1969), 2.98 to 3.14 for those caught off Newfoundland (Kennedy and Steele, 1971) and 2.91 to 3.27 for those caught in the Saint Lawrence River estuary (Vaillancourt et al., 1985). The study by Kennedy and Steele (1971) was done in the summer feeding period while that by Lux (1969) was performed throughout the year. Lux noted that the slope of the length-weight equation was lower in the fish immediately after spawning. The decline seen post-spawning was probably due to the loss of gonad weight, since body weights included gonad weights. Gonad weights may, therefore, mask a change in the fish weight. Indeed, Vaillancourt et al. (1985) reported that the lowest slopes were observed among the immature fish in May and June, while the highest slopes were among the pre-spawning mature fish. The increased slope was attributed to the greater fecundity of larger fish.

The present study used only gonad free weights to calculate the length-weight relationship (Table 2). The slope was lower in the first year of the study compared to the literature values or the following year. However, as already pointed out, the first year group included individuals from late in the non-feeding period, and these might be expected to exhibit greater effects of starvation than fish earlier in the fast.

Condition factor declined in the non-feeding period whether calculated with or without the gonads (Table 3). That condition factor declined even when gonad weights were included indicates that the decline is not due entirely to gonad development. Approximately 71% of the decline in condition factor was attributed to the effects of starvation, while only 29% was due to gonad development.

Rauck (1975) reported that the condition factor of small female plaice (*Pleuronectes platessa*) decreased from August and September to April or May, then increased. The decline coincided with the non-feeding period of this species (White and Fletcher, 1985). Larger (reproductive) female plaice showed low condition factors just at the time of spawning, but the increase in gonad weight masked any other decline. Male plaice showed similar, but dampened, annual fluctuations. Bridges et al. (1976) reported that the condition factor of winter flounder was lowest in April, May and June, and rose to a peak at about November. The condition factors they reported were higher than those of the present study (ranging from 1.78 to 2.25). They do not report whether gonad weights were included. These flounders were captured in Casco Bay, Maine, and the different condition factors may reflect differences in growth due to population, temperature or other factors. Different populations of flounder, even from adjacent bays, exhibit different patterns of growth (Howe and Coates, 1975).

The condition factor reported for plaice, which also undergo a period of winter starvation and spawn in the spring (White and Fletcher, 1985), increased from June to September, then decreased until March or May. Condition factor declined in the winter in mature female plaice as well as in the males. White and Fletcher also estimated the hepatosomatic index (liver wt/body wt x 100) in plaice and found that it followed the same trend as the condition factor. They attributed the increase in the index during the feeding period to an increase in the number of hepatocytes as well as an increase in lipid and glycogen, and the decrease in the winter non-feeding period to the mobilization of these lipid and glycogen reserves. Therefore, although few animals were measured for liver lipid content in the present study (Fig. 8), it is likely that winter flounder also exhibit a general rise in liver lipid levels during feeding followed by a decrease during fasting. Dawson and Grimm (1980) found that the carcass and not the liver was the main store of lipid reserves in the plaice, due to its larger relative size, but the liver lipid stores were mobilized in response to starvation. They estimated that 64% of body lipid and 40% of body protein was utilized during the period of non-feeding. From estimations of lipid and protein levels in the eggs, only 14% of the lipid and 33% of the protein went into egg production. Therefore, the majority of the lipid mobilized was to provide energy during starvation, rather than to provide energy for reproduction. The finding in the present study that muscle lipid seemed to decline less in non-reproductive than in reproductive fish during the winter non-feeding period suggests that lipid is mobilized to some extent for reproduction, but the extent to which such mobilization occurs requires more detailed study.

The increase in muscle water content observed (Fig. 9) was probably due to protein utilization during the non-feeding period (Love, 1970; 1980). A gradual decline in water content was observed throughout the summer to October, but little variation was observed through the non-feeding period, suggesting that the majority of protein utilization occurred early in the non-feeding period. Protein utilization during starvation in fish generally occurs after lipid reserves have been depleted (Love, 1970; 1980; Jobling, 1980). The results of the present study suggest that protein is utilized early in the non-feeding period to support gonad development, but that lipid reserves are adequate to supply most of the energy requirements of the fish through the period of fasting. The relatively constant muscle water content throughout the winter indicates that little protein utilization occurred to supply energy during starvation. It is likely that longer periods of food deprivation would result in a further increase in muscle water content, to levels such as those observed by Templeman and Andrews (1956) in "jellied" American plaice.

Intestinal structure in the winter flounders from Passamaquoddy Bay was similar to that described for flounders caught off Mount Desert Island, Maine (Field et al., 1978), including the two outer muscle layers, a submucosa and a mucosa (Fig. 10). The mucosa contained enterocytes, goblet cells and endocrine cells. Enterocytes were elongated and narrow and, except for microvilli, appeared similar throughout the intestine. In the hindgut, the microvilli were more numerous and longer (Fig. 14), suggesting that these cells possessed greater potential absorptive capacity than those of the upper portions of the intestine. Whether this capacity was for osmoregulatory or digestive requirements is not known. However, Ferraris and Ahearn (1984) suggested that the fish hindgut may serve a scavenging function to obtain as much as possible from the luminal contents. The finding of Dabrowski and his colleagues (1986) that seawater adapted rainbow trout have higher levels of free amino acids throughout the intestine compared to freshwater adapted trout, and thus probably lose a portion of hydrolyzed protein, suggests that such a scavenging role would be adaptive. An increase in the surface area of the hindgut could be the mechanism for such an adaptation.

Enterocytes were similar regardless of their position along the fold, as described previously for the winter flounder (Field et al., 1978; Trier and Moxey, 1980). This is to be expected if, as Trier and Moxey (1980) observed, epithelial cell proliferation occurs all along the mucosal fold, in the absence of crypts of Lieberkuhn.

Folding appeared to be most extensive in the proximal intestine (caeca and foregut), and declined distally. This was reflected in mucosal dry weights, though not wet weights (Fig. 15; Table 5), and especially in the measurements of mucosal perimeter (Table 6). It should be remembered that mucosal weights were obtained from tissues which had undergone up to 10 h of *in vivo* perfusion. Some uptake of water was observed in the *in vivo* uptake studies (Chapter 4), and thus changes in wet weights may be masked by water uptake during absorption.

Proximodistal downward gradients of mucosal mass have been observed in mammals (Williamson, 1978; Karasov and Diamond, 1983a) and fish (Harder, 1975; Reifel and Travill, 1979). The proximodistal gradient is probably maintained by the presence of luminal nutrients (Karasov and Diamond, 1983a). Studies in mammals have shown that luminal nutrients directly stimulate mucosal cell proliferation (Johnson, 1981). Karasov and Diamond (1983a) contended that the gradient of luminal nutrients brought about by the activity of the intestine promotes the gradient of intestinal mass; that is, the proximal intestine is exposed to higher concentrations of nutrients, and thus is stimulated to grow more than the more distal regions. This contention receives some support from the observation in winter flounder (Trier and Moxey, 1980) that mucosal cell proliferation was higher proximally in fed fish; this gradient disappeared after only seven days of food deprivation. Rates of proliferation also appeared to have declined.

The observed hypoplasia of the intestine during starvation in fish (Jobling, 1980; Bogé et al., 1981b; the present study) is probably due to the absence of direct stimulation by food. However, the proximodistal gradients of mucosal mass (Fig. 15) and mucosal surface perimeter (Fig. 16) were not abolished in the January non-feeding period. Kirsch and Meister (1982) found that ingested water was processed in the anterior esophagus and the anterior intestine in European eel (*Anguilla anguilla*). Field and his coworkers (1978) found no variation in transepithelial potential difference or resistance along the length of the winter flounder intestine, suggesting that there were no regional differences in salt uptake, but they excluded the first and last 4 cm of the intestine. It is, therefore, probable that the proximodistal gradient of mucosal mass and surface area was maintained in the absence of luminal nutrients by the requirements for water uptake during salt uptake.

In freshwater rainbow trout starved for four weeks, the reduction of mucosal dry weight was found to be 76%, while after eight weeks, only 16% of the feeding dry weight remained (Bogé et al., 1981b). This contrasts with starvation in plaice, which showed only a 42% decrease in their gut-somatic index after 5 weeks of starvation (Jobling, 1980). Winter flounders in the present experiment starved for approximately twelve weeks showed a reduction of no more than 57% in mucosal dry weight compared to May (feeding) levels.

Structurally, the cells themselves differed little from summer to winter (Fig. 10-14). Microvilli appeared as numerous in non-feeding as in feeding flounders. Although Karasov and Diamond (1983a) noted that microvillar lengths declined in starved rats, apparently no such decline occurred in winter flounder. Gas and Noaillac-Depeyre (1976) reported progressive reduction in the length of microvilli in the first 6 months of starvation in carp. The requirement for continued osmoregulation and therefore ion and water transport may be the reason that microvillar surface area is maintained in winter flounder.

The sparing of intestinal mucosa may be influenced as well by the colder temperatures which the fish encounter during the winter. Winter flounder in Newfoundland do not follow isotherms, but rather stay inshore in the winter, only moving into deeper water when there is danger of ice scouring and turbulence (Van Guelpen and Davis, 1979). McCracken (1963) also found little distributional change in winter flounder throughout the year in Passamaquoddy Bay. Indeed, the presence of antifreeze peptides in these fish (Scott et al., 1986) supports the conclusion that winter flounder need not avoid low temperatures. The observation (Kitchin and Morris, 1971; Groot et al., 1983) that cold acclimated goldfish increase mucosal weight suggests that cold temperature may also have a sparing effect on the mucosa. Whether this is due to an increase in mucosal cell proliferation rate or a decrease in attrition of enterocytes is unknown. In addition, such changes have only been reported in goldfish, and may not be a general phenomenon. Regardless of the reason, however, there does appear to be less of a reduction of mucosal mass in marine fish during starvation than in freshwater fish.

The folding pattern of the intestine changed seasonally as well (Fig. 10-13). Folds tended to be long and narrow in summer, particularly in the upper sections of the intestine, but the height decreased and the folds became more flattened with the cessation of feeding. In rats (Ross and Mayhew, 1984), villus height declined during fasting, but the more important change was in villus shape. The change observed, however, was a thinning rather than a flattening as observed in winter flounder. Gas and Noaillac-Depeyre (1976) reported only a slight decline in fold height in the first seven months of starvation in the carp. However, the loss of intestinal weight was associated with a significant loss of intestinal length in the first few months of starvation. No such loss of length was observed in winter flounder in the present study, although the caeca did become somewhat shorter. Thus, the loss of mucosal mass in winter flounder must be attributed to the loss of mass per unit length, suggesting that more marked changes in the pattern of folding are observed in winter flounder than in carp during starvation.

The number of goblet cells appeared to decrease during the winter non-feeding period compared to the feeding period (Fig. 10-13). Goblet cells function to produce mucus, which acts to protect the mucosa from mechanical and enzymatic damage, the latter by behaving as a diffusional barrier to the digestive enzymes. Mucus can also act as a diffusional barrier to ions (Shephard, 1982) and presumably nutrients. Thus, in the absence of food, and therefore, the absence of the necessity of protecting the mucosa from digestion, and in the face of the continued requirement of osmoregulation, it is not surprising to find that mucus cell numbers decreased. Indeed, MacLeod (1978) observed a decrease in mucus cell numbers in rainbow trout transferred from freshwater to seawater, and the decline in number correlated with the increase in salinity. Similarly, Western (1969) observed that fewer mucus cells were present in the intestine of the marine cottid *Enophrys bubalis* compared to the closely related freshwater species *Cottus gobio*. Thus, the requirements of osmoregulation appear to oppose those of digestion in terms of mucus production, and in the absence of food, the requirements of osmoregulation take precedence. This implies a control system which balances the two functions of the intestine, either in a direct or indirect manner, to determine the number of mucus cells present.

Reduction in the amount of DNA, RNA, protein and water has been observed in rats during starvation (Ju and Nasset, 1959; Steiner et al., 1968; Steiner and Gray, 1969). Jones and Mayer (1973) observed a decline in the activity of seven glucose metabolizing enzymes in rat small intestine during starvation. The decline was not due to the reduced turnover of the enterocytes, but to changes in enzyme turnover. Protein concentrations in the mucosa of winter flounder decreased only in the hindgut. The protein content of the foregut and the midgut changed very little, while that of the caeca increased. It appears, therefore, that the intestine responds to the winter starvation by decreasing the absolute amount of protein (i.e. reducing the mass of the mucosa), but maintaining the relative amount of protein in the foregut and midgut, and even increasing it in the caeca.

Estimations of alkaline phosphatase (AP) activity suggest that this enzyme was decreased with the cessation of feeding by more than would be expected due to the loss of enterocytes. However, because the enzyme activity was estimated at 30°C, it is possible that some portion of the decline represents a change in the enzyme itself: that is, the enzyme produced in winter may have had a lower temperature optimum than that produced in summer, and the decline may be due to a temperature effect rather than a real decline in enzyme activity. Gelman and his colleagues (1984) reported a change in temperature optimum and activity of AP in pike perch (*Lucioperka lucioperka*) from summer (50°C) to winter (°C), though no such changes occurred in bream (*Abramis brama*). They attributed the change to the decreased activity of pike perch in winter compared to summer, since the bream remained active throughout the year. The pike perch is an active predator, and a decrease in activity suggests that it may eat less in winter, and thus the decline in luminal contents could well have been the cause of the reduction in AP activity. It would therefore appear that the winter flounder is more similar to the pike perch than the bream in terms of its intestinal function, and the possibility that the temperature optimum of AP also changed in winter flounder in winter cannot be eliminated. AP has been implicated in osmoregulation (Utida et al., 1968; Oide, 1973) and thus it might be expected that its activity would not decline more than surface area in winter flounder because of the continuing need to osmoregulate. Further study to determine if the observed decline was due to temperature effects or to a real decline in activity in the winter flounder are necessary to assess the seasonal variations in the activity of this enzyme. At present, no conclusions can be drawn.

AP activity in carp, *Cyprinus carpio*, and catfish, *Ameiurus nebulosus*, was found to be higher in the anterior compared to the posterior part of the intestine (Fraisse et al., 1981), whereas leucine aminopeptidase (LAP) activity was evenly distributed. However, in *Perca fluviatilis*, LAP activity was lower in the hindgut (Hirji and Courtney, 1982) and in rainbow trout, while AP was lower in the hindgut, LAP activity was higher (DiCostanzo et al., 1983a).

In summer, AP activity expressed in grams of tissue in the hindgut of winter flounder was higher than in the other sections (Fig. 17), and this may reflect the more numerous microvilli. A proximodistal gradient of activity was observed otherwise (Fig. 17; Fig. 18), with the caeca exhibiting the highest AP activity. The anterior portion of the intestine, therefore, has a much greater capacity for AP activity than the posterior intestine by virtue of a proximodistal gradient of both mucosal mass and enzyme activity. Total activity measurements tend to support this conclusion. The proximodistal gradient in activity observed in summer was abolished in winter (Fig. 18). No proximodistal gradient of LAP activity in winter was observed, and only in the hindgut did LAP activity decrease. The decrease was not observed when activity was standardized to protein content (Fig. 18); the decline in activity therefore follows the decline in protein concentration observed in the hindgut. Total activity estimations (Fig. 20) suggest that LAP activity may have been increased in winter in the other three sections. Levin et al. (1965) observed increases in activity of two mucosal peptidases, glycylglycine peptidase and leucylglycine peptidase, in rats fasted three days, but no change in maltase activity per gram of intestine. These results suggest that the protein handling capacity of the intestine may be spared during starvation.

The remainder of the present study deals with seasonal changes in the capacity of the intestine to transport phenylalanine during the winter non-feeding period and the summer feeding period to assess if the sparing of protein handling extends to the level of amino acid uptake.

Chapter 4

In vivo Uptake of L-Phenylalanine

4.1 Introduction

The uptake of L-phenylalanine was examined *in vivo* to assess uptake under those influences which normally control its operation. Several hormones are known to influence intestinal proliferation (Williamson, 1978) and function (Guidotti et al., 1978; Shotwell et al., 1983; Lerner, 1985) in mammals. When injected into rainbow trout, 17-α-methyltestosterone and 17-β-oestradiol (Habibi et al., 1983) and 11-ketotestosterone (Habibi and Ince, 1984a) were found to stimulate L-leucine uptake by the intestine. Habibi and Ince (1984b), however, were unable to show an effect of gonadectomy on L-leucine transport and suggested that the previous experiments (1984a) could have been pharmacological rather than physiological. Factors influencing intestinal amino acid uptake *in vivo* have received little attention in fish, and the possible role of hormones, neural input, or feedback mechanisms by the solutes themselves, are unknown. The possible existence of a solute effect led to the measurement of plasma phenylalanine concentrations throughout the year.

A major source of error in the measurements of uptake is the unstirred water layer (Barry and Diamond, 1984). This layer is a region of static fluid adjacent to the membrane. It is a gradient, with a motionless layer immediately adjacent to the membrane and layers which are increasingly stirred toward the lumen. However, a model which describes an abrupt change from stirred to unstirred layers provides a reasonable approximation (Barry and Diamond, 1984). An effective unstirred layer thickness can therefore be measured. The unstirred layer behaves as a diffusional barrier between the bulk, or luminal, phase and the microvillar membrane. The measured concentration in the bulk phase is therefore not the same as that at the membrane surface, because transport at the membrane reduces the concentration, and this results in the overestimation of the value of K_t, or the carrier affinity constant. The overestimation is even greater if K_t is calculated by a double reciprocal or Lineweaver-Burk transformation. The estimation of J_{max}, or the maximal rate of uptake, tends to be less subject to error due to the unstirred layer (Barry and Diamond, 1984) because J_{max} is measured at saturating concentrations of solute and the concentration at the membrane will eventually become saturating.

The thickness of the unstirred layer can be altered by a number of treatments. It is greatest *in vivo* compared to *in vitro* preparations (Winne, 1973; Winne, 1976; Thomson and Dietschy, 1980a). Stirring can reduce the unstirred layer thickness to a minimum in *in vitro* preparations, though it cannot eliminate it (Lukie et al., 1974; Westergaard and Dietschy, 1974; Thomson and Dietschy, 1980a; Thomson and Dietschy, 1980b). *In vivo*, perfusion of a solution simultaneously with air (Winne, 1978; Winne et al., 1979) or increasing the perfusion rate (Lewis and Fordtran, 1975; Winne, 1979) can reduce the unstirred layer thickness. Intestinal structure can also strongly influence the thickness of the unstirred layer (Read et al., 1976a; Read et al., 1976b; Read et al., 1977; Thomson and Dietschy, 1977). Because the unstirred layer thickness is greater *in vivo* than *in vitro*, an attempt was made to measure the unstirred layer thickness in the intestine of winter flounder *in vivo* in order to correct the measured affinity constant.

The time required to perfuse the intestine of a single fish is several hours. Circadian rhythmicity has been observed for a number of intestinal functions in mammals, including brush border membrane enzyme activities, villus size (Stevenson et al., 1980), and L-histidine uptake (Furuya and Yugari, 1971). The rhythmicity is related to the timing of food intake. Winter flounder feed between dawn and dusk (Olla et al., 1969), so all *in vivo* perfusions were performed within the same time period, with the initial pre-perfusion begun at approximately 10.00h. The concern that an intrinsic difference in uptake due to rhythmicity would influence the results prompted an examination of uptake over a prolonged period using a series of perfusions of a 5 mM phenylalanine solution. If circadian rhythmicity was found, control experiments at each season would be included to correct for differences in uptake.

4.2 Results

In vivo uptake of phenylalanine was normalized to both surface area and length of the intestinal segment. The surface area measurement was based on the length of the perfused segment, its circumference and the thickness of the wall (allowing calculation of the inner surface area) and therefore was a very rough estimate of surface area; it excludes the area attributable to folding as well as microvilli. Surface area was shown to change with the season (Ch. 3, Tables 7 and 8), so the uptake values were also normalized to segment length. The length of the segment was chosen arbitrarily by placement of the cannulae, and is therefore constant with season.

Uptake measured *in vivo* showed the classic transport curve, a combination of a Michaelis-Menten saturable component and a linear, apparently diffusional, component (see Fig. 7).

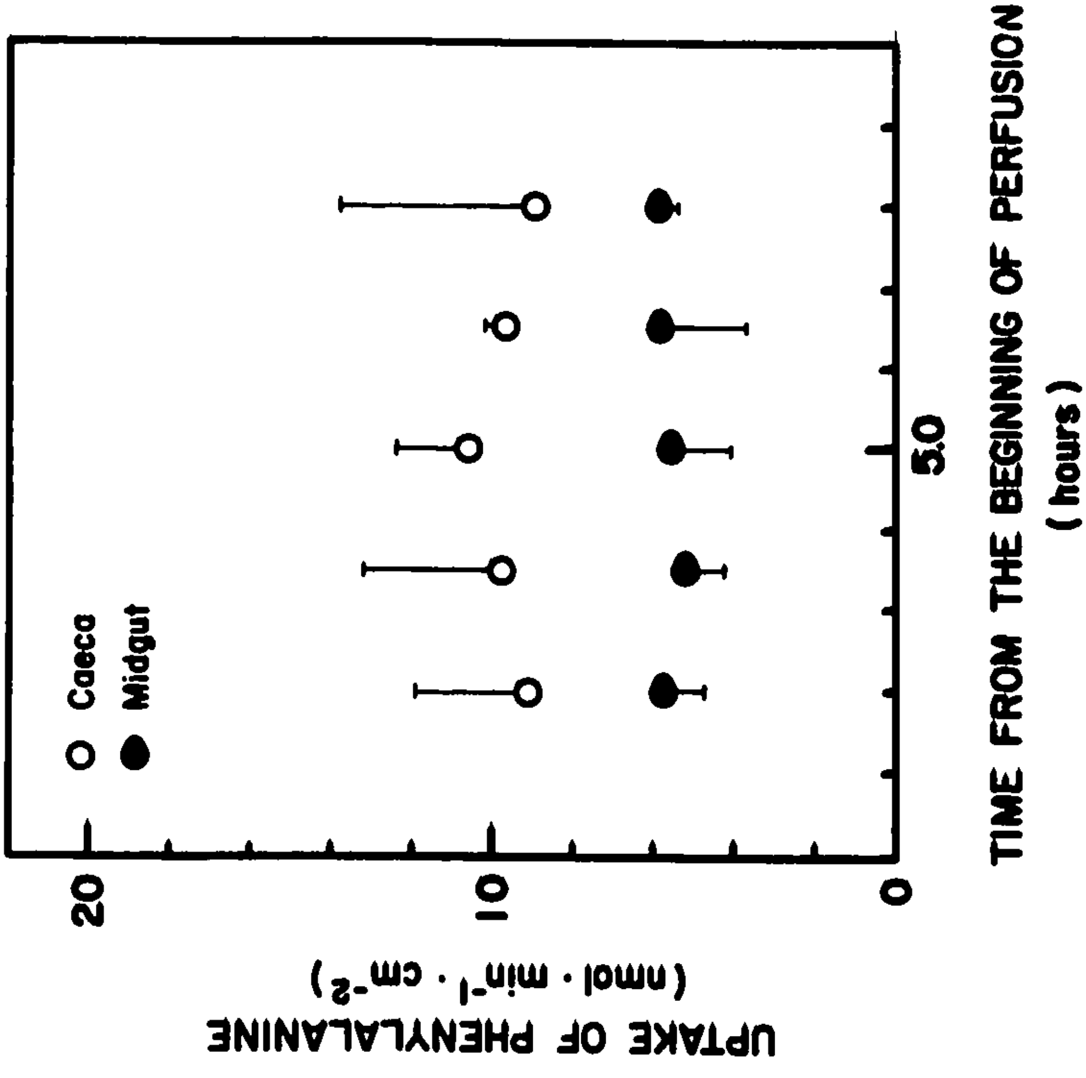
4.2.1 Diurnal Variations in Uptake

Uptake of phenylalanine varied little over a period of 8h in both the caeca and the midgut (Fig. 22), suggesting no important diurnal variations occurred.

4.2.2 Water Movement

Changes in phenylalanine concentration in the perfusate due to movement of water were monitored by the use of phenol red, an unabsorbed marker (Winne, 1978). Water was absorbed from the perfusate in the foregut and midgut, resulting in changes in phenol red concentration of 0.2 to 7%. Water absorption tended to be highest in the caeca in summer, particularly in September, when an increase in phenol red concentration of 21% was observed during perfusion of 15 mM phenylalanine. Secretion of water into the perfusate from the hindgut changed the phenol red concentration by as much as 6%, but absorption as high as 3% was also observed. No other

Figure 22. Variation in *in vivo* L-phenylalanine uptake over a period of 8h in the midgut and caeca. Uptake was corrected for variations in water content of the perfusate through the use of phenol red (mean ± SE; n = 5).


○ Caeca
● Midgut
UPTAKE OF PHENYLALANINE
(nmol · min^{-1} · cm^{-2})
20
10
0
5.0
TIME FROM THE BEGINNING OF PERFUSION
(hours)

pattern of water movement was observed, either with respect to time of day or to concentration of phenylalanine in the perfusate.

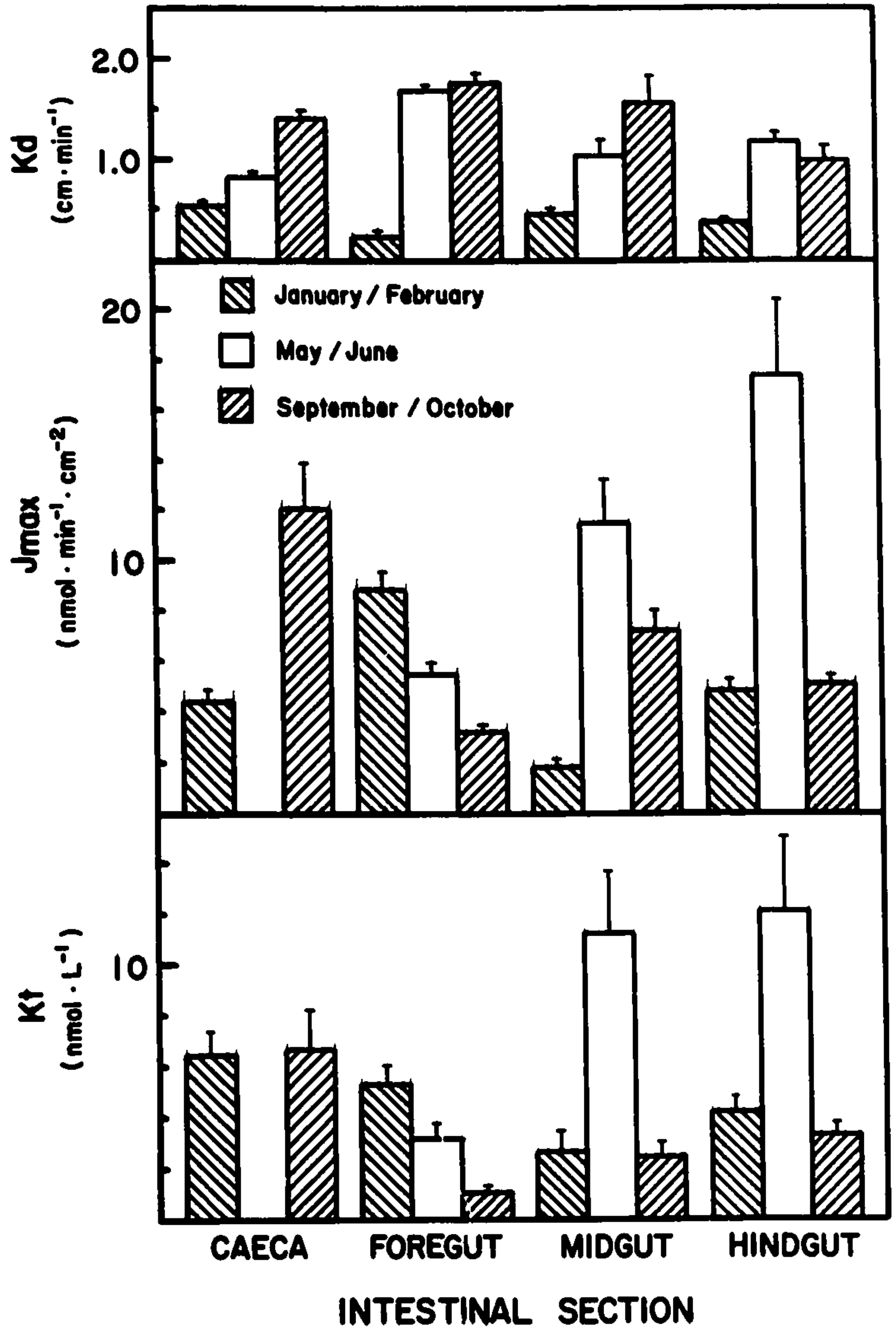
4.2.3 Apparent Diffusional Uptake

The slopes of the diffusional component, K_d, showed similar patterns whether expressed in units of area (Fig. 23) or length (Fig. 24), increasing from January to May to September except in the hindgut. Apparent diffusion of phenylalanine expressed in units of area was significantly lower in January in the foregut than in the midgut or caeca, but not different from the hindgut (Table 9). Although the differences were small, apparent diffusional uptake showed the following pattern: caeca > midgut > hindgut > foregut. The trend reversed at the onset of feeding, with the foregut showing significantly higher apparent diffusional uptake when compared to the other sections, while the caeca, midgut and hindgut did not differ from each other. In September, the foregut still showed higher apparent diffusional uptake, though it was not significantly different from the caeca or midgut. The apparent K_d was lowest in the hindgut.

Within each intestinal section, the apparent K_d was always significantly higher in May than in January (Table 10). The apparent diffusional constants in September were significantly higher than January values except in the midgut. The apparent K_d was significantly higher in September than in May in the caeca, but did not differ in any of the other sections.

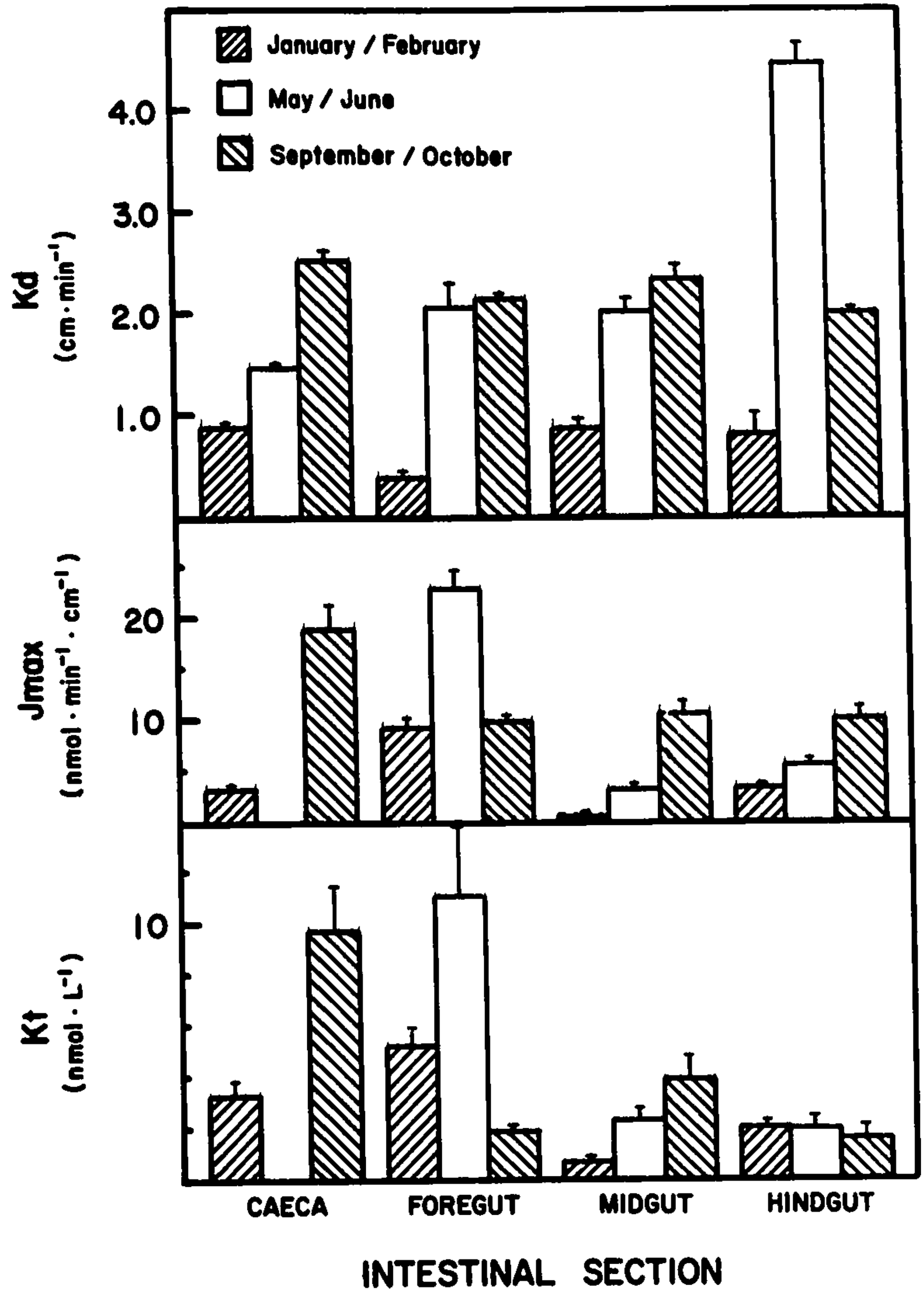
When apparent K_d was expressed in units of length,

Figure 23. Seasonal changes in the apparent diffusion constant (K_d), the maximal uptake rate (J_{max}) and the apparent carrier affinity (K_t) of L-phenylalanine measured *in vivo* (mean ± SE). Uptake is expressed per unit area (cm^2). Measurements were on 7 flounders in January and 6 each in May and September.



Kd (cm · min⁻¹)
2.0
1.0
January / February
May / June
September / October
Jmax (nmol · min⁻¹ · cm⁻²)
20
10
Kt (nmol · L⁻¹)
10
CAECA
FOREGUT
MIDGUT
HINDGUT
INTESTINAL SECTION

Figure 24. Seasonal variation in the apparent diffusion constant (K_d), the maximal uptake rate (J_{max}) and the apparent carrier affinity (K_t) of L-phenylalanine measured *in vivo* (mean ± SE). Uptake is expressed per unit length (cm). Measurements were on 7 flounders in January and 6 each in May and September.



January / February
May / June
September / October
Kd (cm · min⁻¹)
4.0
3.0
2.0
1.0
Jmax (nmol · min⁻¹ · cm⁻¹)
20
10
Kt (nmol · L⁻¹)
10
CAECA
FOREGUT
MIDGUT
HINDGUT
INTESTINAL SECTION

Table 9. Values of t (df=2) for t-tests comparing the *in vivo* apparent diffusion constants of L-phenylalanine (K_d) in different sections of the intestine in units of area (cm^2). Apparent K_d was calculated by regression of the non-saturable portion of the uptake curve. Asterisks indicate significance at the 5% level. Data from Figure 23. (C = caeca, F = foregut, M = midgut, H = hindgut)

		C	M	H
Jan	F	7.70*	5.67*	3.81
	C	-	2.54	10.43*
	M	-	-	4.33
May	F	27.39*	6.72*	5.02*
	C	-	3.42	3.66
	M	-	-	0.58
Sept	F	3.22	0.70	25.05*
	C	-	2.02	16.86*
	M	-	-	5.95*

Table 10. Values of t (df = 2) for t-tests comparing the *in vivo* apparent diffusion constants (K_d) of L-phenylalanine in A: units of area (cm^2) and B: units of length (cm) from non-feeding (Jan), early feeding (May) and late feeding (Sept) periods. Apparent K_d was calculated by regression of the non-saturable portion of uptake. Asterisks indicate significance at the 5% level. Data from Figures 23 (A) and 24 (B).

		A		B	
		May	Sept	May	Sept
Caeca	Jan	475.45*	286.15*	17.71*	15.82*
	May	-	177.13*	-	10.61*
Foregut	Jan	32.89*	15.04*	6.97*	25.37*
	May	-	0.75	-	17.65*
Midgut	Jan	87.57*	1.10	12.39*	9.88*
	May	-	1.62	-	1.91
Hindgut	Jan	82.53*	4.35*	16.41*	16.95*
	May	-	1.01	-	11.27*

the pattern was similar (Fig. 24). The apparent K_d in January was lowest in the foregut, while the other sections did not differ (Table 11). The apparent diffusional constant was higher in May in all sections, with the caeca showing the smallest increase, and the hindgut the largest, becoming almost double that of the midgut and the foregut. By September, the values of apparent K_d were very similar in all sections, but the hindgut was significantly lower than the caeca. Differences in apparent diffusional constants were significant from January to May, and January to September, in all sections (Table 10). The difference was not significant between May and September only in the midgut.

4.2.4 Saturable Uptake

4.2.4.1 Rate of Uptake

Values of J_{max} expressed in units of area are presented in Figure 23. J_{max} was highest in the foregut in January (significantly higher than all other sections; Table 12), while it was lowest in the midgut. The J_{max} of the foregut was lower than the midgut and the hindgut in May, but not significantly so. No saturable component could be calculated for the caeca. By September, the J_{max} had declined, now significantly lower in the foregut than in the mid or hindgut. The highest J_{max} was recorded in the caeca, but the difference was not significant.

The effect of season differed with each section (Table 13). J_{max} declined from January to May to September in the foregut, but the change was not significant. J_{max} in September was significantly higher than in January in caeca,

Table 11. Values of t (df = 2) for t-tests comparing the *in vivo* apparent diffusion constants (K_d) of phenylalanine in units of length (cm) from different sections of the intestine. Apparent K_d was calculated by regression of the non-saturable portion of the uptake curve. Asterisks indicate significance at the 5% level. Data from Figure 24. (C = caeca, F = foregut, M = midgut, H = hindgut)

		C	M	H
	F	8.02*	8.65*	5.27*
Jan	C	-	0.10	1.06
	M	-	-	1.07
	F	2.50	0.19	7.57*
May	C	-	6.04*	13.97*
	M	-	-	10.58*
	F	3.41	1.24	2.29
Sept	C	-	1.00	4.75*
	M	-	-	2.12

Table 12. Values of t (df = 10) for t-tests comparing slopes (apparent K_t; above diagonal line) and adjusted intercepts (J_{max}; below diagonal line) of the active component of L-phenylalanine uptake per cm^2 area measured *in vivo*. Comparisons examine the effect of position in the intestine for non-feeding (January), early feeding (May) and late feeding (September) periods. Equations of the lines were obtained from Eadie-Hofstee transformations of the data. Asterisks indicate significance at the 5% level. May values for the slopes and intercepts from the caeca (nd) could not be calculated. Data from Figure 23.

		Caeca	Foregut	Midgut	Hindgut
Jan	Caeca		0.78	3.12*	2.00
	Foregut	6.71*		2.15	1.20
	Midgut	1.95	8.06*		1.65
	Hindgut	2.50*	4.42*	5.30*	
May	Caeca		nd	nd	nd
	Foregut	nd		3.76*	3.55*
	Midgut	nd	0.19		0.23
	Hindgut	nd	1.05	1.60	
Sept	Caeca		4.30*	2.84*	3.73*
	Foregut	1.37		2.47*	1.73
	Midgut	0.12	2.82*		1.30
	Hindgut	0.72	2.67*	1.36	

Table 13. Values of t (df = 10) for t-tests comparing slopes (apparent K_t; above diagonal line) and adjusted intercepts (J_{max}; below diagonal line) of the active component of L-phenylalanine uptake per cm^2 area measured *in vivo*. Comparisons are for seasonal effects on uptake. Equations of the lines were obtained from Eadie-Hofstee transformations of the data. Asterisks indicate significance at the 5% level. May values for the slope and intercept of the caeca (nd) could not be calculated. Data from Figure 23.

		Jan	May	Sept
	Jan		2.50*	7.31*
Foregut	May	1.66		3.86*
	Sept	1.22	0.56	
Caeca	Jan		nd	0.07
	Sept	4.47*	nd	
	Jan		3.22*	0.11
Midgut	May	2.84*		3.79*
	Sept	7.17*	0.78	
	Jan		2.78*	3.67*
Hindgut	May	1.96		4.60*
	Sept	2.39*	0.46	

midgut and hindgut. There was no significant difference between J_{max} in May and in September in midgut and hindgut. The J_{max} in May was highest in both of these sections, but was significantly higher in the midgut in May than in January; the difference was not significant in the hindgut.

Jmax, expressed in units of length, was affected differently by position in the intestine and season (Fig. 24). The foregut had a significantly higher J_{max} in January than any of the other sections, and all the sections were different from each other (Table 14). The J_{max} of the foregut in May was significantly higher than the midgut, but not the hindgut, and the midgut and hindgut differed. None of the sections differed from each other in September.

The midgut and hindgut both showed significant increases in J_{max} from January to May to September (Table 15). The J_{max} in foregut and caeca was significantly higher in September than in January, but in the foregut, there was no difference between January and May, or May and September.

4.2.4.2 Apparent Affinity for the Carrier

Values of the apparent K_t expressed in units of area are presented in Figure 23. Apparent K_t in January did not differ significantly between any of the sections (Table 12) except the midgut and caeca, where it was higher in the caeca. It rose in May in the hindgut and midgut, but declined in the foregut, where it was significantly lower than the other sections. Its value declined again in all three sections in September. The apparent K_t of foregut and

Table 14. Values of t (df = 10) for t-tests comparing slopes (apparent K_t; above diagonal line) and adjusted intercepts (J_{max}; below diagonal line) of the active component of L-phenylalanine uptake per cm length measured *in vivo*. Comparisons examine the effect of position in the intestine for non-feeding (January), early feeding (May) and late feeding (September) periods. Equations of the lines were obtained from Eadie-Hofstee transformations of the data. Asterisks indicate significance at the 5% level. May values of the slope and intercept for the caeca (nd) could not be calculated. Data from Figure 24.

		Caeca	Foregut	Midgut	Hindgut
Jan	Caeca		1.58	4.94*	2.54*
	Foregut	7.57*		3.26*	4.12*
	Midgut	4.41*	8.82*		3.42*
	Hindgut	3.88*	3.41*	10.99*	
May	Caeca		nd	nd	nd
	Foregut	nd		3.04*	3.58*
	Midgut	nd	2.65*		0.43
	Hindgut	nd	1.14	4.36*	
Sept	Caeca		5.50*	3.00*	4.49*
	Foregut	0.34		2.72*	0.40
	Midgut	0.27	1.37		2.23*
	Hindgut	0.44	0.49	1.34	

Table 15. Values of t (df = 10) for t-tests comparing slopes (apparent K_t; above diagonal line) and adjusted intercepts (J_{max}; below diagonal line) of the active component of L-phenylalanine uptake per cm length measured *in vivo*. Comparisons are for seasonal effects on uptake. Equations of the lines were obtained from Eadie-Hofstee transformations of the data. Asterisks indicate significance at the 5% level. May values of the slope and intercept for the caeca (nd) could not be calculated. Data from Figure 24.

		Jan	May	Sept
Foregut	Jan		1.99	4.87*
	May	1.58		4.11*
	Sept	2.72*	0.01	
Caeca	Jan		nd	2.21
	Sept	5.42*	nd	
Midgut	Jan		1.17	1.23
	May	5.67*		1.70
	Sept	8.02*	4.89*	
Hindgut	Jan		0.12	0.35
	May	3.91*		0.40
	Sept	5.97*	3.74*	

hindgut were not different in September, although the midgut differed from the foregut but not the hindgut. It was significantly higher in the caeca in September than in any of the other sections. The decline that occurred from January to May to September in the foregut (Fig. 23) was significant (Table 13), as was the marked increase from January to May in both the midgut and hindgut. However, while the decline in the apparent K_t of both the midgut and hindgut was significant from May to September, the value fell to the same as that for January in the midgut and below the January value for the hindgut. As in the midgut, the January and September apparent K_t values of the caeca were not different.

Apparent K_t values from data expressed in units of length (Fig. 24) were different between all sections (Table 14) except caeca and foregut in January, midgut and hindgut in May, and foregut and hindgut in September. There was no change with season in the caeca, midgut or hindgut (Table 15). However, the apparent K_t of the foregut was significantly lower in September compared with May or January.

4.2.5 Effective Thickness of the Unstirred Layer

The effective thickness of the unstirred layer of the flounder intestine, using NaCl as the test substrate, was measured in April and May, and thus is valid for non-feeding fish only (see section 2.5.4). The half-time to reach the new potential difference in the midgut ranged from 153 to 264 seconds (temperatures ranged from 6 to 12°C) giving a calculated effective thickness of the unstirred layer of 653 (± 58) um (n = 6) for the midgut. For the hindgut, half-times to reach the new potential difference ranged from 85 to 245 seconds (temperatures from 6 to 8°C) and the effective thickness of the unstirred layer was calculated to be 568 (± 97) um (n = 6). The value of K_t, corrected for the effective thickness of the unstirred layer (section 2.8.1, equation 4), was 2.4 mM (from 2.7 mM) for midgut, and 3.6 mM (from 4.3 mM) for hindgut.

4.2.6 Plasma Phenylalanine Concentrations

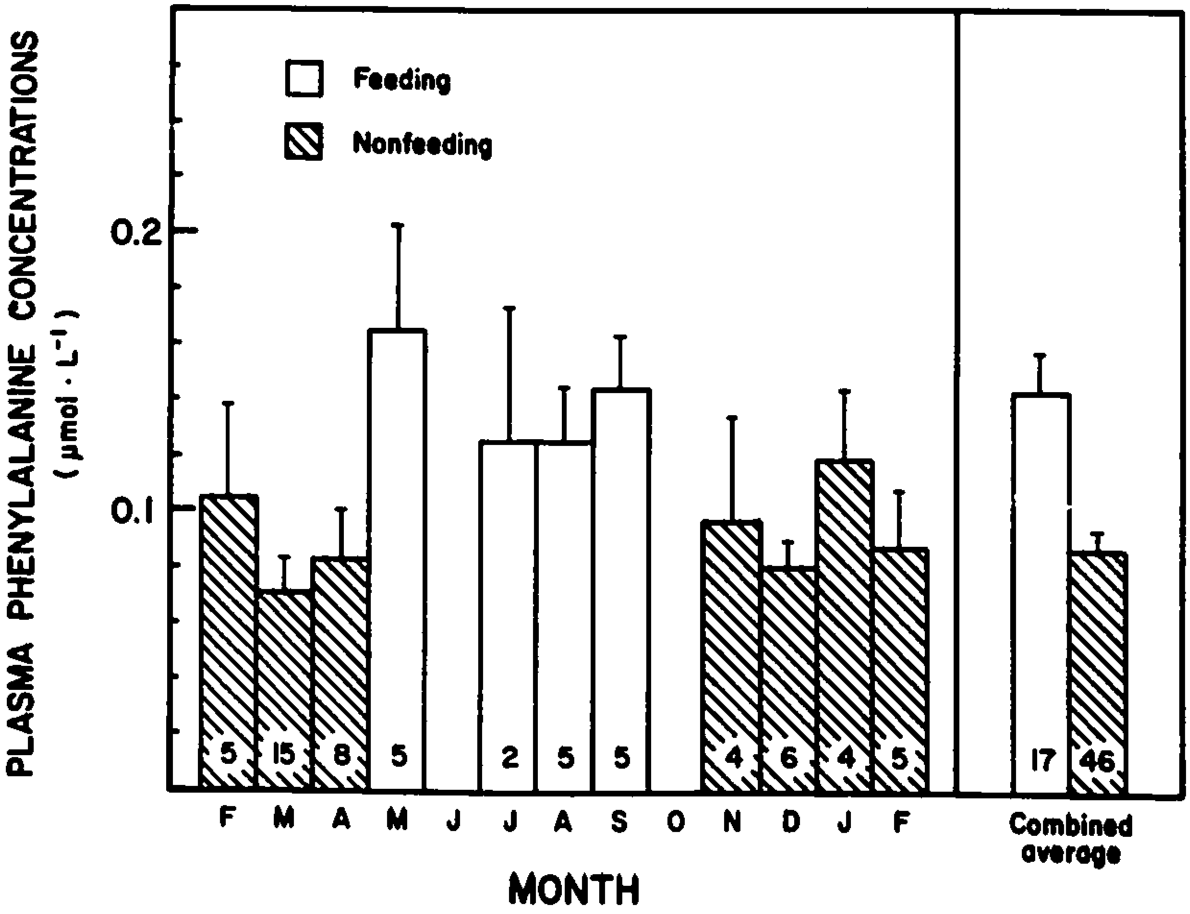
Plasma phenylalanine concentrations were measured in each month except June and October. During the months in which the flounders were not feeding, plasma phenylalanine concentrations were low, between 70 and 115 umol/l (Fig. 25). However, when the fish were feeding, plasma phenylalanine levels rose to 120 to 140 umol/l in July to September. Phenylalanine concentrations were highest in May, at 165 umol/l.

When the values for the months in which the fish were feeding were combined and averaged, then compared to the combined average for non-feeding fish, the feeding fish had plasma phanylalanine concentrations which were significantly higher than non-feeding fish.

4.3 Discussion

Values of the apparent K_t measured *in vivo* in feeding flounders were about 10 mM, or 40% lower than the value of 17 mM reported by Winne et al. (1979) for *in vivo* measurements of L-phenylalanine uptake in rats uncorrected for unstirred layer effects. The only *in vivo* values

Figure 25. Plasma phenylalanine concentrations (umol/l) for each month and combined averages for feeding and non-feeding flounders (mean ± SE). Number in the bar indicates number of fish assayed. L-phenylalanine was estimated through the use of the enzyme phenyalanine ammonia lyase (see section 2.7).



PLASMA PHENYLALANINE CONCENTRATIONS
(µmol · L⁻¹)
0.2
0.1
Feeding
Nonfeeding
5
15
8
5
2
5
5
4
6
4
5
17
46
F
M
A
M
J
J
A
S
O
N
D
J
F
Combined average
MONTH

available for transport of amino acids in fish are for glycine uptake in rainbow trout (Bogé et al., 1979), in which the apparent K_t for midgut and hindgut was 52 mM and 11 mM, respectively. These authors, however, neglected to correct their data for the passive component of transport or the unstirred layer, and therefore their values are probably over-estimations (Ferraris and Ahearn, 1984).

The effects of the unstirred layer on uptake are important, particularly in an *in vivo* study. The values found in the present study, 568 um for the hindgut and 653 um for the midgut, are in good agreement with the values found in the literature. In humans, the unstirred layer thickness was measured to be 632 um in healthy subjects by a method similar to that used in the present study (Read et al., 1977). Winne (1973) suggested that values as high as 1000 um might be expected in studies which use intestinal loops *in vivo*, and reported (1976) an unstirred layer thickness of about 0.5 mm for rat intestine. Mixing the perfusate with air to "stir" the solution *in vivo* in rats reduced the effective unstirred layer thickness by 500 um (Winne, 1978; Winne et al., 1979). Thus, the thickness must be approximately 500 um. That human intestine has a larger diameter than either rat or flounder intestine and yet the measured unstirred layer thickness is similar in all three, indicates that it is not diameter (or lumen size) which determines the unstirred layer thickness, but some other mucosal characteristics. The difference in the unstirred layer thickness between the midgut and hindgut in flounder probably reflects differences in the folding or the amount of mucus in the two regions.

Shephard (1982) reported that mucus presented a diffusional barrier to ions in the esophagus of the flatfish, *Rhombosolea retiaria*, and that this mucus layer could be as thin as 50 um, but was generally 600 to 1200 um thick. The relationship of mucus to the thickness of the unstirred water layer, and whether it contributes to it or is responsible for it, has not been established. In view of the finding in winter flounder that the intestinal structure changes and the number of goblet cells declines in winter, it is likely that seasonal changes in the thickness of the unstirred layer will occur.

Two factors prevented the measurement of the unstirred layer thickness throughout the year to examine seasonal changes (see section 2.5.4). All electrical measurements had to be performed in a different laboratory from that in which the surgery was performed, so the fish was subjected, in transport, to considerable stress. In addition, the water temperature was difficult to control, so that the fish was never subjected to temperatures less than 3°C higher than the ambient, and often considerably more. It was felt that these difficulties were too perturbing to allow accurate and reproducible measurements of unstirred layer thickness in winter flounder.

Although it was not measured, the effect of the unstirred layer cannot be ignored. Karasov and Diamond (1983a) suggested that even in *in vitro* experiments where the unstirred layer is substantially reduced, estimations of the apparent K_t are still over-estimations because it cannot be reduced to zero. Because the apparent K_t depends on both the J_{max} and the unstirred layer thickness, changes in apparent K_t may reflect changes in J_{max} rather than changes in K_t *per se*. Any change in J_{max} is, however, probably real (Karasov and Diamond, 1983a). If apparent K_t changes are in the same direction as J_{max}, then influences of the unstirred layer are probable and no firm conclusions can be drawn. Examination of the kinetic constants for the saturable portion of the uptake of L-phenylalanine in the present study (Fig. 23 and 24) shows that in almost every instance, apparent K_t and J_{max} changed in the same direction. Therefore, changes in apparent K_t cannot be used as reliable indicators of changes in the quality of the carrier mechanisms with season. While the carrier affinity cannot be assessed in this study, the maximal rate of uptake may be considered accurate. Changes in J_{max} will give an indication of any changes in carrier number with season, while the apparent K_d may be used as an indicator of altered membrane permeability.

J_{max} values were standardized to area (Fig. 23), which changed with season, and to length (Fig. 24), which did not. A change in maximal uptake rate per cm^2 would be indicative of changes in carrier number, while a change in J_{max} per cm is indicative of the change due to the change in surface area. The influence of temperature in the *in vivo* studies must also be considered. If temperature is assumed to be the only factor influencing uptake aside from the decreased surface area, then expected values of J_{max} can be calculated for May and September from January values, using the values of Q_{10} (for winter) found in the *in* *vitro* temperature study (Chapter 5). Expected values for foregut (Table 16) were much higher than the measured values in both May and September, suggesting that there was a decline in carrier number per cell in summer. Conversely, this may be stated as an increase in relative carrier number in winter. A similar pattern, though less marked, occurred in the hindgut. The measured J_{max} was only slightly lower than expected in May, suggesting that carrier numbers were maintained in this section at the beginning of feeding, but declined as the feeding period progressed. Relatively high numbers of carriers in the hindgut would be important if, as Ferraris and Ahearn (1984) suggest, it behaves as a scavenger. If marine fish are less effective than freshwater fish at absorbing free amino acids (Dabrowski et al., 1986), then relatively high numbers of carriers in the hindgut at a time when reserves are depleted and feeding has resumed would be adaptive. Slightly more carriers may be present in the midgut in May and slightly fewer in September, suggesting that there is little change in carrier number with season in this section, and that the changes in J_{max} are largely attributable to temperature and surface area changes. The same appears to be true in the caeca.

The above conclusions have been made based on the assumption that the ability of the carrier to transport substrate does not change with season. However, the same effect as a decrease in carrier number could be achieved by a reduction in the efficiency of existing carriers, possibly through alterations in membrane composition. Changes in membrane composition with adaptation to new temperatures have

Table 16. Expected values of J_{max} for A: May and B: September calculated from January measured values, based on Q_{10} values calculated for 3 to 15°C from winter *in vitro* studies (Chapter 5). Measured values are those obtained in the *in vivo* uptake studies for the period indicated. J_{max} could not be calculated for the caeca in May.

A: May

	J_{max}		
Section	Measured (January)	Expected (May)	Measured (May)
Caeca	4.43	-	-
Foregut	8.94	48.38	5.46
Midgut	1.89	6.29	11.56
Hindgut	4.99	24.30	17.51

B: September

	J_{max}		
Section	Measured (January)	Expected (Sept)	Measured (Sept)
Caeca	4.43	10.05	12.13
Foregut	8.94	84.95	3.31
Midgut	1.89	9.38	7.35
Hindgut	4.99	37.59	5.23

been documented (Smith and Kemp, 1971), but the effects on the carriers themselves has received no attention.

Similar calculations can be performed for the apparent diffusional constant (Table 17). The expected value of the apparent K_d is lower in every section in both May and September than the measured value. These results suggest that there was an increase in membrane permeability to phenylalanine in the summer feeding period. Again, this may be due to changes in membrane constituents.

No proximodistal gradient was observed for the apparent diffusion constant, nor for J_{max} (Figs. 23 and 24).

Plasma phenylalanine concentrations were lowest when the fish were not feeding (Fig. 25). They showed a peak in May, corresponding to the onset of feeding. Plasma phenylalanine levels, therefore, reflected the nutritional status of the fish. Squires et al. (1979) observed a similar pattern for plasma phenylalanine levels in winter flounder caught off Newfoundland, but the peak in plasma levels was in July. These differences probably reflect population differences. It is not known if amino acids exert feedback control on their uptake. Karasov and Diamond (1983a) suggested that uptake of glucose, which is not essential, but serves as a source of calories, will be regulated by the caloric requirements of the body. Uptake of nutrients which are essential, such as calcium or iron, is controlled by a complex feedback system related to their levels in the body. In the case of amino acids, the situation is less clear. Some amino acids are essential and might be expected to be regulated by their own levels. Amino acids are also considered to be essential as

Table 17. Expected values of apparent K_d for A: May and B: September calculated from January measured values, based on Q_{10} values calculated for 3 to 15°C from winter *in vitro* studies (Chapter 5). Measured values are those obtained in the *in vivo* uptake studies for the period indicated.

A: May

	Apparent K_d		
Section	Measured (January)	Expected (May)	Measured (May)
Caeca	0.55	0.53	0.83
Foregut	0.24	0.24	1.71
Midgut	0.48	0.81	1.13
Hindgut	0.39	0.34	1.20

B: September

	Apparent K_d		
Section	Measured (January)	Expected (Sept)	Measured (Sept)
Caeca	0.54	0.51	1.43
Foregut	0.24	0.24	1.78
Midgut	0.48	0.97	1.58
Hindgut	0.39	0.33	1.02

nitrogen sources. In addition, they provide calories. Intestinal amino acid uptake, therefore, may be selectively regulated with respect to essential amino acids, or loosely regulated to obtain as much as possible from the intestine, with more selective regulation elsewhere. Obviously, in the present study, the flounder intestine responded to low levels of body phenylalanine, resulting in peak uptake in May concomitant with the highest plasma levels. Whether the response seen here after winter depletion was due to the need for calories, the need for nitrogen, the need for essential amino acids or a combination of the three remains to be examined.

Chapter 5

In vitro Uptake of L-Phenylalanine

5.1 Introduction

In vitro uptake studies on stripped mucosal sheets allow the assessment of the mechanism of transport and external factors which affect it. Temperature effects on the mucosa are far-reaching, involving changes in fatty acid composition, enzyme activities (Smith and Kemp, 1971) and protein synthesis (Morris and Smith, 1967). In goldfish, phenylalanine was found to be one of a group of lipophilic amino acids which were termed non-adaptors; that is, they showed no change in transmural accumulation from fish adapted to different temperatures (Smith, 1970), but uptake changed in the same direction as temperature. Uptake *in vivo* could not be performed at a constant temperature throughout the year without considerable additional stress to the flounder, so an investigation of the effect of temperature on phenylalanine uptake was obviously necessary, and the *in vitro* preparation was the method of choice for such an investigation. Temperature effects on stripped mucosal sheets from summer (feeding) and winter (non-feeding) flounders were examined. It is predicted that the response to temperature will be different in fish acclimatized to different temperatures if membrane constituents change. The linear, non-saturable portion of uptake (K_d) should reflect any such changes in the membrane.

Fish intestines appear to require sodium and specifically the energy of the sodium gradient for amino acid transport in the same way as mammalian intestines (Eveloff et al., 1980; Ferraris and Ahearn, 1984). They also appear to possess a sodium-independent, non-saturable component of uptake (Ferraris and Ahearn, 1984). Changes in sodium-dependence or in the relative contribution of the sodium-dependent and -independent components could conceivably be part of the adaptive processes of seasonal change in the flounder intestine. Thus, the effect of sodium ions on the uptake of phenylalanine was examined in winter and summer fish.

Regional differences have been noted in function of mammalian and fish intestine. The carriers for neutral amino acids in rabbit ileum appear to differ in specificity from those present in the jejunum (King et al., 1981; Stevens et al., 1982). Competition studies were used to establish the existence of regional carrier differences in the flounder intestine.

Few studies have established carrier specificity in fish (Ferraris and Ahearn, 1984). Alanine uptake by winter flounder brush border membrane vesicles is inhibited by phenylalanine (Eveloff et al., 1980) so alanine was an obvious choice as a potential competitive inhibitor of phenylalanine uptake. In addition, alanine is an important component of the antifreeze peptide produced by winter flounder (Scott et al., 1986), and thus its uptake may be favoured. In rat small intestine, L-alanine uptake was competitively inhibited by L-leucine, L-methionine and L-phenylalanine, suggesting that they may share a common transport mechanism. However, two mechanisms for methionine uptake have been hypothesized in goldfish (Kitchin and Morris, 1971). If this is the case, and the second system is shared by phenylalanine as in rabbit jejunum (Stevens et al., 1982), then the inhibition of phenylalanine uptake by a mixture of methionine and leucine should be more than the average inhibition caused by the two amino acids separately. In hamster small intestine, L-histidine uptake is inhibited by methionine and phenylalanine (Navab et al., 1984), and therefore the effect of histidine was also examined. Arginine, which is probably transported by the basic amino acid carrier, should have no effect on the uptake of phenylalanine. Taurine, a prevalent dietary component of marine fish, has been shown to be transported in winter flounder intestine by the β-alanine transporter and not by an A- or ASC-like transporter (King et al., 1986). If this is the case, neither inhibition of phenylalanine uptake by taurine should be observed nor greater inhibition of phenylalanine uptake by a mixture of taurine and leucine than is observed in the presence of taurine alone.

5.2 Results

The *in vitro* uptake of L-phenylalanine was estimated using mucosal sheets stripped of their musculature. These sheets were mounted on glass tubes (Fig. 5) and immersed in solutions of labelled phenylalanine. Solutions were stirred with a magnetic stir bar and vigourously bubbled with oxygen to reduce the unstirred layer (see Ch. 2, section 2.6).

5.2.1 Apparent Diffusional Uptake

Values of the apparent diffusional constant of phenylalanine uptake (K_d) measured at three temperatures (15, 8 and 3°C) and in summer (feeding) and winter (non-feeding) flounder intestines are presented in Figure 26. The apparent diffusion constant in the summer was significantly higher (Table 18) at all temperatures in the anterior portion of the intestine (caeca and foregut), except at 8°C in the caeca. At that temperature, the apparent K_d was the same as that measured in the midgut, and was lower than that observed in the foregut. The apparent K_d in winter was highest in the caeca at all temperatures (Table 18, Fig. 26), and declined from caeca to foregut to midgut to hindgut. At 15°C, the apparent K_d in foregut and midgut was the same.

The effect of temperature on the apparent K_d differed with season, and in each section (Table 19). The apparent diffusion constant in the caeca in summer was the same at 15 and 3°C but significantly lower at 8°C, whereas, in winter, the apparent K_d was highest at 8°C and lowest at 15°C. The apparent K_d in the foregut in summer declined as temperature declined. In winter, however, the apparent K_d at 15 and 3°C was the same and lower than at 8°C. The apparent K_d in the midgut declined with temperature in both winter and summer, although in summer, the uptake at 8 and 3°C was statistically the same. In hindgut, the apparent K_d in summer was highest and the same at 8 and 15°C, whereas in winter it was highest at 8°C and the same at 3 and 15°C.

The apparent K_d was generally higher in summer than

Figure 26. Effect of temperature and season (feeding or non-feeding) on the apparent diffusion constant (K_d) of L-phenylalanine measured *in vitro* (mean ± SE). Measurements are from 6 flounder for caeca, foregut and midgut at 15°C (feeding), and 5 fish for all others.

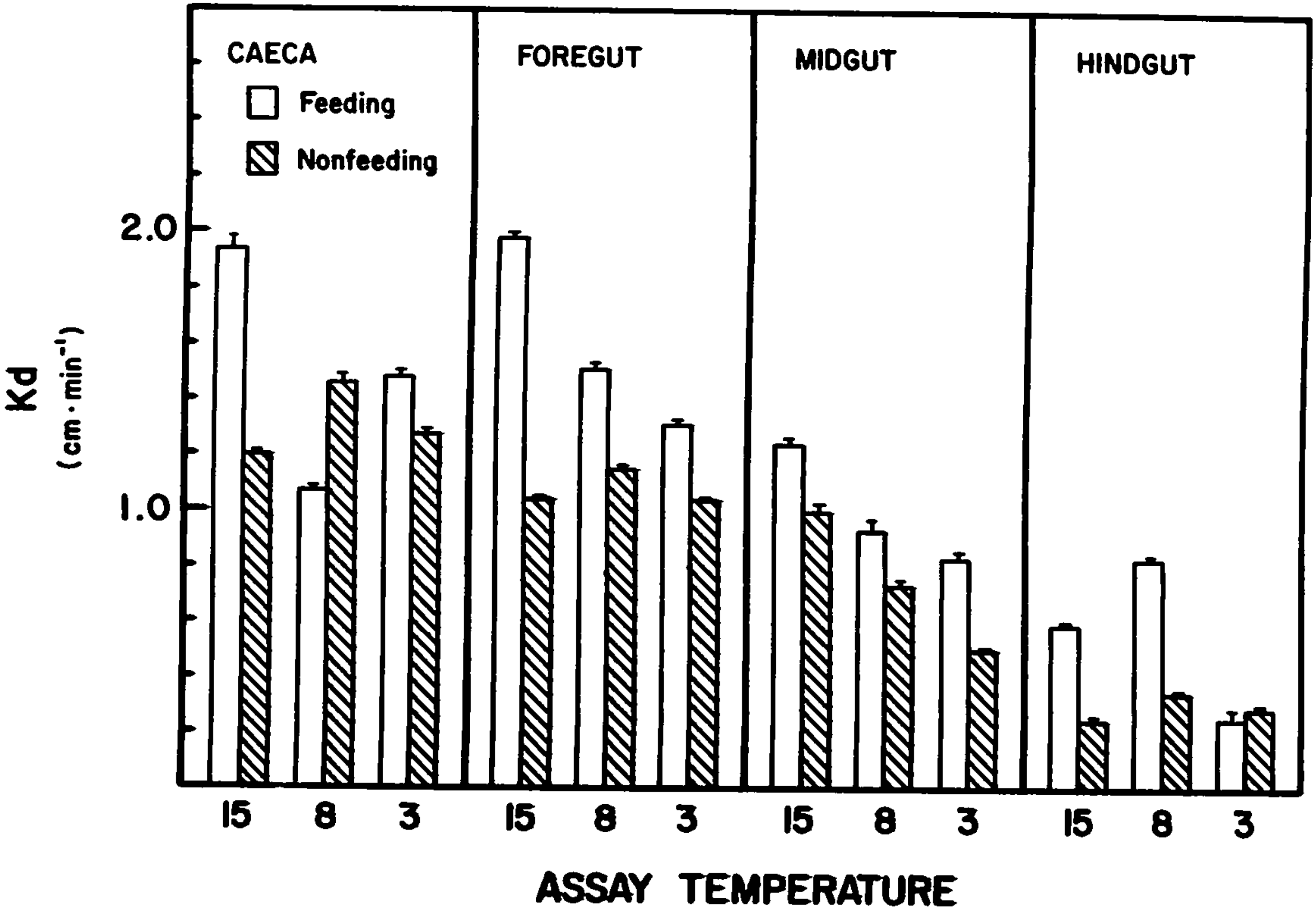

CAECA
FOREGUT
MIDGUT
HINDGUT
Feeding
Nonfeeding
Kd
(cm · min^{-1})
2.0
1.0
15
8
3
15
8
3
15
8
3
15
8
3
ASSAY TEMPERATURE

Table 18. Values of t (df = 2) for t-tests comparing the apparent diffusion constants (K_d) of L-phenylalanine measured *in vitro* from different sections of the intestine. Apparent K_d was calculated by regression on the non-saturable portion of uptake. Asterisks indicate significance at the 5% level. Data from Figure 26. (C = caeca, F = foregut, M = midgut, H = Hindgut)

		Summer (Feeding)			Winter (Non-feeding)		
T°C		C	M	H	C	M	H
	F	0.84	25.61*	72.30*	12.02*	1.44	52.89*
15	M	5.60*	-	24.37*	7.03*	-	25.80*
	H	10.94*	-	-	51.78*	-	-
	F	26.33*	14.11*	40.33*	9.91*	26.64*	67.00*
8	M	3.94	-	2.33	22.43*	-	25.71*
	H	70.23*	-	-	36.22*	-	-
	F	6.89*	16.13*	29.24*	23.62*	256.19*	136.13*
3	M	18.02*	-	12.61*	71.98*	-	38.82*
	H	29.55*	-	-	82.28*	-	-

Table 19. Values of t (df = 2) for t-tests comparing apparent diffusion constants (K_d) at 3, 8 and 15°C in each section of intestine. A: Effect on uptake in summer (feeding). B: Effect on uptake in winter (non-feeding). Asterisks indicate significance at the 5% level. Data from Figure 26. (C = caeca, F = foregut, M = midgut, H = hindgut)

		A		B	
	T°C	8	3	8	3
C	15	7.00*	3.67	8.12*	6.99*
	8	-	17.83*	-	4.54*
F	15	21.40*	34.81*	10.74*	1.32
	8	-	10.50*	-	11.64*
M	15	6.73*	10.76*	9.20*	19.44*
	8	-	2.20	-	18.37*
H	15	3.09	9.44*	6.36*	3.06
	8	-	16.73*	-	5.90*

in winter in all sections (Table 20, Fig. 26) when uptake at the same temperature was compared. The only exceptions were in the caeca at 8°C, where the winter apparent K_d was higher, and in the hindgut, where, at 3°C, the apparent K_d was the same.

Values of Q_{10} between 3 and 15°C for the apparent diffusion constant for caeca, foregut, midgut and hindgut were, respectively, 1.25, 1.41, 1.40 and 2.02 for summer, and 0.95, 1.00, 1.78 and 0.87 for winter experiments.

5.2.2 Rate of Uptake

The maximal uptake rate in summer at 15°C was not significantly different in any of the sections except that the foregut was higher than the hindgut (Fig. 27; Table 21). The J_{max} of the midgut and hindgut at 8°C were significantly higher than that of the caeca, but did not differ from each other or from the foregut. The J_{max} of the caeca at 3°C was significantly lower than that of the hindgut and the foregut. There were no other significant differences.

J_{max} at 15°C in winter was significantly higher in the foregut than in the other sections, which did not differ from each other. The caeca had the highest J_{max} at 8°C, although the difference was only significant between caeca and hindgut. The J_{max} of the hindgut was also significantly lower than that of the midgut, but not the foregut. The caeca had the highest J_{max} at 3°C, while that of the foregut and the midgut were the same, and that of the hindgut was lowest.

Table 20. Values of t (df = 2) for t-tests comparing summer (feeding) and winter (non-feeding) apparent diffusion constants (K_d) of phenylalanine measured *in vitro* at each temperature within each section. Asterisks indicate significance at the 5% level. Data from Figure 26. (C = caeca, F = foregut, M = midgut, H = hindgut)

			SUMMER		
			15°C	8°C	3°C
WINTER	C	15	6.00*	9.77*	11.04
		8	3.81	12.81*	0.62
		3	5.06*	20.72*	6.62*
	F	15	51.58*	27.36*	24.49*
		8	43.08*	19.28*	12.18*
		3	53.31*	28.34*	27.23*
	M	15	6.58*	1.53	4.54*
		8	17.63*	4.84*	2.83
		3	28.99*	11.31*	11.30*
	H	15	21.96*	69.90*	0.19
		8	22.30*	57.99*	2.63
		3	32.80*	90.85*	1.08

Figure 27. Effect of temperature and season (feeding or non-feeding) on the maximal uptake rate (J_{max}) of L-phenylalanine measured *in vitro* (mean ± SE). Numbers of fish as described for Figure 26.

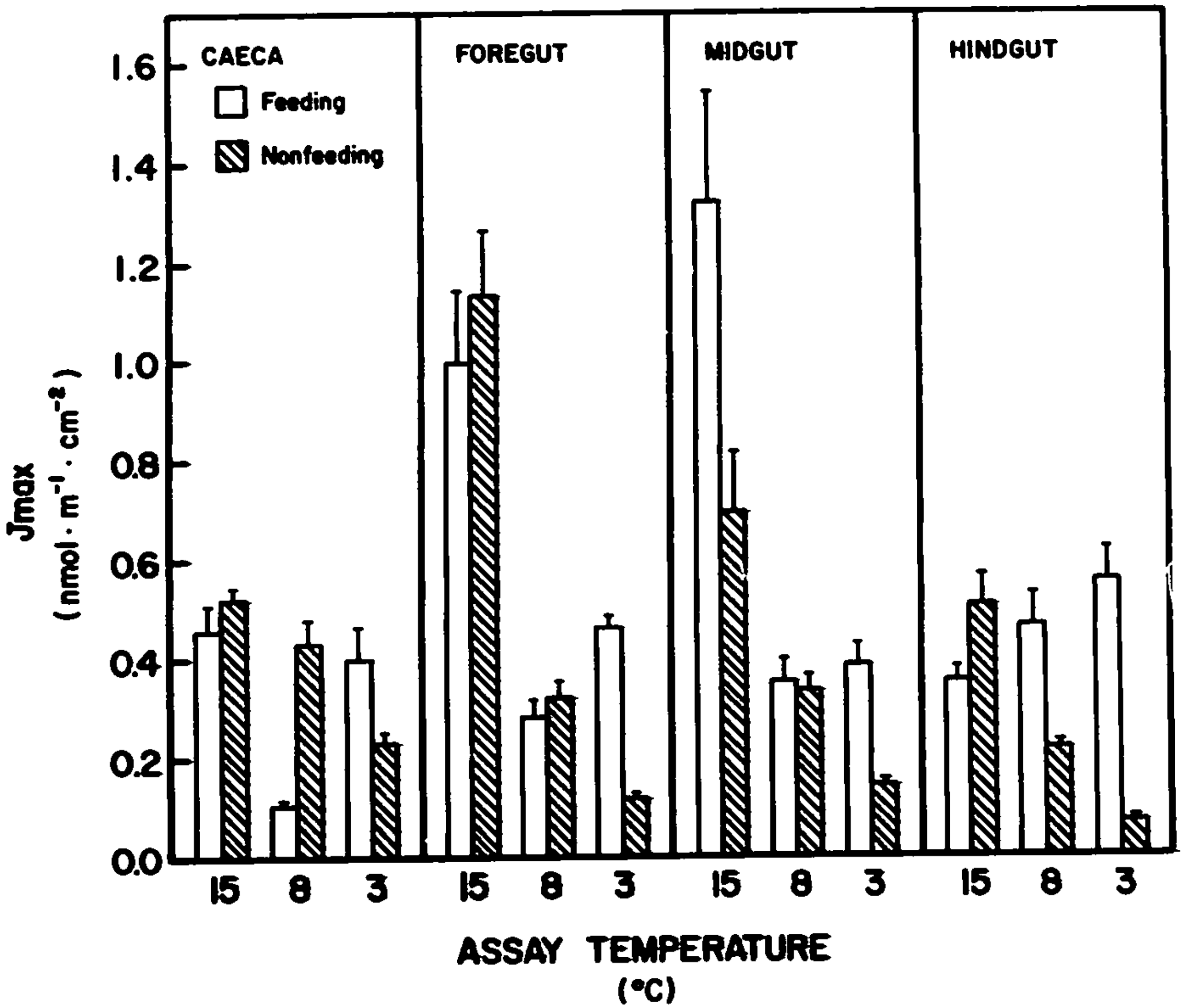

CAECA
FOREGUT
MIDGUT
HINDGUT
Feeding
Nonfeeding
Jmax
(nmol · m⁻¹ · cm⁻²)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15
8
3
15
8
3
15
8
3
15
8
3
ASSAY TEMPERATURE
(°C)

Table 21. Values of t (df = 8) for t-tests comparing slopes (apparent K_t; above diagonal line) and adjusted intercepts (J_{max}; below diagonal line) of saturable uptake of phenylalanine measured *in vitro* for different sections of the intestine. Asterisks indicate significance at a 5% level. Uptake of phenylalanine was measured at 15, 8 and 3°C. Data from Figures 27 and 28. (C = caeca, F = foregut, M = midgut, and H = hindgut)

Temperature = 15°C

		Summer				Winter			
		C	F	M	H	C	F	M	H
Summer	C		1.50	3.05*	0.08	3.68*	1.40	2.10	1.69
	F	1.86		1.56	1.42	0.67	0.36	0.51	0.24
	M	1.29	0.01		2.92*	0.81	2.05	0.86	1.76
	H	1.57	2.73*	1.95		5.70	1.33	2.22	2.09
Winter	C	1.11	3.26*	3.29*	0.16		1.09	0.14	1.91
	F	3.53*	1.18	0.88	4.69*	5.01*		0.85	0.05
	M	0.001	1.92	1.95	0.77	1.35	3.21*		0.93
	H	0.60	2.63*	2.18	0.64	1.07	4.50	0.46	

Table 21. continued

Temperature = 8°C

		Summer				Winter			
		C	F	M	H	C	F	M	H
Summer	C		2.99*	2.65*	2.31*	2.24	1.76	2.67*	2.45*
	F	2.27		0.29	0.38	0.06	1.10	0.85	2.14
	M	2.55*	0.88		0.12	0.34	1.34	1.10	2.10
	H	3.32*	2.17	1.34		0.47	1.39	1.15	1.94
Winter	C	4.08*	2.54*	1.55	0.08		1.05	0.74	1.72
	F	4.50*	1.66	0.65	0.68	0.89		0.44	0.79
	M	4.87*	2.01	0.79	0.71	0.95	0.04		1.53
	H	5.24*	0.07	0.72	1.83	2.33*	2.04	2.33*	

Temperature = 3°C

		Summer				Winter			
		C	F	M	H	C	F	M	H
Summer	C		2.30	2.27	1.10	2.30	3.23*	2.87*	2.81*
	F	2.54*		0.91	1.37	0.52	2.96*	2.00	2.30
	M	1.61	0.74		1.80	0.24	1.19	0.66	0.96
	H	2.56*	0.02	0.46		1.24	2.37*	1.87	1.75
Winter	C	0.29	6.46*	3.61*	3.52*		2.14	1.35	1.95
	F	1.23	7.72*	5.43*	4.29*	2.49*		1.02	0.49
	M	1.16	8.51*	5.33*	4.46*	2.32*	0.75		1.33
	H	2.41*	11.25*	7.20*	6.17*	5.05*	4.60*	4.82*	

J_{max} of the caeca of summer fish declined significantly from 15 to 8°C, but rose again at 3°C (Fig. 27; Table 22). The value of J_{max} at 3°C was not different from that at 8 or 15°C. In winter, J_{max} values did not differ regardless of temperature. When summer and winter uptake rates were compared at the same temperature, there was a difference due to season only at 8°C.

The J_{max} in the foregut in summer at 15 and 3°C did not differ (Fig. 27; Table 22) while that at 8°C was different from both. In winter, the J_{max} measured at each temperature was different from the others. There was no effect of season at 15 or 8°C, but at 3°C, the winter J_{max} was significantly lower than that of summer.

A similar pattern to that in the caeca was seen in the midgut in summer. The value of J_{max} at 8°C was significantly lower than that at 15°C, while the value at 3°C differed from neither (Fig. 27; Table 22). However, in winter, the pattern was the opposite, with J_{max} at 8°C no different from that at 15°C, while those at 3 and 15°C differed, as did those at 8 and 3°C. No difference due to season was observed at 15 or 8°C, but the winter J_{max} at 3°C was significantly lower than that of summer.

There was no effect of temperature on J_{max} in the hindgut in the summer, while there was a significant effect in winter (Table 22). Once again, the effect of season was observed only at 3°C, where the winter J_{max} value was lower than that of summer (Fig. 27).

Table 22. Values of t (df = 8) for t-tests comparing the slopes (apparent K_t, above the diagonal line) and the adjusted intercepts (J_{max}, below the diagonal line) of the saturable component of *in vitro* phenylalanine uptake at 3, 8 and 15°C. J_{max} and apparent K_t were obtained from Eadie-Hofstee transformed saturable uptake data (nmols x min^{-1} x cm^{-2}). Asterisks indicate significance at the 5% level. Data from Figures 27 and 28.

CAECA

			Summer			Winter	
	T°C	15	8	3	15	8	3
Summer	15		0.77	1.97	3.68*	1.46	0.03
	8	6.02*		2.87*	7.68*	2.24	1.57
	3	2.01	1.69		0.07	1.16	2.30
Winter	15	1.11	3.89*	2.23		2.07	5.25*
	8	1.38	4.08*	1.43	0.003		1.47
	3	3.85*	3.70*	0.29	2.05	2.18	

FOREGUT

			Summer			Winter	
	T°C	15	8	3	15	8	3
Summer	15		0.18	1.24	0.36	0.86	1.45
	8	4.33*		1.49	0.01	1.10	3.52*
	3	2.09	5.20*		1.08	0.22	2.96*
Winter	15	1.18	6.43*	4.00*		0.69	1.44
	8	3.55*	1.66	3.82*	5.68*		2.35*
	3	4.70*	1.50	7.72*	6.95*	3.40*	

Table 22. continued

MIDGUT

		Summer			Winter		
	T° C	15	8	3	15	8	3
Summer	15		1.19	3.12*	0.86	2.00	2.21
	8	3.74*		1.86	0.60	1.10	2.64*
	3	1.52	2.00		2.28*	1.27	0.66
Winter	15	1.95	2.18	0.33		1.42	2.05
	8	2.96*	0.79	2.01	1.59		2.55*
	3	4.00*	1.80	5.33*	2.85*	3.69*	

HINDGUT

		Summer			Winter		
	T° C	15	8	3	15	8	3
Summer	15		2.09	1.75	2.09	0.02	1.55
	8	0.24		0.50	0.38	1.94	2.24
	3	1.22	1.49		0.17	1.42	1.75
Winter	15	0.64	0.97	0.67		1.82	2.19
	8	4.33*	1.83	3.67*	3.24*		3.12*
	3	8.44*	4.21*	6.17*	5.95*	6.84*	

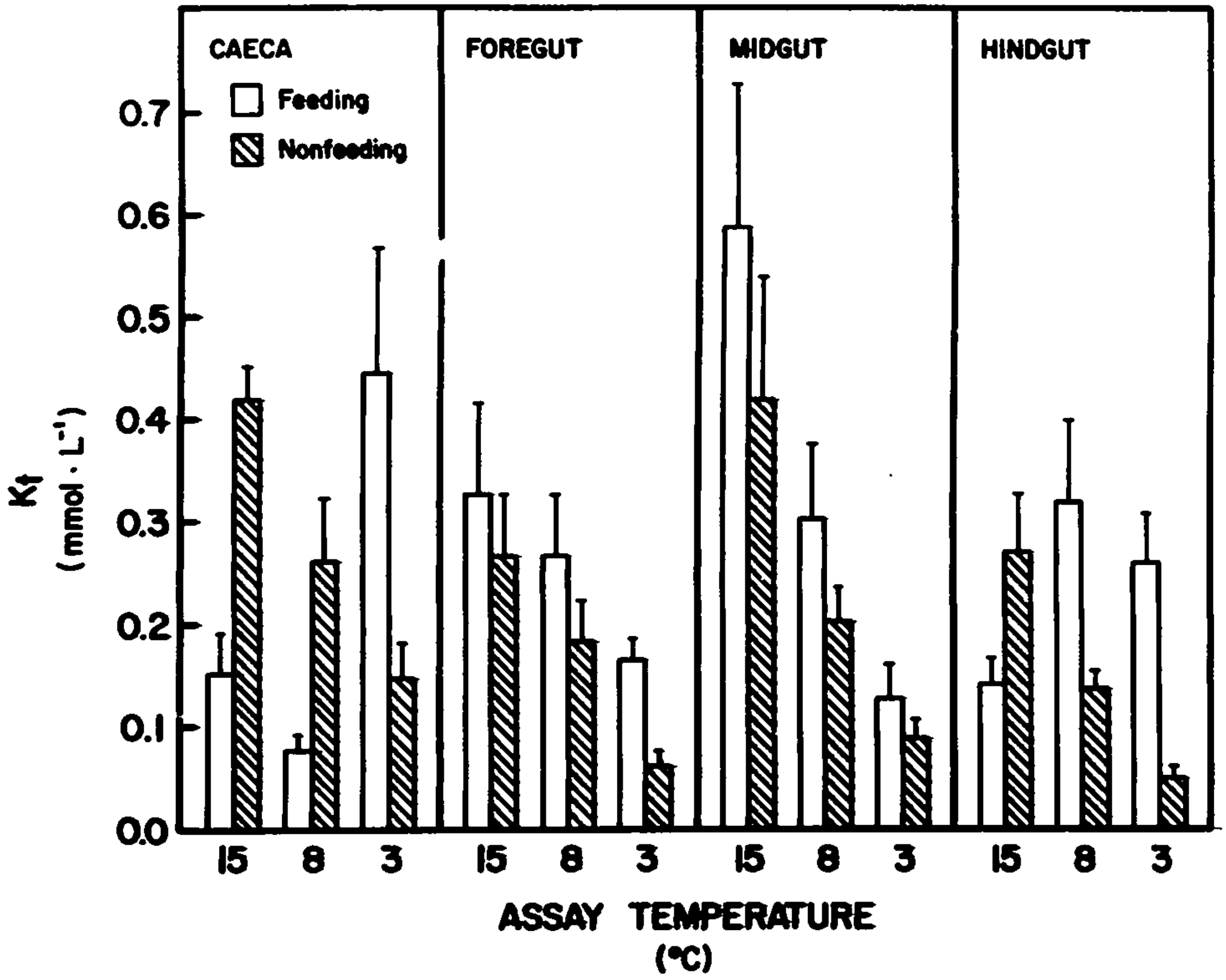
Values of Q_{10} for J_{max} were calculated for summer and winter over the temperature range from 3 to 15°C. Values of the Q_{10} calculated for caeca, foregut, midgut and hindgut were, respectively, 1.12, 1.89, 2.82 and 0.68 for summer, and 1.98, 6.53, 3.80 and 5.38 for winter.

5.2.3 Apparent Michaelis Constant

Examination of the affinity constants showed that the intestinal sections behaved differently in summer than in winter (Fig. 28). At 15°C in summer, the apparent K_t was significantly lower in the caeca than in the midgut (Table 21), as was that of the hindgut. The apparent K_t of the caeca at 8°C was significantly lower than that of the other sections, and the other sections did not differ from each other. At 3°C, the apparent K_t had increased in the caeca, but was not different from that of the foregut, midgut or hindgut. The apparent K_t of the sections was the same at each temperature in the winter animals.

The apparent K_t of caeca during the summer at 3°C was significantly higher than that at 8°C, but there were no other differences (Fig. 28; Table 22). In winter, the apparent K_t at 3°C was significantly different from that at 15°C, but not from 8°C, and that at 8°C did not differ from that at 15°C. Only at 15°C was there a significant effect of season on the apparent K_t in the caeca, with the summer values lower than the winter value. The values at 8 and 3°C, however,

Figure 28. Effect of temperature and season (feeding and non-feeding) on the apparent carrier affinity (K_t) of L-phenylalanine measured *in vitro* (mean ± SE). Numbers of fish as described for Figure 26.


CAECA
Feeding
Nonfeeding
FOREGUT
MIDGUT
HINDGUT
K_t (mmol · L^{-1})
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
15
8
3
15
8
3
15
8
3
15
8
3
ASSAY TEMPERATURE (°C)

were very close to significance.

The apparent K_t for phenylalanine in the foregut from summer, feeding fish showed no temperature effect (Fig. 28; Table 22). In winter, the apparent K_t at 8°C was significantly higher than at 3°C, but there were no other differences. There was no significant effect of season on the apparent K_t at 15 or 8°C, but the value at 3°C was significantly lower in winter than in summer.

The apparent K_t in the midgut was significantly lower at 3°C compared to 15°C, but did not differ from 8°C (Fig. 28; Table 22). In winter, the value of the apparent K_t was different at 8 compared to 3°C. While lower at 3°C, the difference was not significant between 3 and 15°C. There was no effect of season on the apparent K_t in the midgut.

There was no effect of temperature on the apparent K_t in the hindgut in summer (Fig. 28; Table 22). However, in winter, the same pattern that was observed in the foregut and midgut occurred, with the apparent K_t at 3°C significantly lower than 8°C but not lower than 15°C. As in the midgut, there was no significant effect of season.

5.2.4 Effect of Removal of Sodium

The saturable component of phenylalanine uptake disappeared in the absence of sodium, leaving only the linear, apparent diffusional component. Linear regression of these data (Table 23) result in intercepts which are not significantly different from zero. Because the intercepts of

Table 23. Values of the slope (apparent K_d) and intercept of the linear regression for the *in vitro* uptake of L-phenylalanine in the absence of sodium measured in summer (feeding) and winter (non-feeding) fish. In the absence of sodium, the saturable component is abolished, leaving only the linear, non-saturable portion. Intercepts were not significantly different from zero.

		Slope (K_d)	Intercept	r
Summer	Caeca	2.36	0.07	0.99
	Foregut	2.26	0.09	0.99
	Midgut	1.11	0.20	0.99
	Hindgut	0.57	0.03	0.99
Winter	Caeca	1.91	0.08	0.99
	Foregut	2.15	0.03	0.99
	Midgut	1.43	0.09	0.99
	Hindgut	0.52	0.03	0.99

the lines for the diffusional components reflect the J_{max} of the active component, an intercept that is not different from zero indicates no active component.

The absence of sodium from the bathing medium affects the apparent K_d of phenylalanine uptake when measured at 15°C (Fig. 29). Sodium-free phenylalanine uptake in summer was highest in caeca, followed by the foregut, then midgut and hindgut. Uptake in the absence of sodium in winter was highest in foregut, then caeca, midgut and hindgut. These differences are significant (Table 24). Sodium-free uptake in winter was higher in all sections of the intestine than uptake in the presence of sodium (Table 25, column 2). It was higher in summer in the caeca and foregut, but there was no difference in uptake between sodium-free and sodium-containing media in the midgut or hindgut (Table 25, column 1).

Uptake from sodium-free solutions was significantly higher in caeca, foregut and hindgut in summer than in winter (Table 25, column 3). In the midgut, sodium-free uptake was higher in winter than summer.

5.2.5 Effect of Competitive Inhibitors

The presence of other amino acids on the uptake of L-phenylalanine was different in the different sections of the intestine (Fig. 30). All of the amino acids tested in the caeca, except arginine, significantly inhibited phenylalanine uptake. Only alanine and the combination of taurine and leucine significantly inhibited phenylalanine uptake in the foregut. No amino acids significantly inhibited phenylalanine uptake in the midgut and hindgut. Uptake in the presence of

Figure 29. Effect of sodium-free medium on the apparent diffusional component of L-phenylalanine uptake measured *in vitro* (mean ± SE, n = 5). Both feeding and non-feeding uptake was measured at 15°C.

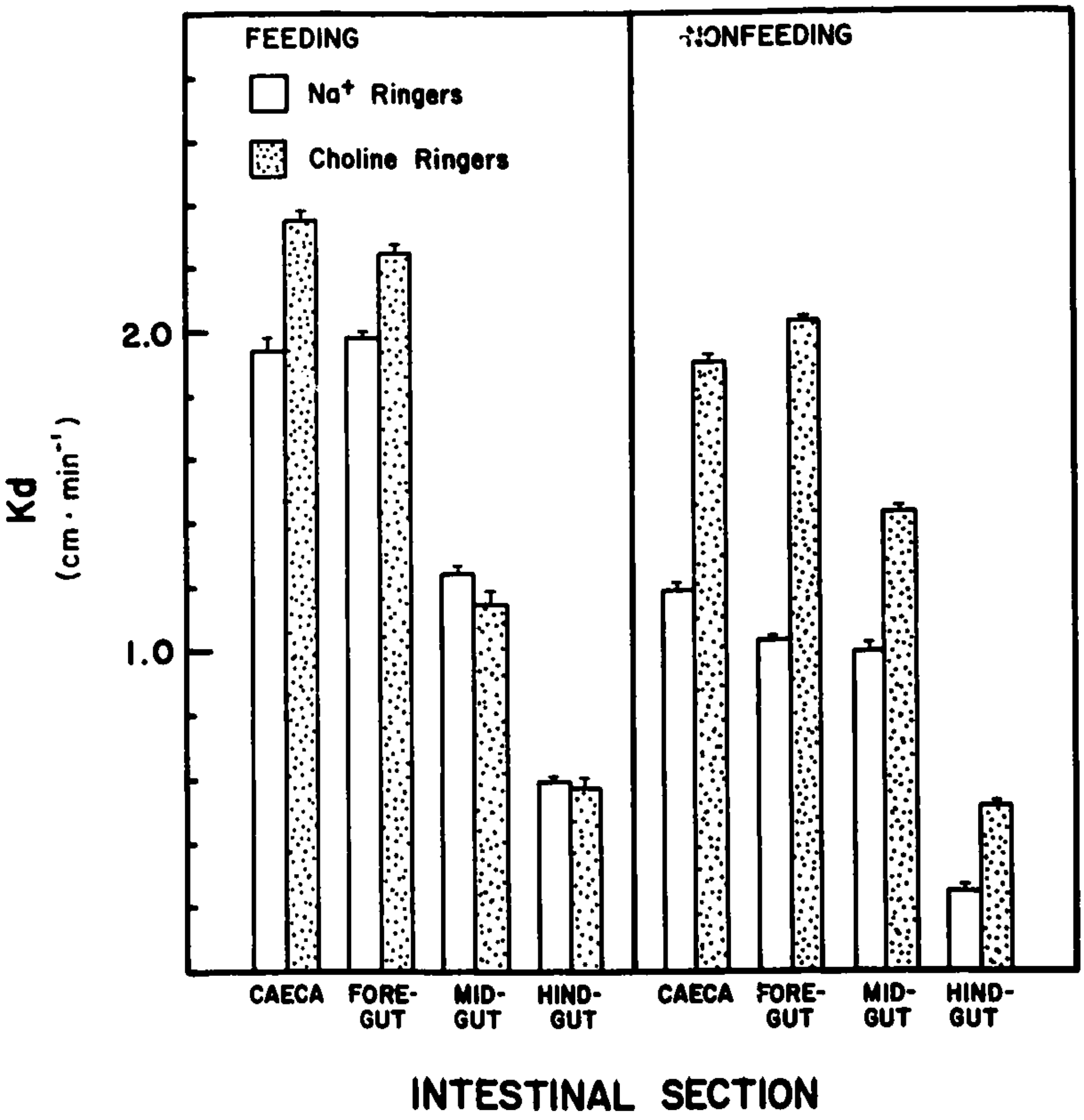

FEEDING
NONFEEDING
Na+ Ringers
Choline Ringers
Kd
(cm · min-1)
2.0
1.0
CAECA
FORE-GUT
MID-GUT
HIND-GUT
CAECA
FORE-GUT
MID-GUT
HIND-GUT
INTESTINAL SECTION

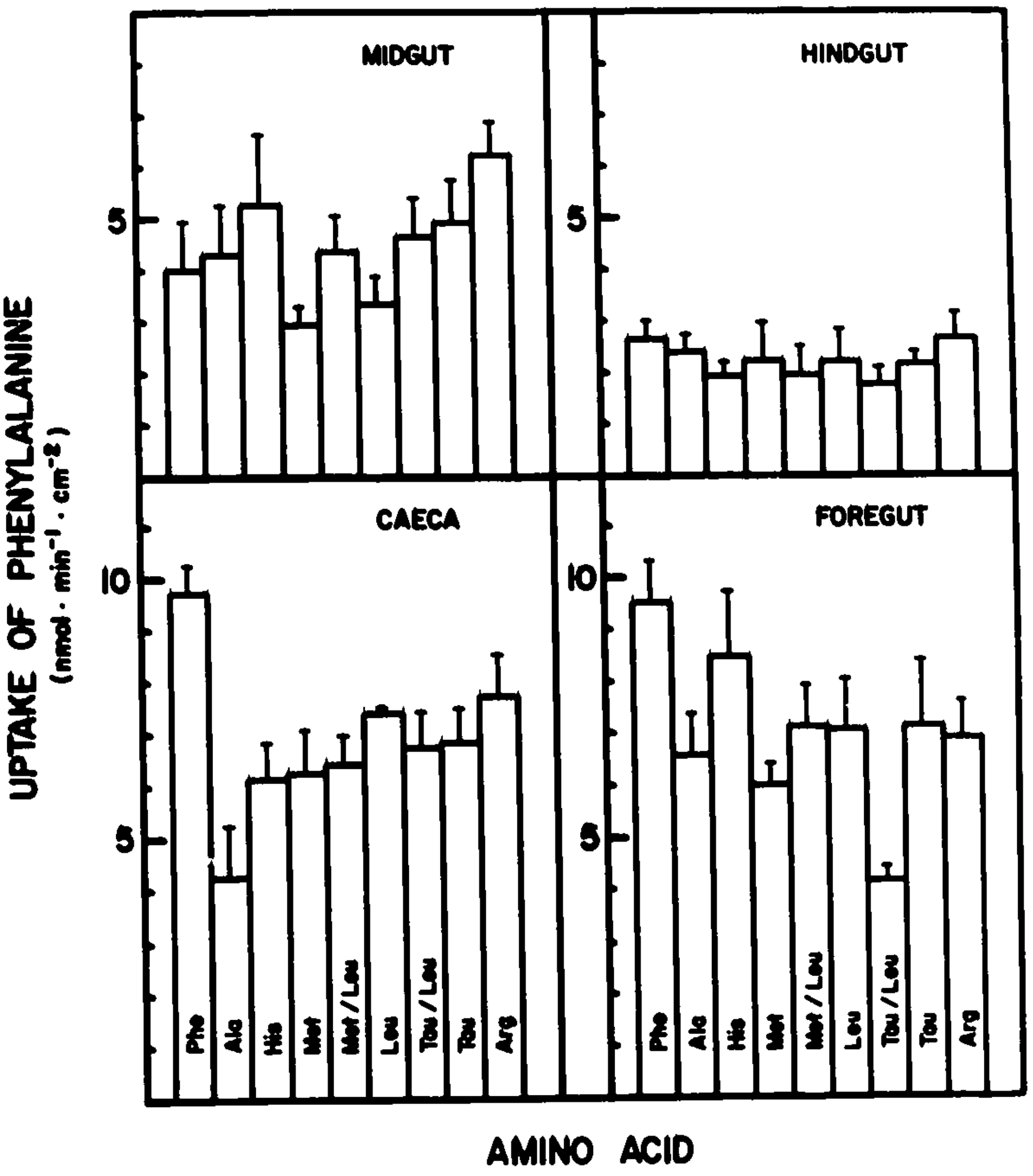
Table 24. Values of t (df = 2) for t-tests comparing *in vitro* uptake of L-phenylalanine in the absence of sodium in the different sections of the intestine. Asterisks indicate significance at the 5% level. Data from Figure 29. (C = caeca, F = foregut, M = midgut, H = hindgut)

		C	M	H
	F	3.20*	19.92*	50.65*
Summer	M	22.06*	-	9.47*
	H	56.50*	-	-
	F	8.17*	20.55*	56.72*
Winter	M	16.73*	-	33.21*
	H	68.46*	-	-

Table 25. Effect of the absence of sodium on *in vitro* uptake of phenylalanine. 1: Values of t (df = 5) for t-tests comparing sodium-free uptake with uptake in the presence of sodium in summer (feeding) flounder. 2: Values of t (df = 5) for t-tests comparing sodium-free uptake with uptake in the presence of sodium in winter (nonfeeding) flounder. 3: Values of t (df = 2) for t-tests comparing sodium-free uptake from winter and summer flounder. Asterisks indicate significance at the 5% level. Data from Figure 29. (C = caeca, F = foregut, M = midgut, H = hindgut)

	1	2	3
C	5.40*	30.87*	17.13*
F	5.96*	30.54*	2.99*
M	1.63	11.55*	5.49*
H	0.54	13.17*	18.67*

Figure 30. Effect of various amino acids or combinations of amino acids on the uptake of 5 mM L-phenylalanine measured *in vitro.* Competitors were present at a concentration of 10 mM, unless in a mixture where concentrations were 5 mM each. Osmolarity was maintained by the addition of mannitol (mean ± SE; n = 5).


MIDGUT
HINDGUT
CAECA
FOREGUT
5
5
10
5
10
5
Phe
Ala
His
Met
Met / Leu
Leu
Tau / Leu
Tau
Arg
Phe
Ala
His
Met
Met / Leu
Leu
Tau / Leu
Tau
Arg
UPTAKE OF PHENYLALANINE
(nmol · min^{-1} · cm^{-2})
AMINO ACID

inhibitors was measured at 5 mM phenylalanine, at which concentration apparent diffusional uptake represents a significant proportion of total uptake. This apparent diffusional component may mask a significant inhibition of the saturable component, and the order of inhibition is herewith presented.

The order of inhibition in the caeca, from greatest to least, was: Ala (56% inhibition) > His (37%) > Met (36%) > Met/Leu (34%) > Tau/Leu (30%) = Tau (30%) > Leu (24%) > Arg (21%). In foregut, the order was: Tau/Leu (56%) > Met (37%) > Ala (31%) > Arg (27%) > Leu (25%) = Tau (25%) = Met/Leu (25%) > His (11%). Only two amino acids inhibited phenylalanine uptake in the midgut: Leu (17%) and Met (3%). The order in hindgut was Tau/Leu (32%) > His (26%) > Met/Leu (25%) > Tau (17%) > Leu (14%) = Met (14%) > Ala (7%) > Arg (0%). Inhibition of phenylalanine uptake by a mixture of Met and Leu was greater in the caeca than the expected inhibition by 5 mM Leu and 5 mM Met alone (average = 29%). It was also greater than expected for hindgut (expected = 14%), but not for foregut (23%) or midgut (1%). In foregut, hindgut and caeca, inhibition by a mixture of taurine and leucine was higher than expected, assuming that 5 mM taurine inhibits less than 10 mM taurine. No inhibition by a taurine/leucine mixture was observed in midgut.

5.2.6 Total Uptake by the Intestine

Values of total uptake of phenylalanine by each part of the intestine were calculated for a 30 cm (standard length) flounder (Table 26). These calculations are based on the

Table 26. Total uptake per cm^2 (A), and total uptake per section (B), of L-phenylalanine in the intestinal sections (A and B) and whole intestine (B) of winter flounder. Uptake was calculated from *in vivo* and *in vitro* kinetic constants, previously calculated surface areas (Table 7), and a phenylalanine concentration of 5 mM. *In vitro* uptake was measured at 15°C in summer (feeding) and at 3°C in winter (non-feeding), and therefore corresponds, respectively, to the September and January *in vivo* values.

A

Phenylalanine Uptake ($nmol/min/cm^2$)

	In Vivo			*In Vitro*	
Section	Jan	May	Sept	Winter	Summer
Caeca	2.73	4.16	7.15	6.39	9.71
Foregut	1.21	8.55	7.92	5.19	9.88
Midgut	2.42	5.63	7.92	2.49	6.19
Hindgut	1.94	6.01	5.12	1.46	2.96

B

Phenylalanine Uptake (umol/min)

	In Vivo			*In Vitro*	
Section	Jan	May	Sept	Winter	Summer
Caeca	118.5	408.3	438.5	278.0	594.9
Foregut	87.5	1214.5	844.2	375.0	1053.1
Midgut	117.4	384.2	501.4	121.2	391.6
Hindgut	21.7	102.7	57.3	16.3	33.1
Total	345.1	2109.8	1841.4	790.4	2072.7

kinetic values estimated in the present study, both *in vivo* and *in vitro*, and the surface areas in Table 8. The total uptake was lowest in the hindgut and, except in January *in vivo*, highest in the foregut. Measured *in vivo*, midgut and caeca values were very similar. Measured *in vitro*, caeca absorbed more phenylalanine than midgut in both summer and winter. Uptake by the entire intestine was lowest in winter (January), but the *in vitro* value was more than twice that measured *in vivo*. The summer value *in vitro* was similar to that estimated *in vivo* in May, although it was measured in August and September. The September value measured *in vivo* was lower than both.

5.3 Discussion

The apparent diffusion constant was generally highest in the caeca and foregut, and showed a proximodistal gradient in summer (feeding) and winter (non-feeding) regardless of the temperature (Fig. 26). Thus the anterior intestine was more permeable to phenylalanine at all times of the year than the posterior intestine.

There was a tendency for the apparent diffusion constant to decline with temperature. The decline was less marked in winter than in summer, suggesting that, except in the midgut, the mucosa was less influenced by temperature change in winter than in summer. Q_{10} values around 1.5 are expected for processes such as diffusion (Hoar, 1983) and most of the values for summer fish were near 1.5. Winter values of Q_{10}, however, except in the midgut, were less than 1.0. Thus, as temperature decreased, the apparent diffusion constant increased slightly in caeca, foregut and hindgut. In the case of the midgut, temperature effects were more marked in winter than in summer. As temperature declined, the apparent diffusion constant declined as well, and by more than would have been observed in tissues from summer fish.

The apparent diffusion constant in nearly every section and at every temperature was lower in winter than in summer. Smith and Kemp (1971) observed a decrease in membrane unsaturation as temperature increased in goldfish. Leray et al. (1984) observed an increased degree of unsaturation (specifically, increased docosahexanoic acid, 22:6(n-3)) and increased membrane fluidity in intestinal brush border membranes of rainbow trout exposed to increased salinity. These findings suggest that a marine fish such as winter flounder may have a greater percentage of unsaturated fatty acids in its membranes compared to a freshwater fish, and that this percentage increases with reduced temperature. DiCostanzo et al. (1983b) could find no change in membrane fluidity of intestinal brush border membranes from rainbow trout over a temperature range from 4 to 39°C, although the membranes were composed of different ratios of saturated and unsaturated fatty acids. They suggested that the already high degree of unsaturation of trout membranes relative to those of mammals were adequate to prevent phase changes above 0°C. Apical uptake of Na^+ and Cl^- did differ, however, being much greater through membranes with a higher proportion of (n-3) unsaturated fatty acids. It is surely not coincidence that these are the same fatty acids which are increased upon exposure of rainbow trout to increased salinity.

The results of the present study suggest, therefore, that any changes which occurred in membrane components from summer to winter may have occurred to support continued salt transport in the tissue. Such changes may have a differential effect on the apparent diffusion constant of phenylalanine; on the one hand decreasing and reversing its sensitivity to temperature (except in midgut), while decreasing its absolute rate on the other. That the response in the midgut was different from that observed in the caeca, foregut and hindgut suggests a differential adaptation of the intestinal regions. An examination of seasonal variations in salt uptake in the different regions of the flounder intestine is required to ascertain if these alterations are indeed related to salt transport.

Apparent diffusion of leucine, methionine and valine represented only 57, 27 and 44% respectively of total uptake at 5 mM in rainbow trout (Ingham and Arme, 1977). At the same concentration, apparent diffusion represented 84 to 97% of total phenylalanine uptake in winter flounder. The difference may reflect membrane differences (marine versus freshwater fish), species differences, or amino acid differences.

The foregut and midgut had high values of J_{max} in the summer, indicating that they were the major sites of active phenylalanine uptake at that time. No proximodistal gradient was observed in the winter. It would appear, therefore, that the phenylalanine carrier(s) is (are) distributed similarly to leucine aminopeptidase in winter (see section 3.2.7.2), but that this distribution may be a result of stimulation by luminal contents in summer.

When J_{max} is expressed as a function of surface area, the decline in surface area (Table 8) is not reflected in the maximal uptake rate. That is, if carrier number or efficiency remains the same, then no change in J_{max} per cm^2 would be observed. No difference in J_{max} measured in summer or winter fish was observed at 8 and 15°C. However, when measured at 3°C, J_{max} was lower in all intestinal sections from winter compared to summer fish. The lack of significance in the caeca may reflect the high variance of the summer assays, rather than a real lack of difference. Thus the phenylalanine transporter appears to be no different in winter compared to summer when measured at high temperatures, but is much less effective at the ambient winter temperature (3°C) in winter than in summer. These results suggest that the compositional changes which appear to have occurred in the membrane have a negative effect on the ability of the phenylalanine transporter to function at the ambient temperature.

An additional mechanism of control of the saturable transport component may be the activity of the Na^+,K^+-ATPase at the basolateral membrane. Csaky and Gallucci (1977) noted that the decreased ability of *Rana pipiens* intestine to transport phenylalanine and 3-O-methylglucose in winter was correlated with a decline in Na^+,K^+-ATPase activity.

The importance of the Na^+,K^+-ATPase to salt transport in addition to organic solute transport suggests that this would not be a site of control in the winter flounder. The major route of salt transport in the winter flounder appears to be through the cotransport of Na^+, Cl^- and K^+ (Musch et al., 1982; Frizzell et al., 1984; O'Grady et al., 1986). In the model presented by O'Grady and his colleagues (1986), the low intracellular Na^+ concentration maintained by the activity of the Na^+,K^+-ATPase creates a driving force for Na^+ entry into the cell. The Na^+-K^+-$2Cl^-$-cotransporter couples Na^+ entry with Cl^- entry at the apical membrane, allowing Cl^- to accumulate above its electrochemical equilibrium. The electroneutrality of the transport effectively uncouples transport from the electrical potential of the cell. K^+ enters the cell against its electrochemical gradient, and is quickly recycled back to the lumen via apical K^+ channels. K^+ is also transported into the cell at the basolateral membrane by the action of the Na^+,K^+-ATPase. A small portion of the K^+ may exit basolaterally through a KCl transporter, but most exits through the apical K^+ channel.

The accumulation of Cl^- above its electrochemical equilibrium in the cell creates the driving force for Cl^- exit at the basolateral membrane. Most of the Cl^- appears to exit through a Cl^--selective channel. The cotransport of Na^+, Cl^- and K^+ and the recycling of the K^+ to the lumen accounts for the observed serosa negative transmural potential difference, rather than the permselective tight junction proposed by Field et al. (1978). It appears to be the activity of the

Na^+,K^+-ATPase and the subsequent efflux of K^+ through apical K^+ channels which accounts for a portion of the observed short circuit current, while the remainder appears to be due to the difference between net Na^+ and Cl^- fluxes across the tissue.

Maintenance of Na^+,K^+-ATPase activities and appropriate membrane fluidity for salt transport at a time when osmoregulation is still important but feeding has ceased would obviously be advantageous. Thus the decreased apparent diffusion constant and reduced J_{max} in winter may be due entirely to membrane alterations which allow continued osmoregulation at the expense of digestive capacity.

The unstirred layer probably exerted an influence on the apparent K_t for the *in vitro* studies as well as the *in vivo* studies (Karasov and Diamond, 1983a), in spite of stirring and bubbling with oxygen. Thus the estimated apparent K_t values are probably over-estimates, and conclusions must be drawn cautiously. Indeed, the apparent K_t followed the direction of the changes of the J_{max} in most assays, satisfying Karasov and Diamond's criterion for non-reliability (see section 4.3). Nevertheless, the values were similar in magnitude to those listed by Ferraris and Ahearn (1984) for uptake of other amino acids by fish intestines, which range generally from 0.1 mM to 3.0 mM, with one much lower (0.03 in killifish for cycloleucine) and a few much higher. Cartier et al. (1979) found an apparent K_t for phenylalanine uptake across emusculated mucosal sheets of tench intestine to be 2.9 mM. The authors estimated kinetic values by the use of Lineweaver-Burk plots, and therefore introduced an additional over-estimation, and performed the experiment at 20 to 25°C. Nevertheless, their value is nearly 5 times higher than the highest measured apparent K_t (15°C summer midgut) of the present study, and suggests that there may be a difference between freshwater and marine fish with respect to amino acid uptake. It is unlikely that the difference results from a response to dietary demands because both tench and winter flounder are omnivorous.

Apparent K_t values for phenylalanine uptake in mammals tend to be higher than those for fish (Ferraris and Ahearn, 1984). In guinea pig intestinal rings, an apparent K_t of 5.33 mM was measured (Sepulveda and Robinson, 1978). Stevens et al. (1982) estimated apparent K_t values for phenylalanine of 0.23 mM for one carrier and 8.8 mM for a second in rabbit jejunal brush border membrane vesicles. Smith et al. (1981) reported apparent K_t values for L-phenylalanine of 2.3 mM for guinea pig, 3.6 mM for hamster, 1.3 mM for pig, 2.4 mM for rabbit and 2.5 mM for rat. However, while apparent K_t values may be higher, mammals tend to have higher uptake rates (J_{max}) than fish (Karasov and Diamond, 1983a) and so can process more food in the same time, an essential requirement of their endothermy.

Apparent K_t values in the present study were generally lower in winter than in the summer, but because these changes parallelled the changes in J_{max}, it cannot be concluded that the observed change was real. Use of brush border membrane vesicles overcomes some of the difficulty caused by the unstirred layer, but it cannot be concluded that carrier affinity in disrupted membranes is the same as that *in vivo.* Until the difficulty in measurements caused by the unstirred layer can be overcome, conclusions about the carrier affinity and how or if it changes with season in intact membranes will have to wait.

No saturable component of uptake was observed in the absence of sodium. A similar observation was made in killifish for cycloleucine (Miller and Kinter, 1979) and in goldfish for valine and methionine (Kitchin and Morris, 1971). Sodium-free uptake of L-phenylalanine in feeding flounders was higher in caeca and foregut than uptake in the presence of sodium. In the winter (non-feeding), the same pattern was observed in all the intestinal sections. Increased diffusional uptake in the absence of sodium cannot be explained by unstirred layer effects (Yuasa et al., 1986) in this instance because apparent diffusional uptake in the presence of saturable uptake was estimated at saturating substrate concentrations. The presence of a sodium-independent carrier which is inhibited by sodium is not a physiologically sensible explanation in a marine fish. It is possible that sodium interacts with the membrane in such a way as to reduce its permeability to phenylalanine. This possibility requires further examination. It is clear, however, that the caeca and the foregut behave differently in summer in the absence of sodium than the midgut and hindgut, and that the difference disappears in winter.

Competition studies were performed on the premise that amino acids which are transported by the same carrier will inhibit the uptake of the counter solute. Competition studies revealed differences in the sections of the intestine of the winter flounder. All the amino acids tested inhibited phenylalanine uptake in the caeca and foregut, suggesting that phenylalanine shares carriers with all of them. The common carrier for arginine and phenylalanine may be system y^+, which handles neutral amino acids in the presence of sodium (Christensen, 1984), or the sodium-independent carrier described in rabbit ileal mucosa (King et al.,1981). The latter seems less likely in view of the results in sodium-free media, and the fact that no such carrier is present in the hindgut or midgut. In the caeca and hindgut, the greater inhibition than expected by a mixture of methionine and leucine suggests that two neutral carriers exist for phenylalanine. Whether they are similar to the carriers described by Stevens et al. (1982) remains open to question. A second carrier of this nature does not exist in the other sections. Phenylalanine does appear to share a common carrier with taurine, which is different from the one it shares with leucine. This is in contrast to the findings of King and her colleagues (1986), which suggested that taurine transport in winter flounder is by the β -system only. However, they tested taurine against α-methylaminoisobutyric acid and L-cysteine sulfinic acid, which are thought to be specific for the A and ASC systems, respectively. Stevens et al. (1982) demonstrated a neutral amino acid transporter in rabbit jejunal mucosa which carried L-neutral amino acids and did not resemble either the A or the ASC system. Therefore, it is possible that taurine and phenylalanine share a neutral amino acid carrier which was not inhibited by King's test inhibitors. Maintenance of levels of taurine, necessary for cell volume regulation, must be achieved by dietary input because the kidney of winter flounder does not appear to reabsorb taurine (Schrock et al., 1982). While the diet of flounders includes a large proportion of invertebrates which may be expected to contain taurine, it also contains other material which does not, including algae (Kennedy and Steele, 1971; Wells et al., 1973). Thus mechanisms which ensure the uptake of taurine whenever it is present are expected to be in place, and multiple carriers would be one such mechanism.

Chapter 6

General Discussion

Winter flounder from Passamaquoddy Bay stop feeding in the winter at a time when they are producing antifreeze peptides and developing gonads for spring spawning. However, the fish bury themselves in the sediments at this time (Fletcher et al., 1981) and remain relatively inactive. Low temperature serves to further depress metabolism. This reduction in activity and metabolism may provide the energy needed to meet these new demands. However, the reduction of condition factor even considering gonad weights (Table 3) suggests that depletion of bodily energy reserves occurred to meet energy demands in addition to that required for reproduction. Winter flounders also exhibited other symptoms of starvation: increase in muscle water content (Fig. 9), indicating utilization of protein; reduction in body lipid stores (Fig. 8); and a marked decrease in the mass (Fig. 15) and the surface area (Fig. 16) of the intestine. The decrease in mucosa during starvation may have been ameliorated by the continued necessity for the gut to participate in osmoregulation, and possibly by the cold temperature *per se*. Starvation reduces the turnover rate of individual enterocytes in rat (Jones and Mayer, 1973), and reduces the rate of proliferation in winter flounder (Trier and Moxey, 1980). Microvillar length and number decreases in rat (Karasov and Diamond, 1983a) and carp (Gas and Noaillac-Depeyre, 1976). Surface area is thus reduced, but the extent of the reduction is not easily quantified because of the size and vast number of microvilli. Winter flounder in the present study apparently did not reduce the length or number of micrivilli during the winter fast, and the decline in mucosal mass was less than that observed in freshwater rainbow trout for a fast of shorter duration (Bogé et al., 1981b). There was also no reduction in intestinal length as was observed in carp (Gas and Noaillac-Depeyre, 1976). These differences in intestinal response to starvation between freshwater and marine fishes is further support for the central role of the gut in osmoregulation.

It appears that the continued need to osmoregulate may also protect the saturable portion of uptake in the flounder intestine. Loss of Na^+,K^+-ATPase activity in January and February in *Rana pipiens* resulted in very low, presumably diffusional, levels of phenylalanine uptake (Csaky and Gallucci, 1977). Uptake of phenylalanine in winter flounder, measured both *in vivo* and *in vitro*, had a saturable and non-saturable, linear component throughout the year. Thus, maintenance of Na^+,K^+-ATPase activity for osmoregulation appears to allow continued saturable transport.

The maintenance of a capacity for uptake of amino acid in winter when no food is consumed may at first glance seem a waste of valuable energy reserves. Maintenance of the capability to process food may reflect a number of factors. First, although mucosal turnover is reduced in starvation and probably cold, some cell sloughing occurs. Second, levels of antifreeze peptides are high in the intestines of several polar fish (O'Grady et al., 1982) and winter flounder (A.L

DeVries, personal communication). An ability to absorb rather than lose these peptides would seem advantageous when the fish is fasting. Finally, amino acids are present in low concentrations in seawater (Stewart, 1979), higher near the sediment than in the water column, and the flounder may be taking advantage of a source of nitrogen which is a part of the seawater ingested for osmoregulatory purposes.

Effects of starvation on intestinal uptake patterns are variable. Semi-starved rats exhibit increased glucose and L-histidine uptake (Kershaw et al., 1960), while semi-starved hamsters did not (Hindmarsh et al., 1967). Uptake of L-valine by starved rats increased when presented alone or in a mixture of free amino acids, but did not when presented as a casein hydrolysate (Steiner and Gray, 1969). Uptake of glucose and galactose, but not of methionine or proline, increased in the ileum of starved rats (Sanford and Smyth, 1974). Glycine uptake increased in 4- and 8-week starved rainbow trout (Bogé et al., 1981b). Collie (1985) observed increased proline uptake at low concentrations, but similar uptake at high concentrations in starved compared to fed coho salmon. The response of the transporting function of the intestine to starvation appears, therefore, to vary with species, with position in the intestine, with presentation of the solute and with concentration of the solute. Maintenance of an ability to transport solutes in winter flounder may thus be due to the same factors which result in increased uptake in other species. The identity of such controlling factors has not as yet been ascertained.

In vitro and *in vivo* methods both showed a lower winter apparent diffusional constant (K_d) in all sections compared to summer. The J_{max} measured *in vitro* was also lower at 3°C in winter than in summer, though the difference disappeared at the higher temperatures. *In vivo*, however, J_{max} from the foregut and hindgut was higher than expected, and the J_{max} of the caeca and midgut was not different in winter compared to summer when the change in temperature is considered. These results suggest that the cell membrane is altered as an adaptation to winter conditions. While the decline in apparent K_d indicates that the membranes were less permeable to phenylalanine in winter than in summer, the different results for J_{max} using the two methods could be explained by differential changes in the basolateral and brush border membranes. Potentially, alteration of membrane components could influence carrier function by altering its conformation. Mammalian studies indicate that the major carrier for basolateral amino acid efflux resembles the L-system (Mircheff et al., 1980), while the brush border membrane may have several carriers, none of which resemble the classical systems (Christensen, 1984). Changes in membrane structure could affect different carriers differently, and the possibility that basolateral and brush border membranes differ (LeGrimellac et al., 1982), and may therefore respond differently to seasonal change, suggests that differential adaptation would be observed. Adaptation may involve a decline in brush border carrier activity (as indicated by the *in vitro* experiments) or an increase in basolateral activity or both. The basolateral activity could also be increased by low blood levels of phenylalanine, which effectively increases the gradient driving phenylalanine efflux. Another possibility which could account for the observed increase in J_{max} *in vivo* is that backflux of phenylalanine was reduced over summer levels because of decreased apical membrane permeability. Further studies are needed to separate these possibilities

The two methods employed to study the transfer of phenylalanine in this thesis, *in vitro* and *in vivo*, showed some differences. Saturable uptake was generally higher *in vivo* than *in vitro* (c.f. Figs. 23 and 27), while values of apparent K_d (c.f. Figs. 23 and 26) were similar. When converted to total intestinal uptake, *in vitro* values were slightly higher than *in vivo* values (Table 26), although this varied with the section. That *in vitro* uptake in the caeca was always higher than *in vivo* (for the same season) may be methodilogical. *In vivo*, the caeca were perfused as a group, and exchange of the perfusate between the intestinal and caecal lumena may have been restricted.

Total uptake was highest in the foregut except in January when measured *in vivo*. The January value reflects a low uptake per cm^2 in spite of the large surface for absorption in the foregut. Both total uptake and total uptake per cm^2 varied not only from winter to summer, but through the summer as well, suggesting a system in continual change as a result of the changing needs of the fish.

Total uptake in the foregut was highest *in vitro* as well, reflecting the greater surface of mucosa in that region (Table 8). While winter uptake per cm^2 was highest in the caeca, foregut and caeca exhibited a high and similar rate of phenylalanine uptake per cm^2 in summer. Fed coho salmon exhibited a much higher rate of proline uptake per mg tissue in caeca than in the anterior or posterior intestine (Collie, 1985). Proline is not an essential amino acid in fish, while phenylalanine is. The uptake of these two amino acids may therefore differ, with uptake of phenylalanine a more general property of all parts of the intestine. However, the results with leucine aminopeptidase, which is thought to be involved in the transport of peptides, indicate that protein constituents are handled by foregut and caeca equally well. A second possibility is that the pyloric caeca of salmon are adapted to a preferential uptake of protein constituents. Pyloric caeca occur in many fish species and may number from 1 to 1000, but Fänge and Grove (1979) state that their presence is not related to the relative gut length or general feeding niche of the species. However, some consistencies are observed. Stomachless fish, which tend to be herbivorous and have large relative gut lengths, never have pyloric caeca (Fänge and Grove, 1979). The existence of pyloric caeca in rainbow trout, a relatively active carnivore, increase the length of the gut to three times body length instead of approximately one half body length when only the intestine is considered (Bergot et al., 1975). Bluefin tuna (*Thunnus thynnus*) are active carnivores and while the intestinal length is only about one third of the body length, the caecal mass is enormous, and is estimated to increase gut length approximately 1000-fold (McLeese, 1982). These observations are very general and for only a few species, but they suggest that the number of pyloric caeca may be related to the activity and feeding habits of fish. de Groot (1971), in a study of 133 species of Pleuronectiformes, including winter flounder, found that the number of pyloric caeca was related to diet, and especially to the size of the diet. However, the flatfish with the largest numbers of caeca tended to be those whose primary dietary component was fish, whereas those with fewer caeca tended to eat molluscs, crustaceans and polychaetes, which have a higher percentage of refractive components. The pyloric caeca may be an adaptation which allows for the rapid uptake of nutrients, especially amino acids and presumably peptides, from diets which are rapidly degraded by intestinal enzymes. Removal of amino acids and peptides would prevent tissue water loss in the anterior intestine as proteins were broken down and the lumen became hyperosmotic to the blood. For fish with diets composed of more refractive materials, the rapid initial uptake would not be as important; instead, a longer intestine would be advantageous to allow time for enzymes to penetrate tissues partially protected by chitin or cellulose. A preferential uptake of the products of protein digestion such as amino acids and peptides would be predicted in the pyloric caeca if this hypothesis is correct.

A large proportion of the diet of the winter flounder is algae (Kennedy and Steele, 1971; Wells et al., 1973) but they also ingest small, whole clams and considerable quantities of sand and mud (Kennedy and Steele, 1971; Fletcher et al., 1981; personal observation of gut contents). A longer intestine to allow penetration of digestive fluids into the digesta is expected, and indeed, the relative gut length is greater than is usual for carnivorous fish. The pyloric caeca may be vestigial with respect to protein digestion in the flounder, and their relatively large diameter compared to the narrow caeca of species such as salmon, allowing entry of larger food particles, may represent a transition stage from protein handling to a more general absorptive function. That the Soleidae and Cynoglossidae (families of Pleuronectiformes) do not possess pyloric caeca and consume similar diets to the winter flounder (de Groot, 1971) indicates that this may be the case.

Total uptake measured *in vivo* was less than half that measured *in vitro* in January, although summer values were similar. This suggests that there may be other factors operating to reduce uptake at this time. Hormonal and neural factors undoubtedly play a role in controlling uptake. The rapid proliferation of mucosa at the onset of feeding (50% more surface area in May than in January) followed by a subsequent decline through the feeding period despite the continued presence of luminal nutrients implies that mucosal proliferation and possibly uptake itself has an important external control system. The nature of this system has yet to be elucidated.

Competition studies suggest that the uptake of phenylalanine by the caeca, foregut and hindgut is achieved through at least two carriers, one which it shares with taurine. Carrier mediated uptake is sodium dependent. All of the sections of the intestine do not behave the same way in response to seasonal change or in the characteristics of phenylalanine uptake. The differences observed in the midgut compared to the foregut indicate that, although there is little structural basis for a division, there is a functional basis. The diffusional component is an important route of phenylalanine uptake in all sections, especially in winter when the saturable component is reduced. The continued presence of the saturable component of uptake is probably due to the maintenance of ion gradients for salt uptake.

The major factor influencing phenylalanine uptake in winter is starvation. Reduction of the mucosal surface area due to loss of direct luminal stimulation by food results in a concomitant loss of transporting ability. The loss of mucosa is less than might be expected from comparisons to freshwater fish during starvation and this is probably due to the continued need to osmoregulate. Some portion of this amelioration may also be due to reduced winter temperatures. A further decline in uptake in addition to that due to surface area loss is probably attributable to changes in membrane constituents to protect osmoregulatory function in winter. The majority of this decline is due to a reduction of the apparent diffusion constant.

Further studies should be performed to determine the nature of the membrane changes and to ascertain if ion transporting capabilities do change with season. In the meantime, studies assessing intestinal function in winter flounder should be interpreted with care, in view of the continuously changing nature of the intestinal mucosa in these fish.

Chapter 7

Conclusions

1. Winter flounder from Passamaquoddy Bay, New Brunswick, fasted from late October to early May. Liver and muscle lipid reserves were depleted and muscle protein was utilized as an energy reserve. Ten percent of the 14% decline in condition factor in winter could be attributed to starvation while 4% could be attributed to gonad development.

2. The intestinal mucosa of the winter flounder atrophied in the winter non-feeding period. No decrease in intestinal length occurred. The decline in mucosal mass was not as great as observed by Boge et al. (1981b) for freshwater rainbow trout fasted for a shorter duration, indicating a sparing of mucosal mass. Maintenance of a decreasing proximodistal gradient, which is thought to be the result of the presence of luminal nutrients, suggests that the sparing of mucosal mass is a result of the continued need of the flounder to transport salts and water for osmoregulation.

3. Structurally, enterocytes were no different from non-feeding and feeding flounder. Numbers of microvilli did not appear to decrease during fasting although this phenomenon has been observed in fasting mammals and freshwater carp. This again is associated with a continued need to osmoregulate. The number of goblet cells appeared to decline in non-feeding fish. In the absence of large

quantities of pancreatic hydrolytic enzymes, the need to protect the mucosal surface is reduced, while the absence of large quantities of mucus increases access of ingested seawater to the enterocytes.

4. Uptake of phenylalanine may be divided into an apparent diffusional component which is linear and non-saturable, and a component which is saturable.

5. Uptake of phenylalanine declined in non-feeding flounder, due in large part to the decrease in mucosal mass. However, a decrease in addition to that due to mass was also observed because of a decrease of the apparent diffusion constant (K_d) for phenylalanine. Decreased apparent K_d values indicate that seasonal changes in the membrane occurred with season. Observed differences in maximal uptake rate (J_{max}) measured *in vivo* and *in vitro* suggest that the brush border and basolateral membranes may have been altered differentially. The affinity of the carrier for phenylalanine (K_t) was affected by the presence of the unstirred water layer, and could not be used as a reliable indicator of seasonal change.

6. No preferential uptake of phenylalanine occurred in the caeca, nor was leucine aminopeptidase activity higher in the caeca. These results indicate that in winter flounder, the caeca can transport protein constituents, but no better than

the other sections of the intestine.

7. Leucine aminopeptidase activity decreased only in the hindgut and may have increased in the caeca, foregut and midgut during the winter non-feeding period. Total LAP activity was similar in all sections of the intestine. The maintenance of the ability to transport protein constituents may be a response to starvation.

8. Removal of sodium from the medium abolished the saturable portion of uptake and increased the apparent diffusional constant. Midgut and hindgut responded differently in summer, though not in winter, than foregut and caeca.

9. Carrier specificities for L-phenylalanine differ in the 4 sections of the intestine. The presence of a shared carrier for taurine and L-phenylalanine is suggested for the caeca, foregut and hindgut. At least two carriers for L-phenylalanine uptake appear to exist in those three sections. A single carrier of narrow specificity appears to transport L-phenylalanine in the midgut.

10. The intestine of winter flounder is in a constant state of flux, with changes in surface area and phenylalanine transporting ability throughout the year. In addition, the different areas of the intestine respond differently. Future studies of intestinal function in winter flounder

should consider these complexities.

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