ZINC METABOLISM IN THE WINTER FLOUNDER (PSEUDIPLEURINECTES AMERICANUS)

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ZINC METABOLISM IN THE WINTER FLOUNDER

(PSEUDOPLEURONECTES AMERICANUS)

by

© Margaret Ann Shears, B.Sc. (Hon.)

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ABSTRACT

The gastrointestinal uptake of Zn^{2+} was studied in the winter flounder using an *in situ* technique, an equilibrium dialysis technique and a non-absorbed marker technique.

The entire digestive tract of the flounder was capable of absorbing Zn^{2+}, with the uppermost portion of the intestine having the highest and the stomach the lowest capacity. A seasonal study revealed that the capacity to absorb Zn^{2+} was greatest during the summer feeding period. Zn^{2+} absorption appeared to involve at least two steps, the first a rapid accumulation of Zn^{2+} by the tissue and the second, a slower transfer of Zn^{2+} into the body. The total amount of Zn^{2+} absorbed increased with increasing loads of Zn^{2+} in the lumen; the transfer mechanism did not appear to be saturated at the highest Zn^{2+} loads tested. However, Zn^{2+} uptake was inhibited by several other metals and by the amino acid, histidine.

The capacity of the digestive tract to absorb Zn^{2+} was not affected by feeding the flounder a high-Zn^{2+} diet or by increasing the body Zn^{2+} load by parenteral injections. In the event of exposure to elevated levels of Zn^{2+} in the diet, it is suggested that elimination mechanisms may play a greater role in Zn^{2+} homeostasis in the winter flounder than limitation of gastrointestinal uptake.

The dynamics of Zn^{2+} turnover in the flounder was investigated by examining the distribution of ^{65}Zn in the tissues following single intramuscular injections of the radiotracer. The tissues exhibited different rates of accumulation and loss of ^{65}Zn, the most rapid being in tissues such as the kidney, liver, gill and gastrointestinal tract.

Retention of ^{65}Zn was examined in live flounder using a whole-body detector. The loss of ^{65}Zn appeared to vary seasonally; when flounder were monitored during the summer feeding period the rate of ^{65}Zn loss (monitored in the area of the peritoneal cavity) increased over that seen in the winter non-feeding period (i.e., $TB_{1/2}$=223 and 1510 days, respectively). Whole-body ^{65}Zn retention patterns were similar in flounder injected with saline or a load of stable Zn^{2+}. Under these experimental conditions the rate of Zn^{2+} loss was not affected by an excess of stable Zn^{2+}.

Experiments conducted to determine the possible site(s) of Zn^{2+} excretion into the digestive tract following i.v. injections of ^{65}Zn indicated that Zn^{2+} could be "secreted" into the lumen contents all along the tract. Other possible sites of Zn^{2+} elimination include the gills, kidney and body surface.

Chromatographic techniques were used to examine the Zn^{2+}-binding proteins in the cytosols of several tissues of the winter flounder. A low molecular weight ^{65}Zn (Zn^{2+})-binding protein, with properties characterizing it as metallothionein, was isolated from the mucosal and liver cytosols of Zn^{2+}-injected flounder. Metallothionein did not appear to be serving the same function(s) in the intestine of the flounder as commonly hypothesized for mammals. The presence of the protein in the mucosal cytosol did not appear to be associated with any enhancement or depression of Zn^{2+} absorption. The role of metallothionein in the normal metabolism of Zn^{2+} in the flounder remains to be resolved. That it could play a role in homeostasis is suggested by the presence of a low molecular weight Zn^{2+}-binding protein, with elution characteristics similar to metallothionein, in the tissues of normal flounder.

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GENERAL INTRODUCTION

Zinc is essential in trace amounts for the well-being of all living organisms. It has been identified as an integral constituent of several metalloenzymes involved in a wide variety of metabolic processes (Riordan and Vallee 1976). Where zinc deficiency has been demonstrated in laboratory animals (i.e., rats and mice), in livestock, and in man, it is characterized by a reduction in growth, a loss of appetite, skin lesions and impaired reproductive development and function (Underwood 1971, 1977).

Fish can obtain zinc from the water, via the gills, and from the diet. However, laboratory studies using the radiotracer ^{65}Zn indicate that under normal conditions the diet represents the major source of input (Hoss 1964; Pentreath 1973 a,b; Renfro et al. 1975). For example, even when Zn^{2+} is present in the rearing water, the growth rates of some fish have been shown to be reduced when they were fed a Zn^{2+}-deficient diet (Ogino and Yang 1978, 1979; Ketola 1979). In addition, these fish exhibited a loss of appetite, erosion of the skin and fins, and developed cataracts.

Mammals and birds have homeostatic mechanisms for maintaining the zinc content of most body tissues. Regardless of whether they are presented with low-(2-4 µg/g) or high-(600 µg/g) Zn^{2+} diets, laboratory rats appear able to closely regulate the zinc concentration in most of their tissues (Reinhold et al. 1967; Ansari et al. 1975). However, zinc does start to accumulate in liver and kidney of rats when fed diets containing 1000 µg/g or higher (Chen et al. 1977). Ruminants also have the ability to maintain the zinc content of most body tissues when fed Zn^{2+}-deficient diets. However, they appear less able to maintain

homeostasis when fed excess dietary Zn^{2+} (Miller 1970). For example, Kincaid et al. (1976) observed a 5-fold increase in liver Zn^{2+} levels in calves fed a high-Zn^{2+} diet (600 µg/g) for 21 days. The mechanisms for maintaining zinc levels in poultry also appear to be very effective. The zinc concentrations of most soft tissues were not significantly depressed when chicks were fed low-Zn^{2+} diets (5 µg/g) (Zeigler et al. 1964). Nor was the zinc concentration of the liver appreciably elevated when they were fed diets containing excess Zn^{2+} (Johnson et al. 1962).

Evidence for zinc homeostasis in fish is largely circumstantial. A number of field studies have demonstrated that, for a given species of fish, tissue Zn^{2+} concentrations tend to vary little between populations exposed to different environmental Zn^{2+} levels (Uthe and Bligh 1971; Portmann 1972; Goodyear and Boyd 1972; Topping 1973; Harms 1975; Eustace 1974; Northcote et al. 1975; Wiener and Giesey 1979). Furthermore, analysis of different species of fish residing in the same locality, has demonstrated the existence of a significant species effect on tissue and whole body concentrations of zinc (Cross and Brooks 1973; Giesey and Wiener 1977; Wiener and Giesey 1979; Milner 1979). It has been suggested that the relatively higher levels of zinc detected in some species of marine fish may be due to their plankton feeding habits (Topping 1973; Windom et al. 1973; Cross and Brooks 1973) or their habits of ingesting bottom sediments while feeding (Eustace 1974). However, Ting (1973) did not observe any significant difference in zinc concentrations of tissues from seven species of marine fish which exhibited four different feeding habits (herbivores, plankton feeders, benthic carnivores or pelagic carnivores). In addition, there are reports of species of fish with similar feeding habits exhibiting significant differences in their whole body zinc concentrations (Wiener and Giesey 1979; Cross et al.

1975; Milner 1979). Within a species, consistent differences in tissue concentrations occur due to the sex of the fish (Fletcher and King 1978; Chernoff and Dooley 1979), the body size (Eisler and LaRoche 1972; Cross et al. 1973; Northcote et al. 1975; Wiener and Giesey 1979; Milner 1979) and the season when sampled (Fletcher and King 1978; Milner 1979), implying some form of physiological control.

While direct accumulation from the water would seem to play only a minor role in the normal metabolism of zinc in fish, a few field studies have attributed elevated zinc concentrations in localized fish populations to high levels of Zn^{2+} in the water (Andersen et al. 1973; Atchison et al. 1977). Differential uptake from water containing elevated levels of zinc has also been demonstrated experimentally (Joyner 1961; Spehar 1976; Milner 1976, 1982; Holcombe et al. 1979; Farmer et al. 1979; Pierson 1981). In general, these laboratory studies show an increase in Zn^{2+} accumulation in the fish with increasing water concentrations. After the initial influx, the stickleback (*Gasterosteus aculatus*) was able to reduce its internal zinc level despite continued exposure to a high concentration of Zn^{2+} in the water (4000 µg Zn^{2+}/L) (Matthiessen and Brafield 1977). However, other species of fish exhibited linear uptake over the entire exposure period or took a much longer time to reach an apparent equilibrium. Variables such as the feeding rate and the sex of the fish also influenced the pattern of Zn^{2+} uptake (Pierson 1981; Farmer et al. 1979). Different tissues within the fish also accumulate zinc to different degrees. After 20 weeks exposure to 1360 µg Zn^{2+}/L, the gill, kidney, liver and operculum bone of the Brook trout (*Salvelinus fontinalis*) contained 4.6, 1.6, 2.9 and 5.4 times more Zn^{2+} than did tissues from control fish (exposed to 2.6 µg Zn^{2+}/L). In contrast, no significant differences were observed in

the Zn^{2+} concentrations in muscle, spleen or gonad (Holcombe et al. 1979). The Zn^{2+} concentrations in most of the tissues and in the whole body declined when fish were transferred from water containing elevated levels of Zn^{2+} to control water, demonstrating that fish have mechanisms for eliminating zinc accumulated from the aquatic environment (Joyner 1961; Holcombe et al. 1979).

The ability of fish to maintain zinc homeostasis when presented with diets varying in Zn^{2+} content has only been examined experimentally by a few investigators. There is some indication that elevated levels of Zn^{2+} in the diet may result in increases in the Zn^{2+} content of fish (Singh and Ferns 1978; Patrick and Loutit 1978). Milner (1976, 1979, 1982) reported that the total amount of Zn^{2+} retained by young plaice (0-group, *Pleuronectes platessa*), fed diets containing different amounts of zinc, increased with increasing Zn^{2+} loads. However, the percentage of retained Zn^{2+} decreased with increasing input level, implying some form of regulation. When the amount of Zn^{2+} in their diet was doubled from 15 to 30 µg/g there was very little difference in the whole body Zn^{2+} content of rainbow trout (*Salmo gairdneri*) or in the Zn^{2+} concentrations of several of their tissues. However, feeding the trout a low-Zn^{2+} diet (5 µg/g) resulted in a lower whole body content of zinc; the Zn^{2+} concentration of the liver was similar at all dietary levels but the Zn^{2+} concentrations of the vertebrae, intestine and eyeball were 2-3 times lower in fish fed the low-Zn^{2+} diet (Ogino and Yang 1978). In agreement with field observations that species of fish with similar feeding habits can vary in their Zn^{2+} content, Jeng and Sun (1981) found that when the common carp (*Cyprinus carpio*) and the silver carp (*Hypothalmichthys molitrix*) were fed the same diets from hatching to adult, the Zn^{2+} concentrations in the former species were several times

higher than in the latter. They concluded that the difference in Zn^{2+} concentration in the two species was not caused by the level of Zn^{2+} in the diet *per se*, but might be due to a higher rate of Zn^{2+} absorption in the common carp. The Zn^{2+} concentrations in the common carp were similar in fish fed 17 or 294 µg/g Zn^{2+} for 8 weeks. However, Zn^{2+} accumulated in the tissues when they were fed diets containing 1007 and 1974 µg/g Zn^{2+}. At these concentrations there appeared to be a linear relationship between the level of Zn^{2+} in the body and the level of Zn^{2+} in the diet. The first evidence of Zn^{2+} accumulation was observed in the digestive tract tissues, followed by the skeletal tissues, then the skin and muscle. Zn^{2+} concentrations in the hepatopancreas, spleen, kidney and rest of the viscera showed no significant change.

To summarize, while available information tends to indicate that fish maintain tissue Zn^{2+} levels, little is known about the extent of regulation or the mechanisms involved. In mammals and birds Zn^{2+} homeostasis is thought to be maintained by mechanisms operating at the sites of absorption and secretion in the gastrointestinal tract (Becker and Hoekstra 1971; Underwood 1971, 1977). It seems reasonable to postulate that similar mechanisms could operate in marine fish since it appears that most Zn^{2+} enters via the digestive tract. The primary aim of the present study was to assess the role of the gastrointestinal tract in Zn^{2+} regulation in fish.

The thesis is presented in three chapters. Chapter I deals with the absorption of Zn^{2+} by the digestive tract of the winter flounder. Chapter II is concerned with the dynamics of Zn^{2+} turnover in the tissues and whole body, and the possible role of the gastrointestinal tract in Zn^{2+} excretion. In chapter III the Zn^{2+}-binding proteins in the gastrointestinal tract, liver, kidney and gill are examined.

CHAPTER I

GASTROINTESTINAL ABSORPTION

INTRODUCTION

The methodology commonly used in studies on the gastrointestinal absorption of Zn^{2+} in mammals has been reviewed by Becker and Hoekstra (1971). The techniques generally fall into three categories, namely *in vivo*, *in situ* and *in vitro*. *In vivo* methods include such techniques as the conventional balance study, which involves analyses of the total stable Zn^{2+} in the diet (input) and in the feces (output), and feeding studies using known amounts of the radiotracer ^{65}Zn, either alone or with non-absorbed markers. When ^{65}Zn is administered, whole-body detectors are often used to monitor the retention of the radiotracer in the animal at various time intervals. *In situ* methods include perfusion of segments of the intestine with ^{65}Zn plus other test substances or injection of the material into ligated segments. *In vitro* methods involve the incubation of everted gut sacs or isolated strips of intestine. These three methods have been used in mammals to determine the site(s) of absorption and to examine factors affecting the extent of absorption. Such factors include exposure time, the amount of Zn^{2+} in the lumen, the presence of other metals or dietary constituents in the lumen and the Zn^{2+} status of the animal.

In situ studies in rats have demonstrated that Zn^{2+} is mainly absorbed from the small intestine, with maximum uptake occurring in the duodenum (Van Campen and Mitchell 1965; Methfessel and Spencer 1973 a). This has been confirmed by examination of the whole-body retention of ^{65}Zn in rats following injection of the radiotracer into different regions of the gastrointestinal tract (Davies 1980). In ruminants the site(s) of Zn^{2+} absorption has been examined in intact feeding animals using non-absorbed markers in conjunction with ^{65}Zn. Using this method

Miller and Cragle (1965) concluded that ruminants absorb Zn^{2+} from the abomasum, secrete it into the first third of the small intestine and absorb it throughout the rest of the small intestine.

Studies of the time course of ^{65}Zn absorption from the intestinal lumen of different species of mammals have demonstrated that both accumulation of Zn^{2+} by the intestinal mucosa and transfer into the body occur rapidly. In rats and mice maximum ^{65}Zn absorption was observed within 30 minutes to one hour following oral administration of ^{65}Zn, following instillation of ^{65}Zn directly into ligated segments of the intestine or following perfusion of the intestine with ^{65}Zn (Methfessel and Spencer 1973a; Smith et al. 1978a; Davies 1980; Jackson et al. 1981). In addition, Davies (1980) observed that the rapid phase of ^{65}Zn transfer in rats was followed by a slower phase. He attributed this slower phase to transfer of ^{65}Zn that had bound to the mucosal tissue, and proposed that the rapid and slow phases were distinct processes. In ruminants, absorption of a ^{65}Zn dose injected directly into the duodenum was very rapid during the first hour and diminished progressively thereafter, with very little absorption occurring after 8 hours (Pate et al. 1970).

The mechanism of Zn^{2+} absorption in mammals is not well understood but the consensus seems to be that it involves binding of Zn^{2+} to specific sites or carrier proteins in the mucosal tissue. This speculation has arisen from the observation that the rate of ^{65}Zn absorption versus load of stable Zn^{2+} (in the intestinal lumen of rats) exhibits saturation kinetics characteristic of a carrier-mediated process (Hamilton et al. 1978; Davies 1980). It has also been inferred from feeding studies in intact animals. The percentage retention of orally administered ^{65}Zn declined in both rats and ruminants with increases in dietary Zn^{2+} (Furchner and Richmond 1962; Miller 1970).

Several nutritional factors influence the extent of Zn^{2+} absorption in mammals. The Zn^{2+} content of the diet appears to influence the amount of Zn^{2+} absorbed (Becker and Hoekstra 1971; Sandström and Cederblad 1980). However, factors which alter the availability of the Zn^{2+} for absorption are also important (O'Dell et al. 1972). The presence of other trace metals has been demonstrated to affect the extent of Zn^{2+} absorption, although the results from different laboratories, using different methods of investigation, are not always in agreement. Van Campen (1969) observed that injection of Cu^{2+} into isolated duodenal segments *in situ* interferred with the absorption of ^{65}Zn in rats. However, addition of Cu^{2+} to the diet had no effect on the total body turnover of ^{65}Zn (injected i.p.) in mice (Cotzias et al. 1962) or rats (Kinnamon and Bunce 1965), contrary to what one might expect if less stable Zn^{2+} was being absorbed. Results from an *in situ* study in mice have led to speculation that there are also analogous mucosal binding sites for uptake of Fe^{3+} and Zn^{2+} (Hamilton et al. 1978). In the same study, these authors also found that Cd^{2+} impaired uptake and transfer of Zn^{2+} in mice on an iron-deficient diet but had no significant effect in iron-replete mice. Feeding Cd^{2+} to calves and goats reduced the amount of ^{65}Zn they absorbed (Hiers et al. 1967). However, Cd^{2+} had the opposite effect on the uptake of Zn^{2+} by strips of incubated rat intestine, and on *in vitro* transfer in perfused gut sacs (Sahagian et al. 1966, 1967). When these techniques were used Cd^{2+} greatly enhanced Zn^{2+} uptake into the intestinal tissue and the subsequent transmural movement. Conflicting results have also been reported on the effect of calcium on Zn^{2+} absorption. High calcium had no definite effect on the absorption of dietary Zn^{2+} in rats (Forbes and Yohe 1960) or man (Spencer et al. 1965). However, Hoekstra (1964) observed Ca^{2+} antagonism

in rats fed certain diets. In diets containing cereals, it is a matter of dispute whether the antagonistic effect of calcium is due to the formation of insoluble calcium-phytate-zinc complexes (Solomons 1982).

As would be expected if Zn^{2+} homeostasis operates via changes in Zn^{2+} absorption, the extent of Zn^{2+} absorption in mammals appears to be influenced by the Zn^{2+} status of the animal. Ruminants exhibiting signs of clinical Zn^{2+} deficiency absorbed and retained a greater % of an oral dose of ^{65}Zn than did controls (Miller 1970). On the other hand, elevating the Zn^{2+} status of the animal, either by injections of stable Zn^{2+} or feeding high Zn^{2+} diets, has been found to lower the % of ^{65}Zn absorbed. For example, Richards and Cousins (1975a) found that the % of an oral dose of ^{65}Zn transferred to the carcass was reduced in rats which had been injected (i.p.) with a Zn^{2+} load. Intraperitoneal injections of Zn^{2+} also resulted in a decrease in the % of ^{65}Zn and total stable Zn^{2+} transferred to the vascular perfusate when rat intestine was perfused *in situ* (Smith et al. 1978a; Smith and Cousins 1980). In rats fed a Zn^{2+}-deficient diet, the % of ^{65}Zn and total stable Zn^{2+} transferred to the perfusate was increased markedly.

There is little information in the literature on the nature of gastrointestinal absorption of Zn^{2+} in fish. In the present study, Zn^{2+} absorption in the winter flounder was investigated using an *in situ* technique (Section A), an equilibrium dialysis technique (Section B) and a non-absorbed marker technique (Section C). Briefly, the *in situ* technique was used to determine the site(s) of Zn^{2+} uptake in the gastrointestinal tract and to describe the time course of absorption. This method was also used to examine several of the factors which might affect the extent of Zn^{2+} uptake, i.e. the level of stable Zn^{2+}, the presence of other metals and dietary constituents such as amino acids and sugars.

Since the winter flounder in Newfoundland only feeds for part of the year (Fletcher and King 1978) seasonal variation in Zn^{2+} uptake was also investigated. In addition, the effect of different amounts of Zn^{2+} in the diet, and of the Zn^{2+} status of the fish (artificially elevated by Zn^{2+} injections) on *in situ* uptake of Zn^{2+} were examined. The equilibrium dialysis technique was used to examine the binding of Zn^{2+} to soluble proteins of the intestinal mucosa. The effect of other metals on Zn^{2+} binding was also investigated using this technique. Studies with diets containing a non-absorbed marker were carried out to determine the site(s) and extent of Zn^{2+} absorption in intact, feeding winter flounder.

MATERIALS AND METHODS

Section A. *In situ* technique

An *in situ* technique used to study gastrointestinal uptake of Zn^{2+} in mammals (Hamilton et al. 1978; Van Campen 1969) was modified to study similar parameters in fish. The method was used to investigate the site(s) of uptake and several factors which might affect Zn^{2+} uptake, such as time, Zn^{2+} load, other metals, dietary constituents (amino acids, fructose), season, level of Zn^{2+} in the diet and Zn^{2+} status of the fish.

Winter flounder (*Pseudopleuronectes americanus*) (25-45 cm long, 250-1000 g) were collected by divers equipped with SCUBA (Fletcher 1977). In the laboratory, the fish were maintained in aquaria (250-40,000 L) supplied with flowing seawater under conditions of ambient temperature and photoperiod. They were fed chopped capelin (*Mallotus villosus*) throughout the feeding period from April to October (Fletcher and King 1978). Except where indicated, the experiments were conducted during this

period. The fish were not fed for 5-7 days prior to surgery to allow the intestine to empty.

Fish were placed in anaesthetic (0.5 g MS222, tricaine methanesulfonate, in 4 L seawater) for approximately 10 minutes and a (1:2) dilution of the same solution or clean seawater was passed over the gills during surgery. An incision was made in the body wall to expose the gastrointestinal tract. A segment of the tract was then tied off and a fixed volume (0.50-0.55 mL) of saline solution (1% NaCl) containing the radiotracer ^{65}Zn (New England Nuclear), and, where indicated, $ZnCl_2$ as well as other test substances, were injected into the ligated region. The incision was sutured and the fish returned to the aquarium. After a specified time period the fish was killed by a blow on the head, bled from a caudal blood vessel and the ligated segment dissected out of the body. The contents in the lumen of the ligated segment were emptied into a counting vial. The tissue was placed in a separate vial and both samples were counted in a gamma scintillation counter (Packard Model 578). Where indicated, the radioactivity was also determined in the blood, kidney, liver and other tissues.

The results were expressed as the amount of Zn^{2+} accumulated in the intestinal tissue (based on the cpm ^{65}Zn in the tissue) and the amount of Zn^{2+} transferred across the digestive tract into the body. The latter was computed by subtracting the sum of the cpm ^{65}Zn in the intestinal tissue and in the intestinal lumen contents from the total cpm ^{65}Zn injected into the tied-off segment. When a load of $ZnCl_2$ was injected with the isotope, the results were converted to µg of Zn^{2+} based on the specific activity of the ^{65}Zn injection.

Site(s) of uptake

To determine the site(s) of zinc uptake in the gastrointestinal tract of the flounder, ^{65}Zn (plus $ZnCl_2$ where indicated) was injected into the following segments: the stomach, the upper intestine (which included the pyloric appendages), the mid portion of the intestine and the lower intestine (excluding the rectum). In addition to examining the site(s) of uptake in flounder during the summer feeding period, the site(s) of uptake was also examined in fish maintained in heated seawater (8°C) and fed a minimum amount of capelin during what normally is the non-feeding period (November-March) and in flounder maintained under ambient conditions in March (water temperature ~0.6°C and not fed).

Factors affecting Zn^{2+} uptake:

In examining these parameters, unless otherwise specified, only one segment of the intestine, the uppermost portion including the pyloric appendages, was tied off.

Time

To describe the time course of Zn^{2+} uptake from the intestinal tract, ^{65}Zn and a 52 µg Zn^{2+} load were injected into the ligated intestine and left for periods ranging from 1 to 48 hours (n=5-7 fish per time period).

Zn^{2+} load in lumen

The effect of the amount of Zn^{2+} in the intestinal lumen on both the accumulation of Zn^{2+} in the tissue, and the transfer into the body, was examined for a fixed time period (5-7 hours) at loads of stable Zn^{2+} ranging from 5 to 530 µg (minimum of 5 fish for each Zn^{2+} load).

Other metals

To test the effect of other metals on Zn^{2+} uptake, ^{65}Zn, stable Zn^{2+}

(5 µg) and 0 (control, n=35) or 200 µg loads (as its chloride) of Cu^{2+} n=15; Mg^{2+} n=10; Ca^{2+} n=10; Ni^{2+} n=9; Fe^{3+} n=10; Mn^{2+} n=5; Hg^{2+} n=9; Co^{2+} n=6; Cd^{2+} n=10; Cr^{2+} n=10 (n = number of fish) were injected into the ligated intestine for a 4-5 hour exposure period.

The effect of Cu^{2+} on Zn^{2+} (5 µg) uptake was further examined using copper loads ranging from 10-200 µg (n=5 fish per load). The statistical significance of the effects of these metal loads on Zn^{2+} accumulation in the intestinal tissue and on Zn^{2+} transfer into the body was determined by analysis of variance and the least significant difference (lsd) test (Steel and Torrie 1960).

Amino acids, fructose

To test the effect of amino acids on Zn^{2+} uptake, stable Zn^{2+} (26 µg), ^{65}Zn and 0 (control) or 0.1 M loads of amino acids (proline, methionine, alanine, serine, histidine and glutamic acid) were injected into the ligated upper intestine (n=4 fish per test group; time period = 6-7 hours). The experiment was repeated using the same load of three of the amino acids (0.1 M methionine, proline and histidine) and one tenth of the above Zn^{2+} load for a shorter time period (1 1/2 hours) (4-5 fish per test group).

The effect of the addition of fructose (1.0 M) on Zn^{2+} uptake (35 µg Zn^{2+} load) was also examined (time period=4-5 hours; n=5 fish per test group).

Season

The seasonal uptake of Zn^{2+} (55 µg Zn^{2+} load, exposure period=5-7 hours) was examined at monthly intervals over a two-year period. Fish used in this study were collected from Conception Bay, Newfoundland. They were held in the laboratory without feeding for approximately one week before being tested.

Level of Zn^{2+} in the diet

To test the effect of diets with varying Zn^{2+} content on Zn^{2+} uptake, fish were fed food pellets (40% moisture, comprised of Silver Cup Fish Feed, manufactured by Murray Elevators, Murray, Utah. Guaranteed Analysis -- crude protein 38%, crude fat 5%, crude fibre 7%, ash 15%, with additions of cod liver oil (2%), carboxymethyl cellulose (3%) and distilled water) containing 60 µg Zn^{2+}/g wet weight (control) or pellets with $ZnCl_2$ added (600 µg Zn^{2+}/g wet weight). Both groups were fed the control diet for 2 weeks, followed by the control or high Zn^{2+} diet for 5 weeks, at a rate of approximately 2% of their body weight per day. After allowing 6 days for the gut to clear, uptake of Zn^{2+} (^{65}Zn plus a 52 µg Zn^{2+} load) from the ligated intestine, was examined in the two groups.

Body load

The effect of intravenous injections of 20-25% of the total body load of Zn^{2+} in the flounder (based on an estimated whole body concentration of 15 µg Zn^{2+}/g wet weight) or of an equivalent volume of saline solution (1% NaCl) on Zn^{2+} uptake from the ligated intestine was examined from 1 day to 5 months after the injection.

Analysis of stable Zn^{2+} content of tissues

Stable Zn^{2+} concentrations were determined on nitric acid digests of the tissue using flame atomic absorption (Fletcher and King 1978). Precautions were taken to prevent metal contamination of the samples. All glassware was washed, soaked overnight in 50% nitric acid and rinsed thoroughly with distilled water. All acids used for digestion were of Analar grade (BDH Chemicals Ltd.). Tissue samples (1-5 g wet weight) were weighed into flasks, dried at 90-100°C for 2-3 days, reweighed and

digested with 9 mL of nitric acid. Following the addition of the acid the digestion was allowed to proceed at room temperature for several days and then heated until white fumes evolved. The digests were diluted to 25 mL with distilled water. Digestion blanks were included in each run of samples. The concentration of Zn^{2+} was determined by flame atomic absorption (Varian Tectron AA 5) coupled to a Varian A-25 recorder. An air-acetylene flame was used to determine the Zn^{2+}; a standard curve was run with each analysis.

Determination of pH of lumen contents

The pH of the lumen contents (ie. of the supernatant obtained after centrifugation of the lumen contents) was determined using a micro-electrode pH meter (Radiometer).

Section B. *In vitro* technique

An equilibrium dialysis technique was used to study the binding of Zn^{2+} to the soluble proteins of the intestinal mucosa (Shears and Fletcher 1979).

Winter flounder (*Pseudopleuronectes americanus*) (30-40 cm long, 300-600 g) were obtained by divers equipped with SCUBA (Fletcher 1977) and held in [redacted] L aquaria under ambient conditions of temperature and light. They were fed chopped capelin throughout the feeding period of April to October (Fletcher and King 1978).

To prepare the mucosal supernatant, the fish were killed by a blow on the head and a section of the intestine from the pyloric caeca to the rectum was removed. The contents were gently squeezed out with forceps and the lumen flushed with ice cold saline (1% NaCl). The tissue was

kept ice cold throughout the mucosal cytosol isolation procedure. The intestine was cut open lengthwise and the mucosa scraped from the underlying tissue with a glass slide (Van Campen and Kowalski 1971). The mucosal tissue was homogenized in 4-5 volumes of buffer (175 mM NaCl, 10 mM TES [tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]pH 7.4) using a motor-driven, glass teflon, Potter Elvejhem homogenizer. The homogenate was spun at approximately 65,000 g for 2 hours (International Equip. Co. centrifuge model B-60) and the resultant supernatant tested for ^{65}Zn binding. The protein concentration of the supernatant was determined by a modified Lowry (Hartree 1972) or Biuret method (Layne 1957). Zinc concentrations were determined on 5% TCA (trichloroacetic acid) precipitated samples using an atomic absorption spectrophotometer (AA5 Varian Tectron) (Fletcher et al. 1975).

The binding of ^{65}Zn to the mucosal supernatant was determined by equilibrium dialysis (Hughes and Klotz 1963) as described by Fletcher and Fletcher (1978). Washed dialysis tubing was filled with one mL of the supernatant (diluted to 1.3-1.6 mg/mL protein) and placed in tubes containing 10 mL of buffer with added ^{65}Zn (approximately 0.1 µCi) and $ZnCl_2$ loads ranging from 0.08 to 5.00 µg Zn^{2+}/mL. Each sample was assayed in duplicate. The tubes were placed on a multipurpose rotator (Scientific Industries Inc. model 150V) and rotated at approximately 60 rpm for 72 hours. The bags were then removed from the outside solution, blotted and the contents emptied into test tubes. Aliquots of the inside and outside solutions were counted for ^{65}Zn in a gamma scintillation counter (Packard Model 578). The counts were used to determine the % of ^{65}Zn bound to the mucosal supernatant, to construct binding curves and

to determine association constants.

The % of metal bound was calculated as follows:

$$\% \text{ Bound} = 100\left[1 - \frac{\text{cpm } ^{65}\text{Zn/mL outside soln.}}{\text{cpm } ^{65}\text{Zn/mL inside soln.}}\right]$$ (Westphal 1971).

From this, the moles/L of Zn^{2+} bound i.e. [Zn^{2+} Bound] at different Zn^{2+}loads was determined. The slope of the line of the Scatchard-type plot [Zn^{2+} Bound]/[Zn^{2+} Unbound] versus [Zn^{2+} Bound] then gives (-K), where K=the association constant (Rosenthal 1967).

In addition to studying the effects of increasing Zn^{2+} loads on ^{65}Zn binding, the effects of 10 μM loads of a number of other metals were also studied ($FeCl_3$, $NiCl_2$, $CoCl_2$, $MgCl_2$, $CuCl_2$, $MnCl_2$, $HgCl_2$, $CrCl_2$, $CdCl_2$ and $CaCl_2$). The effects of increasing Cu^{2+} loads (0.0-1.8 μg Cu^2/mL) on the % of ^{65}Zn bound to the mucosal supernatant was also examined in one experiment using the standard buffer (TES) and repeated using a HEPES buffer (2-[N-2-hydroxyethyl-piperazin-N'-yl]ethanesulfonic acid).

Glassware and dialysis tubing were washed as described by Fletcher and Fletcher (1978). TES and HEPES buffers were purchased from Sigma Chemical Company; all other chemicals used were analytical reagent grade (BDH Chemicals). Radioisotopes (^{65}Zn, Specific activity 2-3 mCi/mg) were obtained from New England Nuclear.

Most of this study was carried out using feeding flounder during the summer months. However, two experiments were carried out on winter non-feeding fish to determine if there were any dramatic seasonal differences.

Section C. *In vivo* technique

In order to study zinc uptake in intact, feeding animals, flounder were fed diets containing the radioisotopes ^{65}Zn and a non-absorbed marker, ^{141}Ce (cerium-141) (New England Nuclear). According to theory, the site(s) and extent of Zn^{2+} absorption in the gastrointestinal tract should be apparent from the changes in ^{65}Zn relative to ^{141}Ce in the lumen contents (Miller and Cragle 1965).

Winter flounder (30-40 cm long; 300-800 g) used in the studies were obtained by divers equipped with SCUBA (Fletcher 1977). They were initially held in 40,000 L tanks under ambient conditions of temperature and light. They were fed chopped capelin from April to October--the feeding period (Fletcher and King 1978).

Preliminary studies were conducted to determine the suitability of radiolabelled cerium as a non-absorbed dietary marker in fish. Winter flounder used in these studies were maintained in individual 10-15 L tanks and fed chopped capelin. In several fish, ^{141}Ce in saline solution was injected directly into the stomach (using a surgical procedure similar to that used in the *in situ* studies). The flounder were dissected 2, 3 and 5 days after the injection. The levels of ^{141}Ce in several internal organs, a blood sample, the gastrointestinal tissue, the contents of the gastrointestinal tract, a water sample and the fecal material which had settled to the bottom of the tank, were determined by counting the samples in a gamma scintillation counter (Packard model 578). The ^{141}Ce levels in several tissues and in the contents of the gastrointestinal tract were also determined 6, 9, 12, 18 and 72 hours after feeding the flounder pieces of capelin which had been injected with ^{141}Ce.

The $^{65}Zn/^{141}Ce$ feeding studies were carried out during the period of July to October. 8-12 flounder were placed in 250 L tanks supplied with flowing seawater under ambient conditions of temperature and photoperiod. The fish were fed chopped capelin (gutted, head and tail removed) daily (at rates of 4 to 6% of their total body weight) for 4 to 5 weeks (total feeding period). Pieces of capelin were radiolabelled by injecting a few microliters of solution containing the two isotopes into the muscle. Radiolabelled capelin was fed to the flounder for the last 18-19 days of the total feeding period.

In another experiment ^{65}Zn and ^{141}Ce were incorporated into food pellets comprised of Silver Cup Fish Feed, cod liver oil (2%), carboxymethyl cellulose (3%) and distilled water (40% moisture). Flounder were fed this diet at a rate of approximately 2.5% of their body weight per day for a total of 4 weeks. Radiolabelled pellets were fed for the last 18 days of the feeding period.

On the day following the last feeding, each fish was killed by a blow on the head, bled from a caudal blood vessel and the digestive tract tied off *in situ* into the following regions: stomach, upper intestine (includes the pyloric appendages), mid intestine (divided into two portions in some instances), lower intestine and the rectum. The contents in the lumen of these regions were emptied into counting vials; each of the gastrointestinal sections were also placed in separate vials. The samples were counted in a dual channel gamma scintillation counter (Packard model 578). Appropriate corrections were made for the spill of energy from the ^{65}Zn into the ^{141}Ce counting channel (principal Photon energy of ^{141}Ce=0.145 MeV; ^{65}Zn=1.12 MeV). The radioactivity in a blood sample, and in the liver, kidney and several other tissues was also determined for each fish.

The data for the lumen contents of each segment were expressed as the ratio of ^{65}Zn to ^{141}Ce in the contents, relative to the ratio of ^{65}Zn to ^{141}Ce in the diet. According to theory, if the ratio of ^{65}Zn to ^{141}Ce in the contents, relative to the ratio of ^{65}Zn to ^{141}Ce in the diet, remains unchanged (i.e. $\frac{^{65}Zn/^{141}Ce \text{ in the contents}}{^{65}Zn/^{141}Ce \text{ in the diet}} = 1$), this indicates that no net absorption or secretion of ^{65}Zn has taken place in the segment; if the ratio of ^{65}Zn to ^{141}Ce in the contents is greater than the ratio of ^{65}Zn to ^{141}Ce in the diet (i.e. $\frac{^{65}Zn/^{141}Ce \text{ in the contents}}{^{65}Zn/^{141}Ce \text{ in the diet}} > 1$), this indicates that a net secretion of ^{65}Zn has taken place in the segment; if the ratio of ^{65}Zn to ^{141}Ce in the contents is less than the ratio of ^{65}Zn to ^{141}Ce in the diet (i.e. $\frac{^{65}Zn/^{141}Ce \text{ in the contents}}{^{65}Zn/^{141}Ce \text{ in the diet}} < 1$), this indicates that a net absorption of ^{65}Zn has taken place. The % net ^{65}Zn absorption or secretion by the flounder was calculated by subtracting the $^{65}Zn/^{141}Ce$ ratio in the rectum contents (i.e. the ratio of ^{65}Zn to ^{141}Ce in the rectum contents, relative to the ratio of ^{65}Zn to ^{141}Ce in the diet), expressed as a %, from 100%. A ratio greater than unity would yield a negative absorption value, indicating net secretion.

The stable Zn^{2+} (where possible) and the dry weights of the lumen contents of the different segments were also determined. The data were expressed as a ratio of the ^{141}Ce in the contents and the methodology described above used to determine the site(s) and extent of absorption and secretion. The % net absorption or secretion of stable Zn^{2+} and dry material by the flounder was calculated by subtracting the appropriate ratio in the rectum contents (i.e. $\frac{\text{dry material}/^{141}Ce \text{ in the contents}}{\text{dry material}/^{141}Ce \text{ in the diet}}$; $\frac{\text{stable } Zn^{2+}/^{141}Ce \text{ in the contents}}{\text{stable } Zn^{2+}/^{141}Ce \text{ in the diet}}$), expressed as a %, from 100%.

The effect of injections (i.p.) of Zn^{2+} ($ZnCl_2$ in 1% NaCl, 25% of the estimated total body Zn^{2+} load) or an equivalent volume of saline (1% NaCl) on the net absorption of Zn^{2+} from the diet was also studied using the non-absorbed marker technique. Flounder were fed chopped capelin for one month before being injected (i.p.) with Zn^{2+} or saline. The feeding was continued for 14 days, with $^{65}Zn/^{141}Ce$ labelled capelin being fed for the last 7 days of the study. The day after the last feeding the radioactivity remaining in the gastrointestinal contents and in selected tissues was determined as described above. The contents of the tract (from the upper, mid and low intestine) were also examined to determine if ^{65}Zn and ^{141}Ce were present in the same compartment. The contents were rinsed from the counting vials into centrifuge tubes with approximately 3 mL of seawater. The samples were spun for 10 minutes at 12,000 g (Sorval Centrifuge). The cpm ^{65}Zn and ^{141}Ce were determined in the resulting supernatants and precipitates. The supernatants were subsequently treated with TCA (trichloroacetic acid, final concentration 12.5%) and the radioactivity determined in the resulting supernatants and precipitates.

The stable Zn^{2+} content of the capelin, the food pellets, the gastrointestinal tract contents and several of the tissues was determined (following digestion with nitric acid) using an atomic absorption spectrophotometer (AA5 Varian Tectron).

RESULTS

Section A. *In situ* technique

Site(s) of uptake

In flounder examined during the summer feeding period, the amount of ^{65}Zn and stable Zn^{2+} accumulated by the gastrointestinal tract tissue and transferred into the body was considerably greater in intestinal areas than in the stomach. The amount of ^{65}Zn and stable Zn^{2+} accumulated by and transferred across the tract declined from the upper to the lower intestine (Fig. 1 A,B). This trend was also observed in fish maintained in heated seawater and fed during February and March, months in which flounder do not normally feed (Fig. 2). However, in normal March fish, which had not been fed since the previous October, there was very little difference in the extent of tissue accumulation between any of the intestinal areas (Fig. 3). Transfer of Zn^{2+} into the body was very low in these flounder and no difference between any of the areas of the intestine was apparent (Fig. 3).

The lumen contents of the different segments of the intestine were alkaline (pH 8.0-8.6) and no trends in pH attributable to the site in the intestine, the Zn^{2+} load, or whether or not the flounder had been feeding, were apparent (Table 1).

Factors affecting Zn^{2+} uptake:

Time

The upper intestinal tissue accumulated approximately 20% of the total amount of ^{65}Zn injected into the intestinal lumen within the first hour. This proportion remained essentially the same throughout the period of observation (48 hours, Fig. 4B). Very little ^{65}Zn was

Fig. 1. The uptake of ^{65}Zn plus Zn^{2+} (1A) and ^{65}Zn (1B) by different segments of the digestive tract. The digestive tract was ligated into sections *in situ*. A load of Zn^{2+} (50 µg) plus ^{65}Zn (A) or ^{65}Zn alone (B) was injected into the lumen of each segment and left for 7-8 hours. ^{65}Zn was counted in the intestinal tissue and in the lumen contents. Stable Zn^{2+} was computed from the cpm and the specific activity of the injected ^{65}Zn. Accumulated Zn^{2+} and ^{65}Zn was that associated with the intestinal tissue. Transferred Zn^{2+} and ^{65}Zn was that computed from the difference between the amount of ^{65}Zn injected and the amount recovered from the tract and the lumen contents. Values plotted are $\bar{X} \pm SE$ (1A, n=7 fish; 1B, n=5 fish).

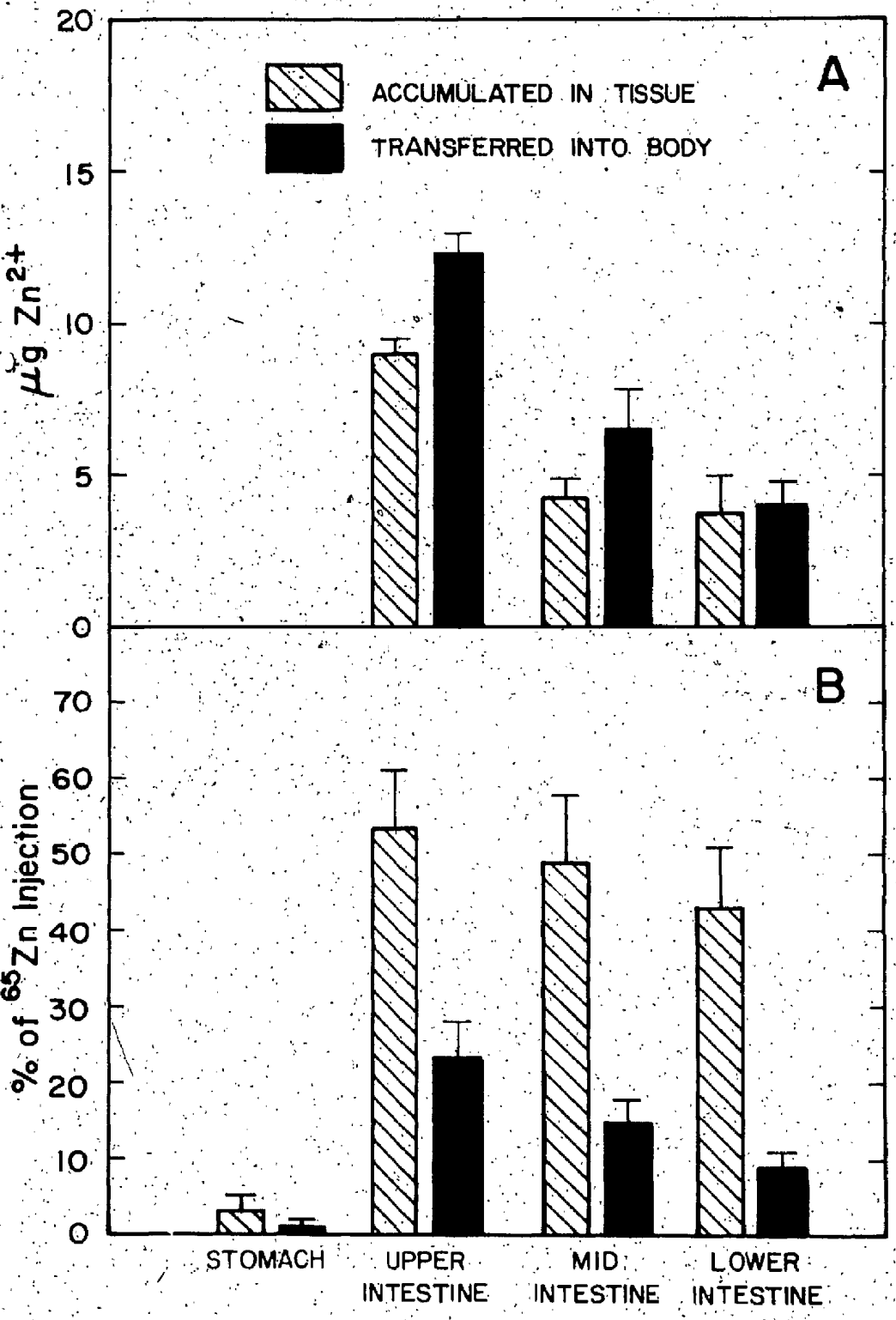

A
ACCUMULATED IN TISSUE
TRANSFERRED INTO BODY
μg Zn2+
20
15
10
5
0
B
% of 65Zn Injection
70
60
50
40
30
20
10
0
STOMACH
UPPER INTESTINE
MID INTESTINE
LOWER INTESTINE

Fig. 2. The uptake of ^{65}Zn plus Zn^{2+} by different segments of the digestive tract of flounder maintained in heated seawater (8°C) and fed during what normally is a non-feeding period (November-March). The uptake studies were conducted in February and March. The digestive tract was ligated into sections *in situ*. Loads of stable Zn^{2+} ranging from 2.5 to 250 µg plus ^{65}Zn were injected into the lumen of each segment and left for 8 hours. ^{65}Zn was counted in the intestinal tissue and in the lumen contents. Stable Zn^{2+} was computed from the cpm and the specific activity of the injected ^{65}Zn. Accumulated Zn^{2+} was that associated with the intestinal tissue. Transferred Zn^{2+} was that computed from the difference between the amount of ^{65}Zn injected and the amount recovered from the tract and the lumen contents. Values plotted are $\bar{X} \pm SE$ (5-7 fish per each Zn^{2+} load).

Fig. 3. The uptake of ^{65}Zn and Zn^{2+} by different segments of the digestive tract of flounder maintained under ambient conditions. The fish were examined in March (water temp.= -0.6°C); feeding had been terminated in October of the preceeding year. The digestive tract was ligated into sections *in situ*. Loads of stable Zn^{2+} ranging from 2.5 to 255 µg plus ^{65}Zn were injected into the lumen of each segment and left for 8 hours. ^{65}Zn was counted in the intestinal tissue and in the lumen contents. Stable Zn^{2+} was computed from the cpm and the specific activity of the injected ^{65}Zn. Accumulated Zn^{2+} was that associated with the intestinal tissue. Transferred Zn^{2+} was that computed from the difference between the amount of ^{65}Zn injected and the amount recovered from the tract and the lumen contents. Values plotted are $\bar{X} \pm SE$ (4-5 fish per each Zn^{2+} load).

Fig 2

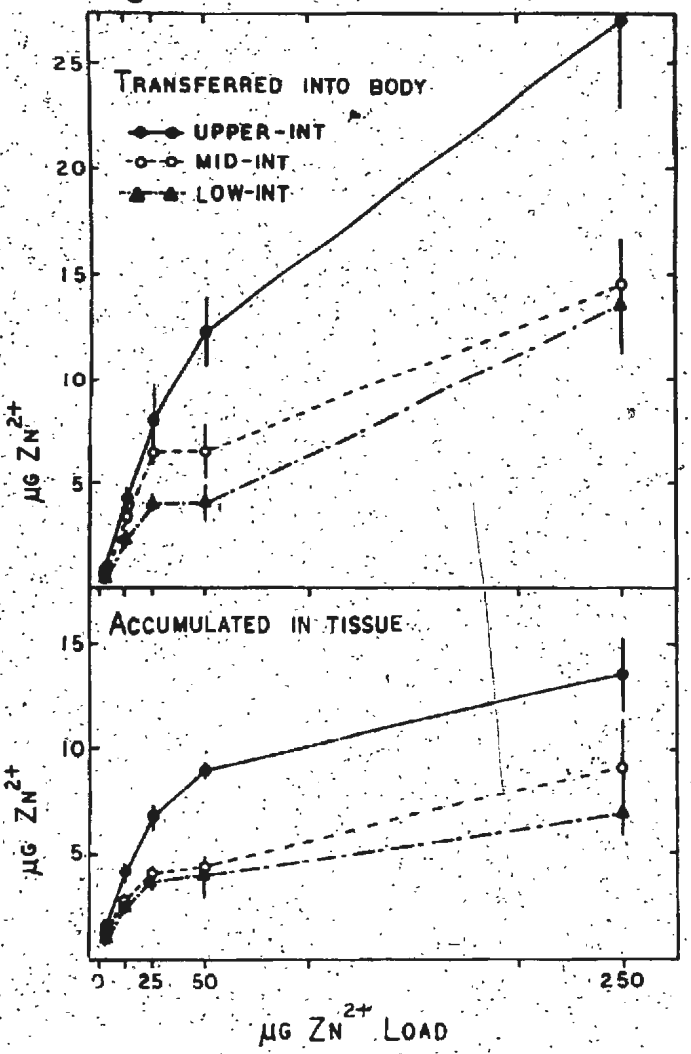

TRANSFERRED INTO BODY
UPPER-INT
MID-INT
LOW-INT
ACCUMULATED IN TISSUE
μG Zn2+
μG Zn2+ LOAD
0
25
50
250
5
10
15
20
25

Fig 3

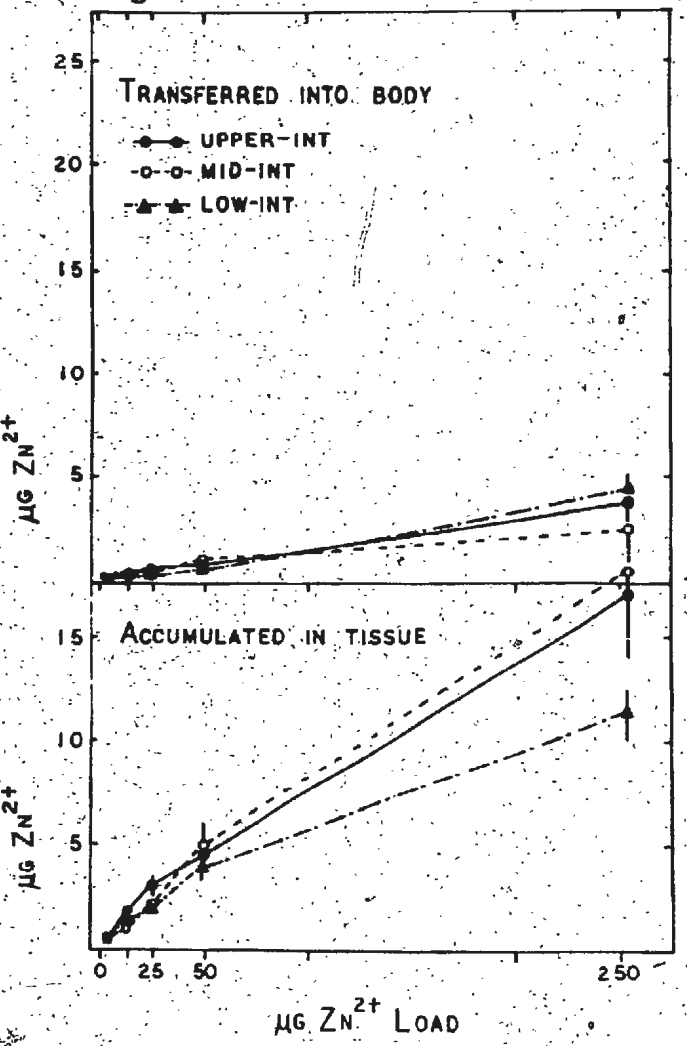

TRANSFERRED INTO BODY
UPPER-INT
MID-INT
LOW-INT
ACCUMULATED IN TISSUE
μG Zn2+
μG Zn2+ LOAD
0
25
50
250
5
10
15
20
25

Table 1. pH of contents[a] of different segments of the digestive tracts of:

A. Fish maintained in heated seawater (8°C) and fed during what normally is a non-feeding period (January-March). The digestive tract was ligated into sections *in situ*. Loads of stable Zn^{2+} ranging from 2.5 to 250 µg were injected into the lumen and left for 8 hours. Values are the mean of 2 fish per Zn^{2+} load.

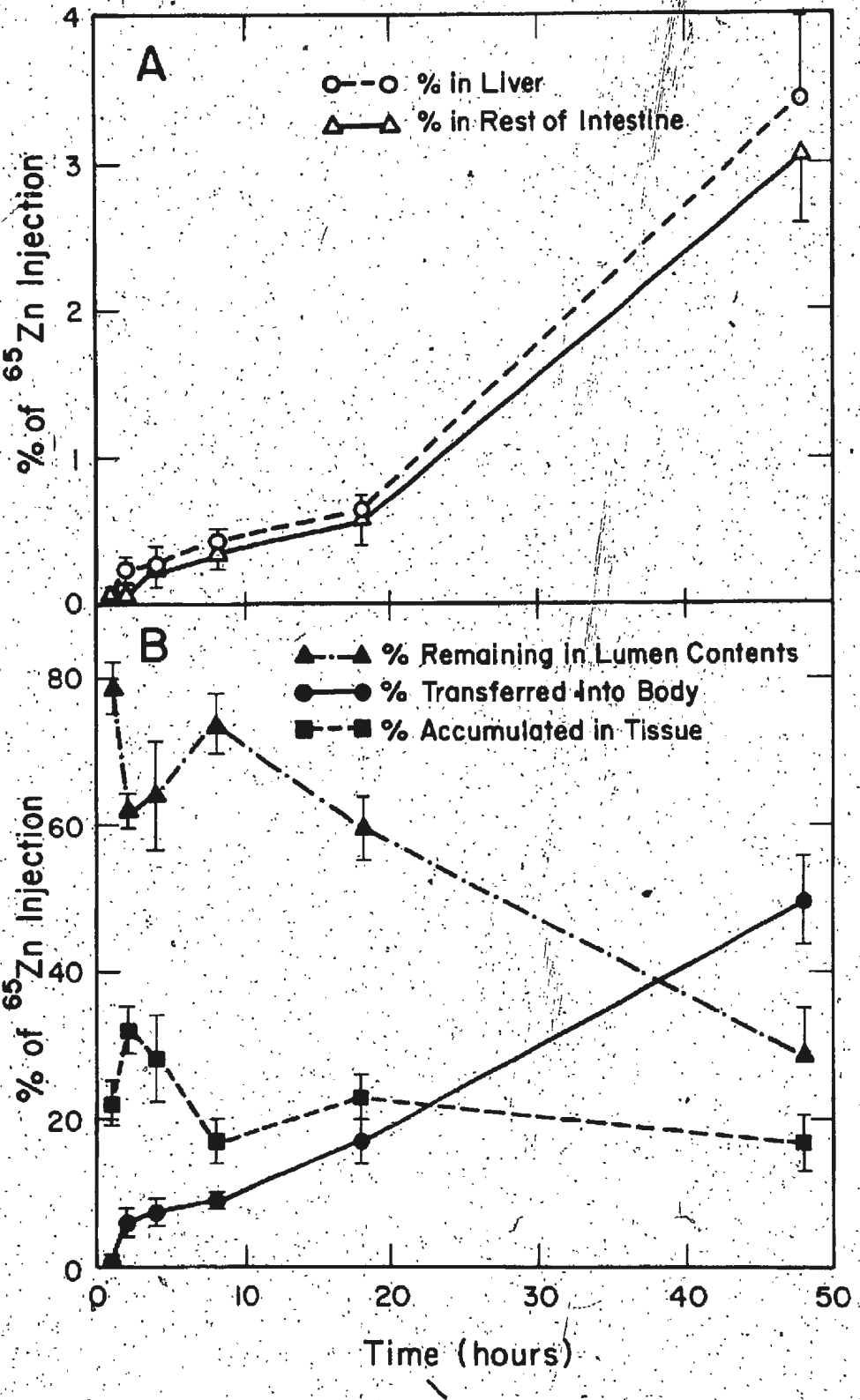
Zn^{2+} load (µg)	pH		
	Upper-intestine	Mid-intestine	Low-intestine
2.5	8.22	8.40	8.43
12.5	8.60	8.50	8.30
25.0	8.05	8.00	8.25
50.0	8.30	8.30	8.45
250.0	8.60	8.50	8.55

B. Fish maintained under ambient conditions of water temperature and not fed from October until March when the study was conducted. The digestive tract was ligated into sections *in situ*. A 25 µg load of Zn^{2+} was injected into the lumen and left for 8 hours. Values represent the $\bar{X} \pm SE$ of 5 fish.

Zn^{2+} load (µg)	Upper-intestine	Mid-intestine	Low-intestine
25	8.39 ± 0.09	8.47 ± 0.04	8.42 ± 0.07

[a]The lumen contents were spun at 3000 rpm for 10 minutes and the pH of the supernatant determined using a micro-electrode pH meter.

Fig. 4. (A) Accumulation of ^{65}Zn by the liver and intestine from a ligated portion of the upper intestine. The upper intestine (including the pyloric appendages) was ligated *in situ* and a load of Zn^{2+} (52 µg) plus ^{65}Zn was injected into the lumen. The liver and the non-ligated portion of the intestine were removed from the fish and counted for radioactivity at the times indicated. Values plotted are $\bar{X} \pm SE$ (5-7 fish per time point), and (B) Time course of ^{65}Zn accumulation by the intestine and its transfer into the body from a ligated upper portion of the digestive tract (see Fig. 4 (A)). The ligated portion of the intestine and its contents were counted for radioactivity at the times indicated. The percentage of ^{65}Zn transferred into the body was the difference between the cpm injected and the cpm recovered in the ligated section of the tract and its contents. Values plotted are $\bar{X} \pm SE$ (5-7 fish per group).


A
% in Liver
% in Rest of Intestine
% of 65Zn Injection
4
3
2
1
0
B
% Remaining in Lumen Contents
% Transferred into Body
% Accumulated in Tissue
% of 65Zn Injection
80
60
40
20
0
0
10
20
30
40
50
Time (hours)

transferred into the body in the first hour of exposure (i.e. no ^{65}Zn was transferred in 3 of the 6 fish examined). Thereafter, the amount of ^{65}Zn transferred from the intestinal segment to the rest of the body increased in a lineaf fashion (coefficient of linear regression, r=0.94, df=33) (Fig. 4B). This was reflected by the % of injected ^{65}Zn appearing in the rest of the intestinal tract and in the liver (Fig. 4A).

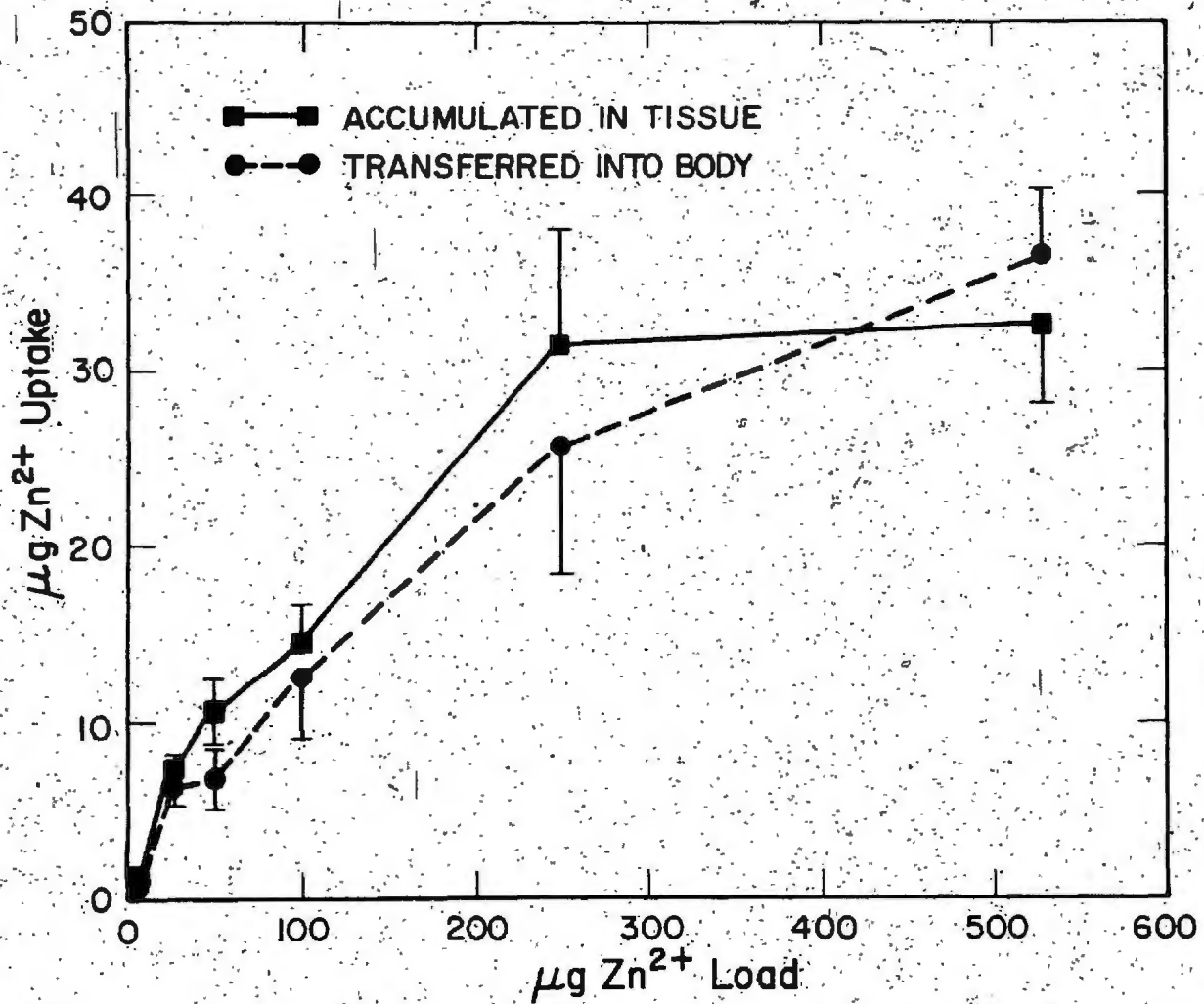
Zn Load

When loads of 5 to 530 µg of Zn^{2+} were injected into the upper intestine, both the amount of zinc accumulated in the tissue and the amount transferred into the body increased with increasing loads (Fig. 5). While the relationship between the amount of Zn^{2+} in the lumen and the amount transferred into the body did not appear to be linear, saturation did not appear to have been reached at the highest load tested. When data from all loads (n=52) were combined, statistical analysis revealed that the amount of Zn^{2+} transferred into the body was directly proportional to the amount of Zn^{2+} accumulated in the intestinal tissue (Fig. 6). The regression line (r=0.93) can be expressed by the equation $y=0.96\ x\ -0.88$, where y=µg Zn^{2+} transferred into the body and x=the µg Zn^{2+} accumulated in the tissue.

Other Metals

200 µg loads of Cu^{2+}, Ni^{2+}, Fe^{3+}, Mn^{2+}, Hg^{2+}, Co^{2+}, Cd^{2+}, and Cr^{2+} significantly ($P < 0.05$, lsd test) depressed tissue accumulation of a 5 µg load of Zn^{2+} in the ligated upper portion of the intestine. The amount of Zn^{2+} transferred across the tract was significantly depressed ($P < 0.05$, lsd test) by Cu^{2+}, Ni^{2+}, Fe^{3+}, Co^{2+}, Cd^{2+}, and Cr^{2+} (Fig. 7 A,B).

Fig. 5. The effect of Zn^{2+} loads on accumulation of ^{65}Zn and its transfer from the intestine into the body. The upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. Loads of stable Zn^{2+} ranging from 5-530 µg and ^{65}Zn were injected into the tied-off segment. Five to seven hours later the ligated segment was removed from the fish and the intestinal tissue and contents counted for radioactivity. Zn^{2+} accumulation was the amount of Zn^{2+} (cpm ^{65}Zn) associated with the ligated segment of intestinal tissue. Zn^{2+} transfer was the difference between the total ^{65}Zn injected and the amount found in the ligated segment and its contents. Values plotted are $\bar{X} \pm SE$ (minimum of 5 fish per group).


ACCUMULATED IN TISSUE
TRANSFERRED INTO BODY
μg Zn2+ Uptake
μg Zn2+ Load
0
10
20
30
40
50
0
100
200
300
400
500
600

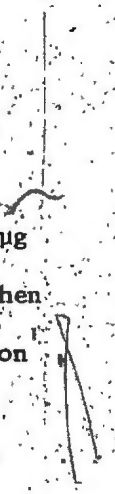


Fig. 6. Relationship between μg Zn^{2+} transferred into the body and μg Zn^{2+} accumulated in the intestinal tissue. Based on data obtained when Zn^{2+} loads from 5-530 μg were injected into the tied-off upper portion of the intestine (see Fig. 5).

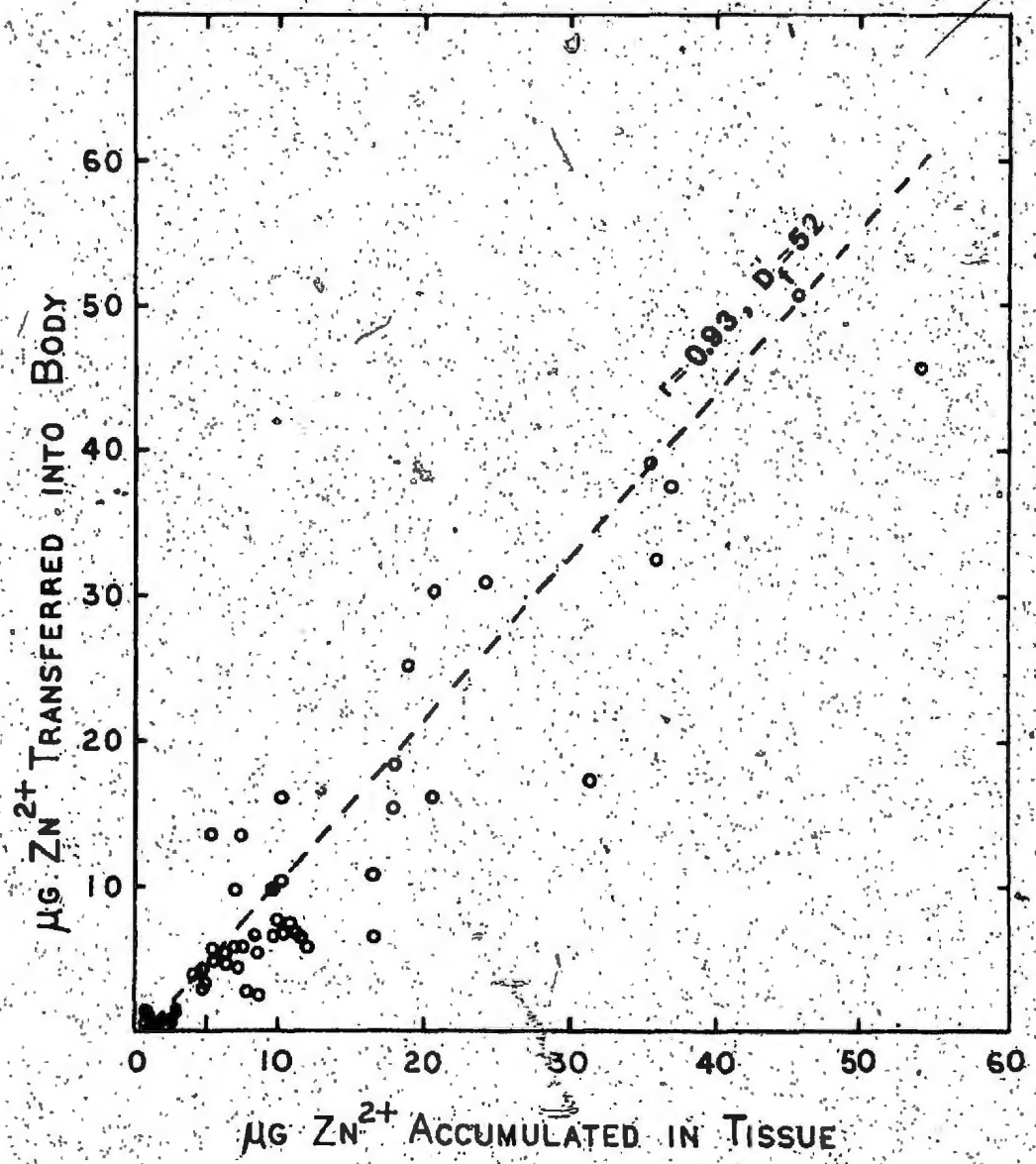
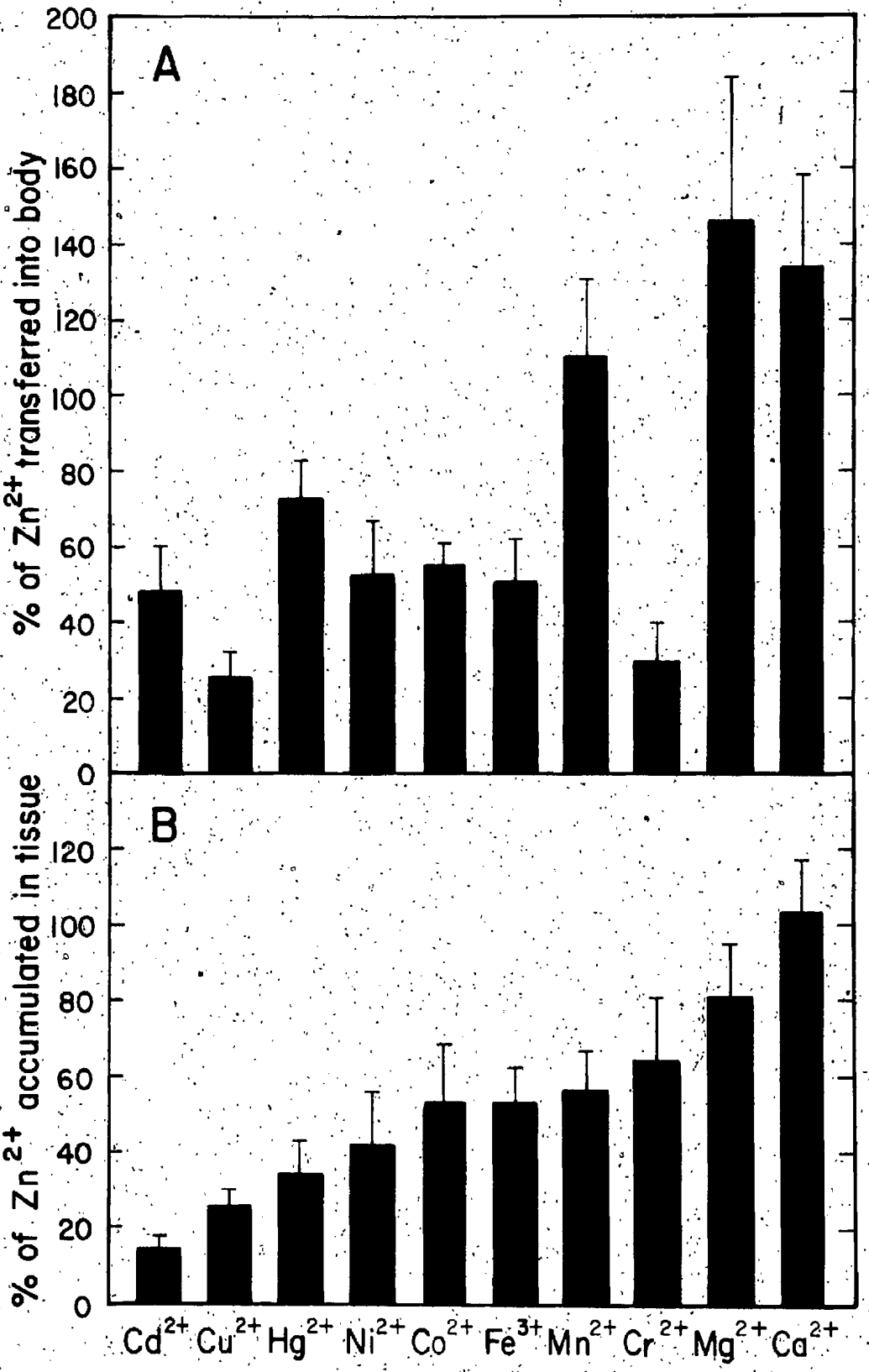

μG. Zn2+ Transferred into Body
μG Zn2+ Accumulated in Tissue
r=0.93, D.F.=52
0
10
20
30
40
50
60

Fig. 7. The effects of metal loads on the accumulation of Zn^{2+} by the intestine and on its transfer into the body. The upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. Stable Zn^{2+} (5 µg) plus ^{65}Zn and 0 (control, n=35) or 200 µg loads of Cu^{2+}, n=15; Mg^{2+}, n=10; Ca^{2+}, n=10; Ni^{2+}, n=9; Fe^{3+}, n=10; Mn^{2+}, n=5; Hg^{2+}, n=9; Co^{2+}, n=6; Cd^{2+}, n=10; Cr^{2+}, n=10 (n=number of fish) were injected into the ligated segment. Four to five hours later the ligated segment was removed from the fish and the intestinal tissue and the contents counted for radioactivity. Zn^{2+} accumulation and transfer was computed from the cpm and the specific activity of the injected ^{65}Zn. Zn^{2+} accumulation was the amount of Zn^{2+} associated with the ligated section of intestinal tissue. Zn^{2+} transferred was computed as the difference between the amount of Zn^{2+} injected into the lumen and the amount associated with the ligated portion of the intestine and its contents. The percentage of Zn^{2+} accumulated or transferred $= \frac{Zn^{2+}\ (\text{load})}{Zn^{2+}\ (\text{no load})} \times 100$, where Zn^{2+} (load) is the amount of Zn^{2+} accumulated or transferred in the presence of a 200 µg metal load and Zn^{2+} (no load) is the amount of Zn^{2+} accumulated or transferred in the absence of a metal load. Values plotted are $\bar{X} \pm SE$.


A
B
% of Zn^{2+} transferred into body
% of Zn^{2+} accumulated in tissue
0
20
40
60
80
100
120
140
160
180
200
Cd^{2+} Cu^{2+} Hg^{2+} Ni^{2+} Co^{2+} Fe^{3+} Mn^{2+} Cr^{2+} Mg^{2+} Ca^{2+}

Copper significantly ($P < 0.05$, lsd test) depressed the amount of zinc accumulated by the tissue (by 33%) and the amount transferred across the intestinal wall (by 57%) at Cu^{2+} loads as low as 5 times the Zn^{2+} load (i.e. 5 µg Zn^{2+}, 25 µg Cu^{2+}) (Fig. 8). Loads of 10 or 15 µg Cu^{2+} had no significant effect on Zn^{2+} uptake.

Amino acids, fructose

Histidine (0.1 M) significantly depressed the amount of Zn^{2+} (Zn^{2+} load 26 µg) accumulated in the intestinal tissue and transferred into the body (Fig. 9). None of the other amino acids tested had any effect on the accumulation or transfer of Zn^{2+} under these experimental conditions. When the experiment was repeated, using the same load of three of the amino acids (0.1 M methionine, proline and histidine) with a lower Zn^{2+} load (one tenth of the amount in the above experiment) for a shorter time period (1 1/2 hours), the amount of Zn^{2+} accumulated and transferred was lower in the presence of histidine but the effect was not statistically different from that of the other treatments. An examination of the pH of the injected solutions and the lumen contents revealed that while the test solutions, with the exception of that containing histidine (pH 7.5), were originally acidic (pH 2.8-4.0) the lumen contents at the end of the experiment were all alkaline (pH 7.9-8.3).

The addition of fructose to the intestinal lumen did not have any significant effect on the accumulation of Zn^{2+} in the intestinal tissue or on its transfer into the body.

Season

There was a seasonal difference in the Zn^{2+} uptake from the gastrointestinal tract of fish recently brought in from the field (i.e. one

Fig. 8. The effects of Cu^{2+} on the accumulation of Zn^{2+} by the intestine and on its transfer into the body. The upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. Stable Zn^{2+} (5 µg), ^{65}Zn and 0 (control) to 200 µg loads of Cu^{2+} were injected into the ligated segment. Four to five hours later the ligated segment was removed from the fish and the intestinal tissue and the contents counted for radioactivity. Zn^{2+} accumulation and transfer were computed from the cpm and specific activity of the injected ^{65}Zn. Zn^{2+} accumulation was the amount of Zn^{2+} associated with the ligated segment of intestinal tissue. Zn^{2+} transfer was computed from the difference between the amount of Zn^{2+} injected into the lumen and the amount found associated with the ligated portion of the intestine and its contents. Values plotted are $\bar{X} \pm SE$ (5 fish per group).

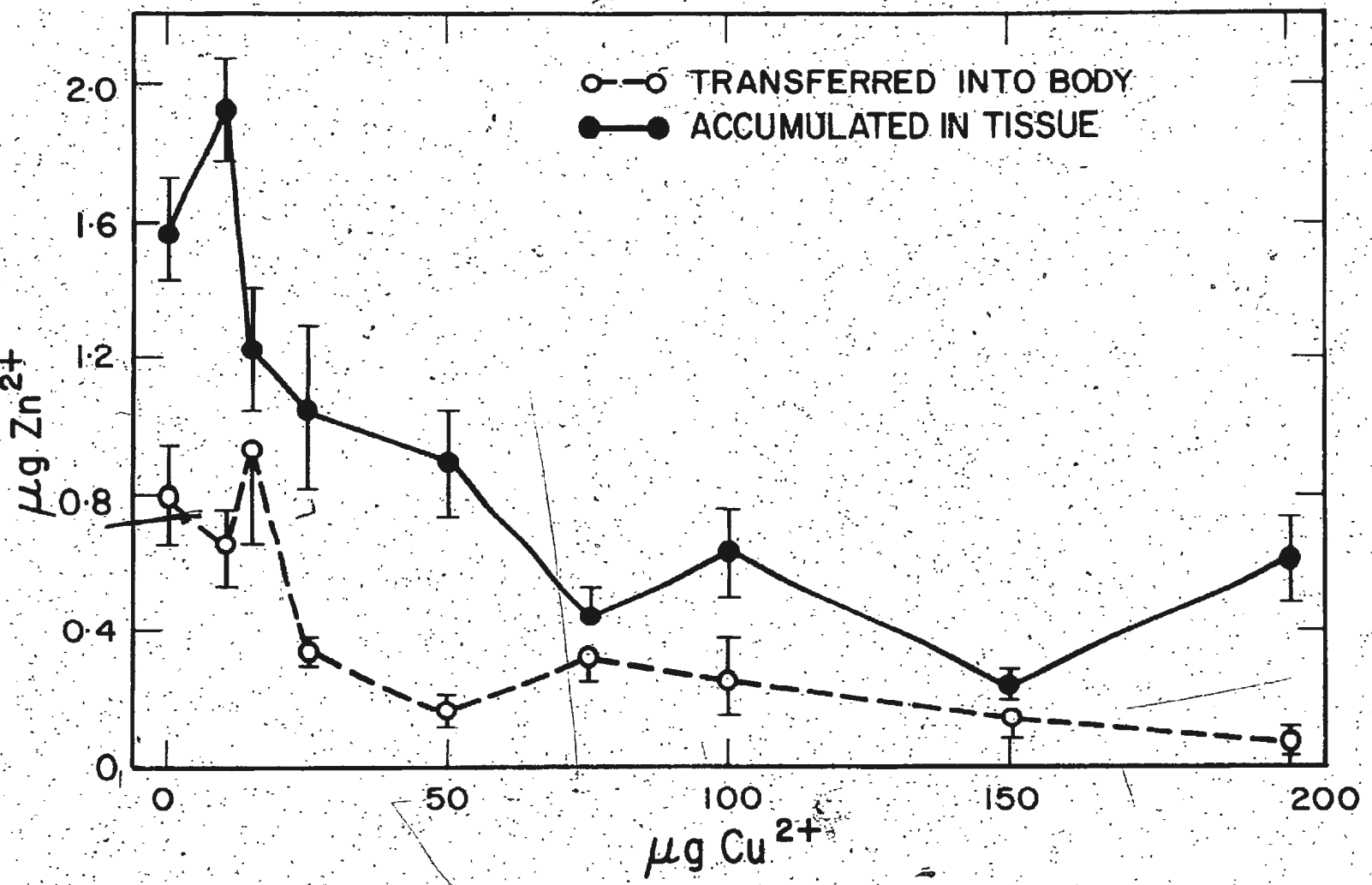
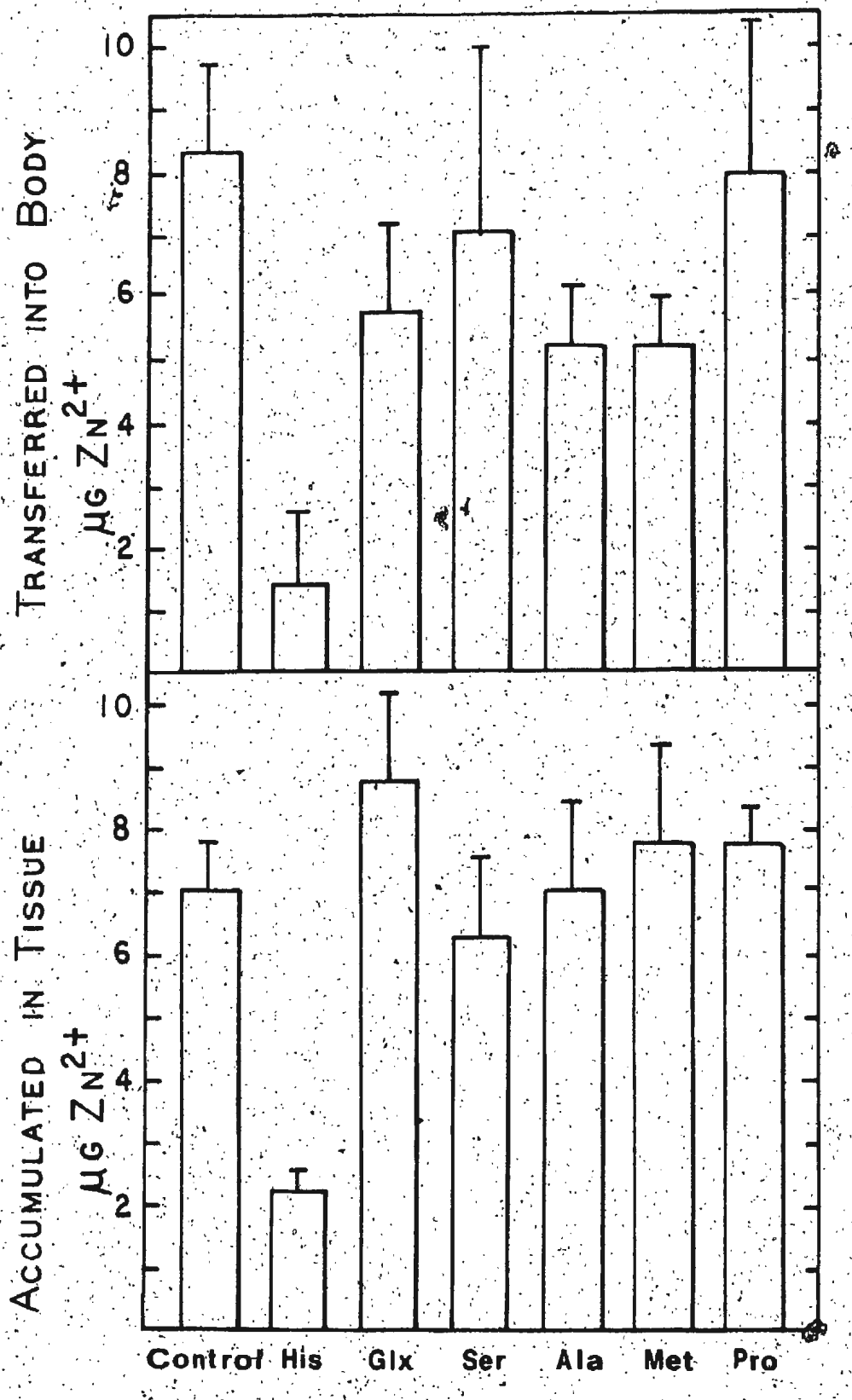

TRANSFERRED INTO BODY
ACCUMULATED IN TISSUE
2·0
1·6
1·2
0·8
0·4
0
μg Zn2+
0
50
100
150
200
μg Cu2+

Fig. 9. The effect of 0.1 M loads of amino acids on Zn^{2+} accumulation by the intestine and on its transfer into the body. The upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. Stable Zn^{2+} (26 µg), ^{65}Zn and 0 (control) or 0.1 M loads of amino acids (histidine, glutamic acid, serine, alanine, methionine and proline) were injected into the ligated segment. Six to seven hours later the ligated segment was removed from the fish and the intestinal tissue and contents counted for radioactivity. Zn^{2+} accumulation and transfer were computed from the cpm and the specific activity of the injected ^{65}Zn. Zn^{2+} accumulation was the amount of Zn^{2+} associated with the ligated section of intestinal tissue. Zn^{2+} transferred was computed as the difference between the amount of Zn^{2+} injected into the lumen and the amount associated with the ligated portion of the intestine and its contents. Values plotted are $\bar{X} \pm SE$. Four fish were examined in each test group.


Transferred into Body
μg Zn^{2+}
10
8
6
4
2
Accumulated in Tissue
μg Zn^{2+}
10
8
6
4
2
Control
His
Glx
Ser
Ala
Met
Pro

week earlier). Both the amount of Zn^{2+} accumulated in the tissue and transferred into the body from the ligated upper intestine peaked during the summer months (Fig. 10B). Peak values for tissue accumulation were at least three times, and transfer into the body six times, greater than the lowest values. Feeding activity, as evidenced by the presence of food material in the digestive tract of at least one fish sampled from the field, was initiated in April and terminated in November. The amount of food material in the gut (expressed as a percentage of the body weight) reached a maximum in July, remained high in August and fell off by September (Fig. 10A). There was also a seasonal fluctuation in the stable Zn^{2+} concentration (μg/g dry weight) of the intestinal tissue (Fig. 11). The stable Zn^{2+} level reached a low in February-March, increasing 20-30% in April. The moisture content of the intestinal tissue varied by one to four percent between different months throughout the year, a peak of 87% moisture being reached in August (Fig. 11).

The accumulation and transfer of Zn^{2+} in flounder held in the laboratory and maintained under ambient conditions in July was similar to that observed in fish maintained in heated seawater (8°C) and fed during what normally is the non-feeding period (November-March) (Table 2). The accumulation of Zn^{2+} in the tissue was approximately twice and the transfer of Zn^{2+} into the body approximately ten times greater than that observed in laboratory fish examined in March. Transferring fish from ambient water (0°C) in April to heated seawater (8°C) resulted in no change in intestinal tissue accumulation of Zn^{2+} but a four-fold increase in transfer was observed (Table 2).

Fig. 10. (A) Seasonal changes in gut content weight. The winter flounder were killed immediately after capture and the contents of the whole digestive tract weighed. The water temperature was recorded in the area where the fish were caught (Fletcher 1977), and (B) Seasonal changes in accumulation of Zn^{2+} in the intestine and in its transfer into the body. Winter flounder were caught at approximately monthly intervals and maintained in laboratory aquariums for seven days without feeding. The upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. Stable Zn^{2+} (55 µg) and ^{65}Zn were injected into the ligated segment. Five to seven hours later the ligated segment was removed from the fish and the intestinal tissue and the contents counted for radioactivity. Zn^{2+} accumulation and transfer were computed from the cpm and specific activity of the ^{65}Zn injected. Zn^{2+} accumulation was the amount of Zn^{2+} associated with the ligated segment of intestinal tissue. Zn^{2+} transfer was computed from the difference between the amount of Zn^{2+} injected into the lumen and the amount found associated with the ligated portion of the intestine and its contents. Values plotted are $\bar{X} \pm SE$. There were no differences in Zn^{2+} uptake in the two years examined so monthly data were consolidated. Four to 19 fish were tested each month.

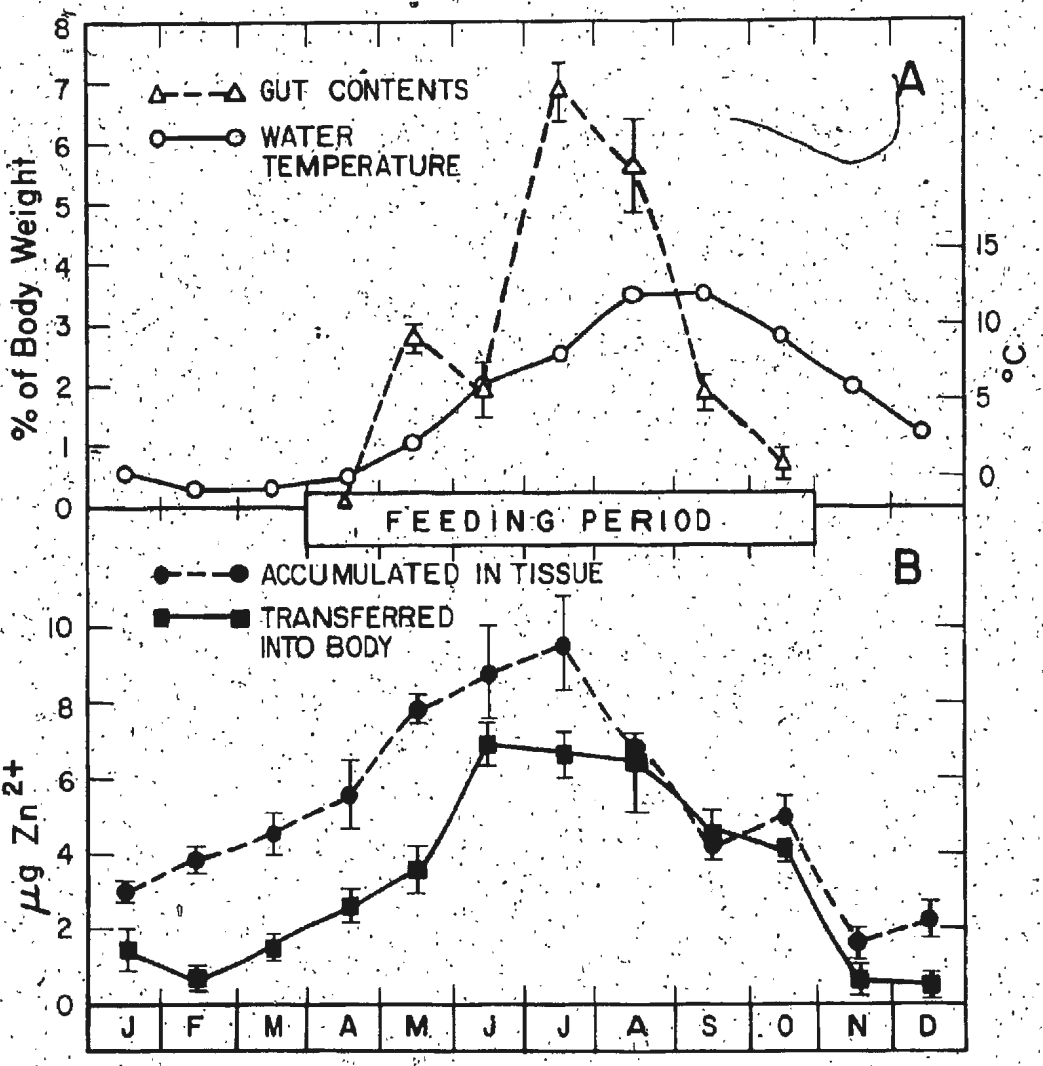

GUT CONTENTS
WATER TEMPERATURE
A
% of Body Weight
°C
FEEDING PERIOD
ACCUMULATED IN TISSUE
TRANSFERRED INTO BODY
B
μg Zn 2+
J F M A M J J A S O N D

Fig. 11. Seasonal changes in the stable Zn^{2+} (µg/g) concentration (as determined by flame atomic absorption) and percentage moisture content of intestinal tissue. The stable Zn^{2+} concentrations (µg/g dry weight) were determined in samples of intestinal tissue taken from the same fish which had been used to examine seasonal uptake of Zn^{2+} (Fig. 10). The % moisture of the samples were also determined ($\frac{\text{dry weight}}{\text{wet weight}} \times 100$). Values plotted are $\bar{X} \pm SE$; 5-15 fish per month.

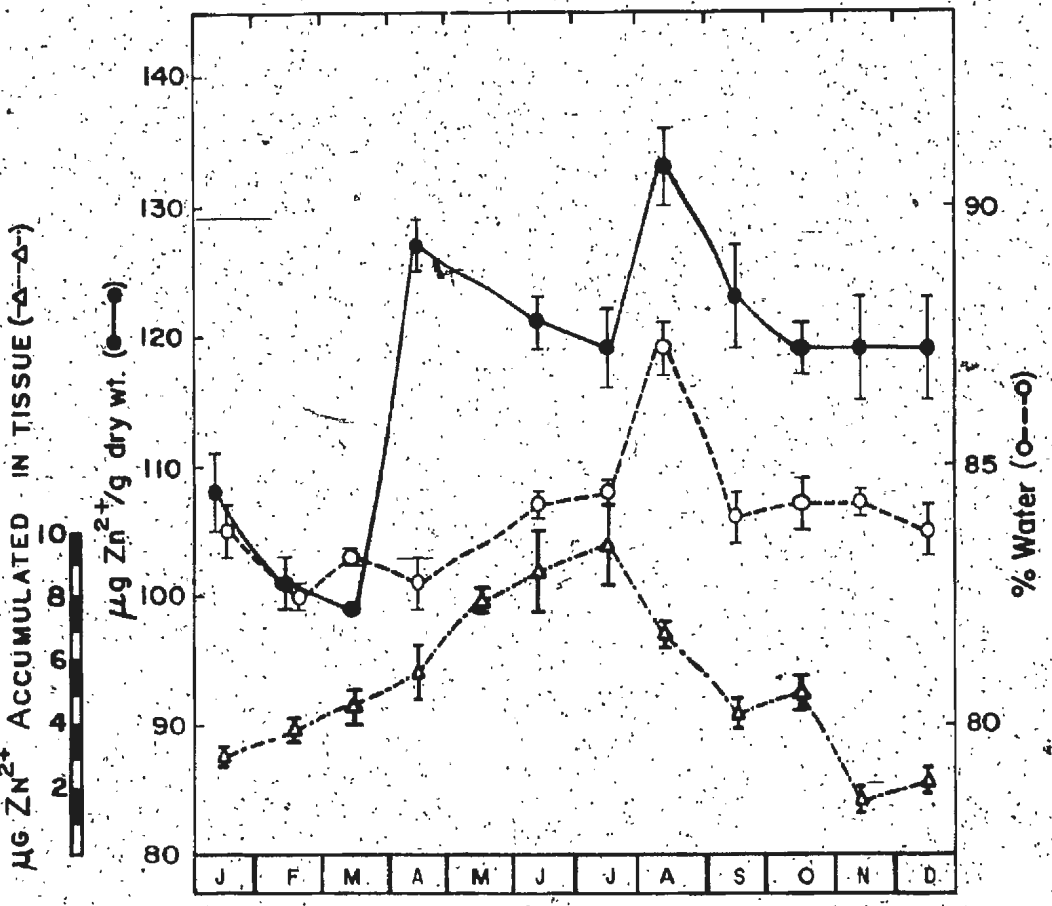

140
130
120
110
100
90
80
90
85
80
J
F
M
A
M
J
J
A
S
O
N
D
μg Zn²⁺/g dry wt. (●—●)
% Water (○—○)
μG Zn²⁺ ACCUMULATED IN TISSUE (-Δ-Δ-)
10
8
6
4
2

Table 2. Zn^{2+} uptake from the upper intestine of winter flounder maintained under different laboratory conditions. The upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. Stable Zn^{2+} (25 µg) and ^{65}Zn were injected into the ligated section. Six to eight hours later (except where indicated) the ligated segment was removed from the fish and the intestinal tissue and contents counted for radioactivity. Zn^{2+} accumulation and transfer were computed from the cpm and specific activity of the injected ^{65}Zn. Zn^{2+} accumulation was the amount of Zn^{2+} associated with the ligated intestinal tissue. Zn^{2+} transfer was computed from the difference between the amount of Zn^{2+} injected and the amount found associated with the ligated portion of the intestine and its contents. Values are expressed as $\bar{X} \pm SE$ (n= number of fish).

	(A) Fish maintained under ambient conditions; uptake examined in March.	(B) Same as (A) but exposure period increased to 24 hours.	(C) Zn^{2+} uptake examined in April. Fish transferred from ambient to heated (8°C) seawater for several days prior to examination.	(D) Fish maintained in heated seawater (8°C) and fed from November-March; Zn^{2+} uptake examined in February-March.	(E) Fish maintained under ambient conditions; Zn^{2+} uptake examined in July.
Zn^{2+} accumulated in intestinal tissue (µg)	3.0 ± 0.5 (n=5)	2.9 ± 0.4 (n=5)	2.9 ± 0.3 (n=9)	6.5 ± 0.7 (n=5)	7.4 ± 0.5 (n=24)
Zn^{2+} transferred into body (µg)	0.7 ± 0.2 (n=5)	1.9 ± 0.5 (n=5)	2.7 ± 0.4 (n=9)	8.2 ± 1.6 (n=5)	6.3 ± 0.7 (n=24)

Level of Zinc in the Diet

The concentration of Zn^{2+} in the diet (60 vs. 600 µg/g wet weight) had no significant effect on the intestinal accumulation of Zn^{2+} or on its transfer into the body (Table 3). In addition, the Zn^{2+} concentrations of the intestine, kidney and liver were not significantly different between fish fed the two diets. It should be noted that three of the control fish (n=11) and four of the experimental group (n=12) had lost weight by the termination of the study. However, weight loss or gain did not consistently influence Zn^{2+} uptake in fish from either group. Also, there was no difference in the Zn^{2+} uptake attributable to the sex of the fish.

Sex, Age (body length, weight)

The relationship between Zn^{2+} uptake and sex or age (body weight, length) of the fish was not studied *per se*. However, fish of both sexes, covering a wide range of weights (250–1000 g) and lengths (25-45 cm) were used in the investigations of the various factors affecting Zn^{2+} uptake. No difference in Zn^{2+} uptake attributable to sex or body size was apparent.

Body Load

When the winter flounder were given an intravenous injection of Zn^{2+}, the intestinal levels of Zn^{2+} reached a peak within 7-11 days (Fig. 12).

Although the concentration of Zn^{2+} in the intestine was significantly higher in Zn^{2+}-injected fish examined 7 days after the injection than it was in the controls, the uptake of Zn^{2+} and ^{65}Zn by the ligated part of the intestine did not differ between the two groups (Tables 4 and 5).

Table 3. The effects of a high Zn^{2+} diet on the uptake of Zn^{2+} from the upper intestine of the winter flounder. Control diet (Zn^{2+} = 60 µg/g wet weight); high Zn^{2+} diet (Zn^{2+} = 600 µg/g wet weight). The fish were fed the respective diets for 5 weeks. The fish were then starved for 5 days to allow the gut to empty. The upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. Stable Zn^{2+} (52 µg) and ^{65}Zn were injected into the ligated section. Seven to nine hours later the ligated segment was removed from the fish and the intestinal tissue and contents counted for radioactivity. Zn^{2+} accumulation and transfer were computed from the cpm and specific activity of the injected ^{65}Zn. Zn^{2+} accumulation was the amount of Zn^{2+} associated with the ligated intestinal tissue. Zn^{2+} transfer was computed from the difference between the amount of Zn^{2+} injected and the amount found associated with the ligated portion of the intestine and its contents. Zn^{2+} concentrations were analyzed in the liver, kidney and unligated part of the intestine. Values expressed as $\bar{X} \pm SE$ (n=number of fish).

Diet	(n)	Zn^{2+} Accumulated in the Intestinal Tissue (µg)	Zn^{2+} Transferred into the Body (µg)	Sex	(n)	Zn^{2+} Concentration (µg/g dry weight) Intestine	Kidney	Liver
Control	11	5.41 ± 0.21	6.47 ± 1.84	Male	5	99.9 ± 3.67	161.0 ± 12.5	128.0 ± 18.7
				Female	3	96.0 ± 2.33	127.4 ± 4.25	131.0 ± 7.36
High Zn^{2+}	12	5.61 ± 0.59	6.72 ± 1.22	Male	7	103.0 ± 2.08	197.0 ± 16.5	108.0 ± 8.06
				Female	5	102.0 ± 4.94	152.0 ± 9.38	170.0 ± 27.7
P		NS*	NS*			NS*	NS*	NS*

NS* = not significant at 0.05 level

Fig. 12. Changes in intestinal Zn^{2+} concentration following Zn^{2+} injections. Winter flounder were injected intravenously with saline or Zn^{2+} equivalent to approximately 20-25% of their total body Zn^{2+}. The intestinal tract was removed from the fish 1 to 27 days following the injection and analyzed for Zn^{2+} (µg Zn^{2+}/g dry weight). Values plotted are $\bar{X} \pm SE$. The number of saline injected fish examined = 34. The number of Zn^{2+} injected fish examined after 1 day = 6, after 7-11 days = 14 and after 16-27 days = 14.

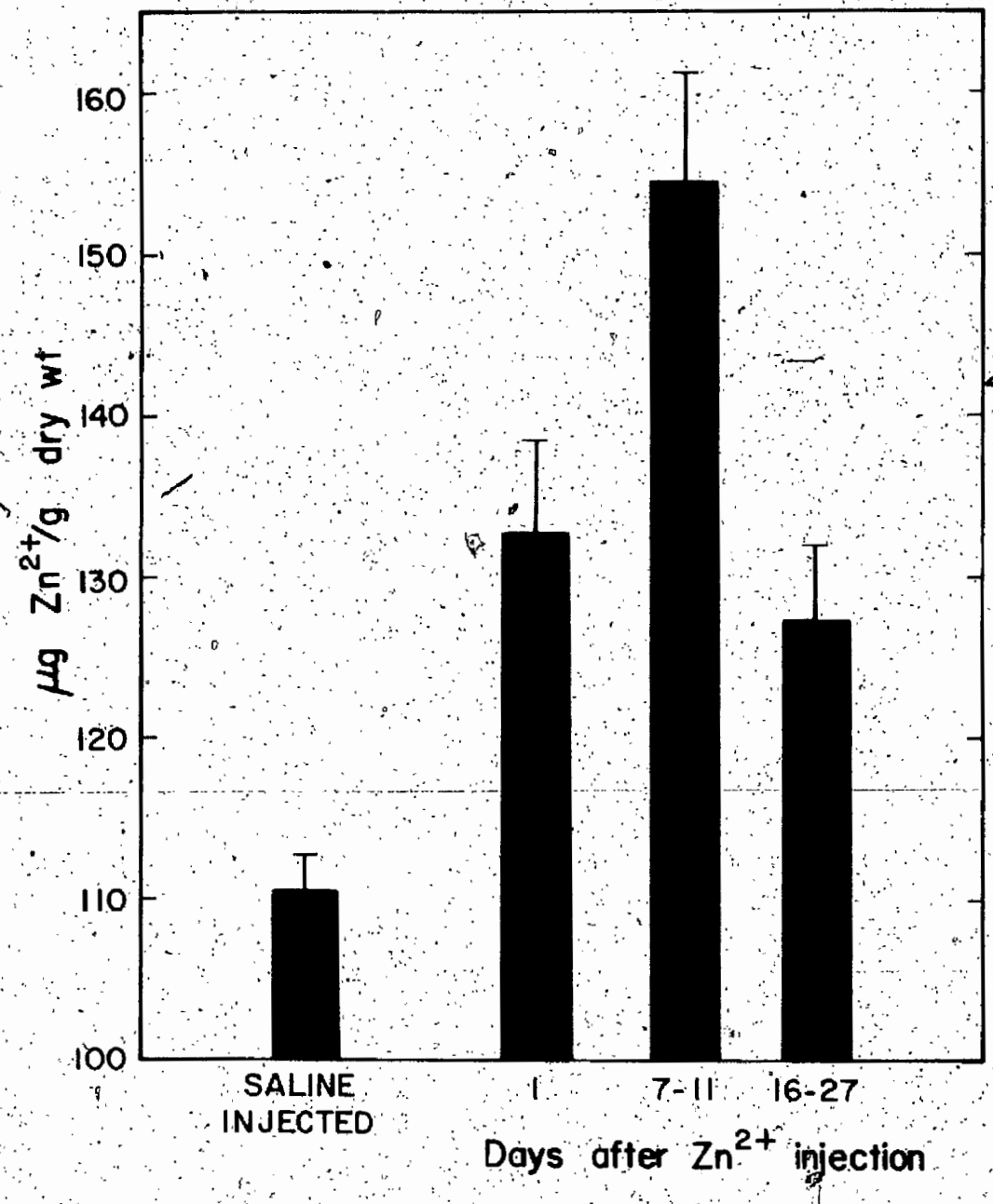

160
150
140
130
120
110
100
μg Zn2+/g dry wt
SALINE INJECTED
1
7-11
16-27
Days after Zn2+ injection

Table 4. The effects of Zn^{2+} injections on the uptake of Zn^{2+} by the intestine. Winter flounder were injected intravenously with saline or Zn^{2+} equivalent to 25% of their total body Zn^{2+}. Seven days following the injection the upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. Stable Zn^{2+} (52 µg) and ^{65}Zn were injected into the ligated segment. Nine to 10 hours later the ligated segment and its contents were removed from the fish and counted for radioactivity. The Zn^{2+} accumulated and transferred were computed from the cpm and specific activity of the injected ^{65}Zn. Zn^{2+} accumulation was the amount of Zn^{2+} associated with the ligated segment of intestinal tissue. Zn^{2+} transferred was computed from the difference between the amount of Zn^{2+} injected and the amount associated with the ligated portion of the intestine and its contents. The intestinal Zn^{2+} concentration was analyzed in the unligated part of the intestine. Values expressed as $\bar{X} \pm SE$ (n=number of fish).

Treatment	(n)	Zn^{2+} Accumulated (µg)	Zn^{2+} Transferred (µg)	Intestinal Zn^{2+} (µg/g dry weight)
Zn^{2+} Injected	10	9.12 ± 0.75	17.4 ± 1.95	151.0 ± 5.22
Saline Injected	11	7.70 ± 0.73	18.4 ± 1.18	128.0 ± 3.43
P		NS*	NS*	<0.01

NS* = not significant at the 0.05 level

Table 5. The effects of Zn^{2+} injections on the uptake of ^{65}Zn by the intestine. Winter flounder were injected intravenously with saline or Zn^{2+} equivalent to 25% of their total body Zn^{2+}. Seven days following the injection the upper portion of the intestine (including the pyloric appendages) was ligated *in situ*. ^{65}Zn was injected into the ligated segment. Nine to 10 hours later the ligated segment and its contents were removed from the fish and counted for radioactivity. The ^{65}Zn accumulated and transferred were computed from the cpm of injected ^{65}Zn. ^{65}Zn accumulation was the percentage of the injected ^{65}Zn associated with the ligated intestinal tissue. ^{65}Zn transferred was the difference between the amount of ^{65}Zn injected into the lumen and the amount associated with the ligated portion of the intestine and its contents, expressed as a percentage of the injected ^{65}Zn. The intestinal Zn^{2+} concentration was analyzed in the unligated portion of the intestine. Values expressed as $\bar{X} \pm SE$ (n=number of fish).

Treatment	(n)	^{65}Zn Accumulated (%)	^{65}Zn Transferred (%)	Intestinal Zn^{2+} (µg/g dry weight)
Zn^{2+} Injected	10	43.0 ± 5.75	19.9 ± 2.59	152.0 ± 5.11
Saline Injected	8	47.5 ± 4.59	20.1 ± 3.13	127.0 ± 2.98
P		NS*	NS*	<0.01

*NS = not significant at 0.05 level

No significant difference in the Zn^{2+} uptake by the intestine was observed between controls and Zn^{2+}-injected fish when examined from 1 day to 5 months following the injections.

Section B. *In vitro* technique

Zn^{2+} binding to the mucosal supernatant proteins was studied using Zn^{2+} loads ranging from 1.16 to 33.0 µg Zn^{2+} per mg protein. Three association constants (K_1, K_2, K_3) could be derived from the Scatchard-type plot of [Zn^{2+} Bound]/[Zn^{2+} Unbound] versus [Zn^{2+} Bound] (Fig. 13). The data for one March ($K=1.29 \times 10^7$) and one April ($K=3.45 \times 10^7$) non-feeding fish were similar to the data obtained for four feeding fish. Therefore all of the data were pooled and summarized in Table 6.

The effects of 10 µM loads of various metals on Zn^{2+} binding were as follows: (mean of two separate trials with each metal; Metal (10 µM load) or Buffer (0 µM load), % Bound: Buffer, 95.6; $ZnCl_2$, 85.7; $CuCl_2$, 79.9; $FeCl_3$, 97.3; $NiCl_2$, 96.2; $CoCl_2$, 96.5; $CaCl_2$, 97.1; $MgCl_2$, 96.8; $HgCl_2$, 95.1; $MnCl_2$, 97.0; $LiCl_2$, 97.3). Another experiment was carried out and the results were as follows: Buffer, 92.0; $ZnCl_2$, 74.5; $CdCl_2$, 89.6; $CrCl_2$, 90.3. Cu^{2+} was the only metal ion tested which appeared to affect the binding of Zn^{2+} to the mucosal cytosol. Therefore the effect of various Cu^{2+} loads on the binding of ^{65}Zn was determined and compared with similar loads of Zn^{2+}. The results of one experiment using the standard buffer (TES) and another using a HEPES buffer are illustrated in Fig. 14. It would appear that Cu^{2+} and Zn^{2+} loads had similar effects on Zn^{2+} binding. Observations on the variation of Zn^{2+} binding between several fish using TES buffer tend to indicate that the

Fig. 13. Scatchard-type plot of Zn^{2+} binding to intestinal cytosol proteins of one summer feeding fish. Association constants were computed on the first slope of the line (loads up to 1.4 µg Zn^{2+}) $K_1=2.47 \times 10^7$; the second slope of the line (loads up to 5.9 µg Zn^{2+}) $K_2=5.09 \times 10^6$; the third slope of the line (loads up to 47 µg Zn^{2+}) $K_3=3.56 \times 10^5$.

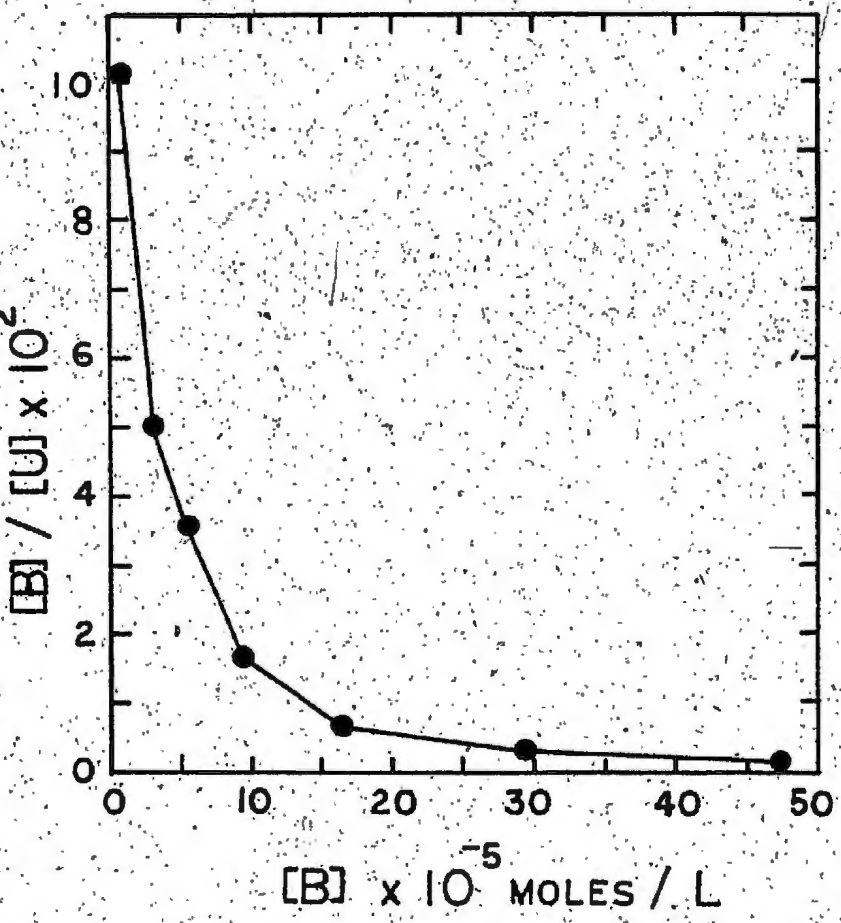

10
8
6
4
2
0
[B] / [U] x 10^2
0
10
20
30
40
50
[B] x 10^-5 MOLES / L

Fig. 14. Effect of increasing Zn^{2+} and Cu^{2+} loads on Zn^{2+} bound to mucosal soluble proteins using TES or HEPES buffers.

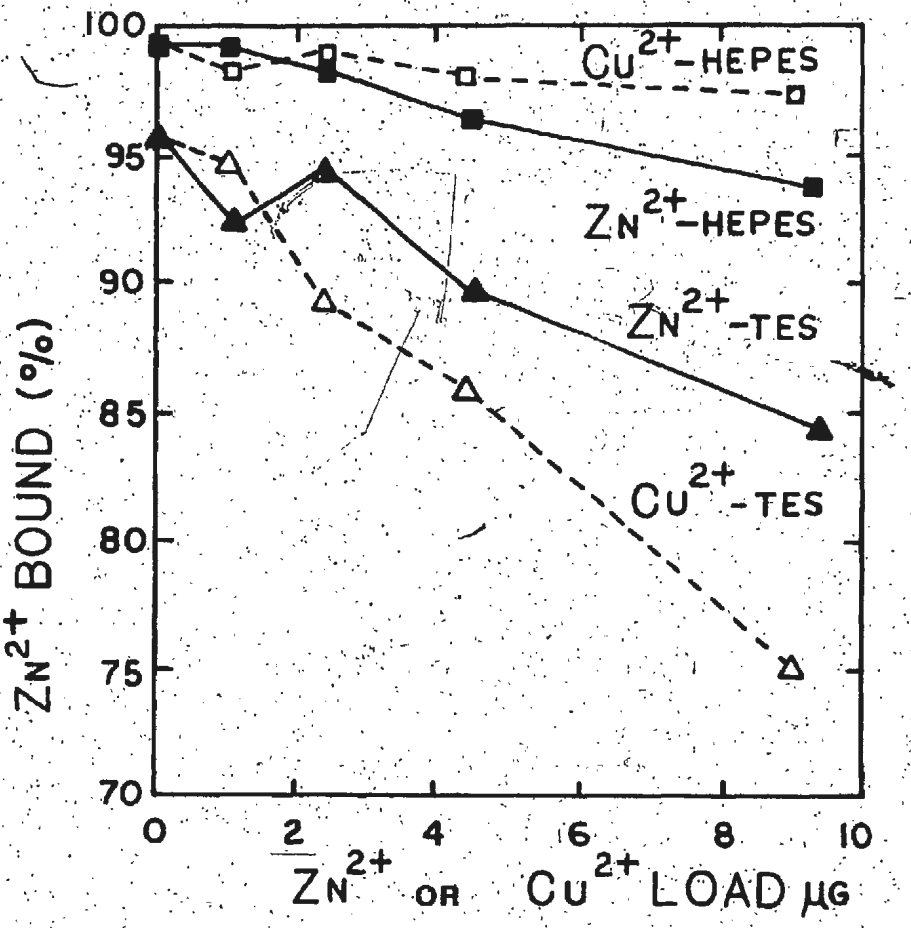

100
95
90
85
80
75
70
0
2
4
6
8
10
Cu^{2+}-HEPES
Zn^{2+}-HEPES
Zn^{2+}-TES
Cu^{2+}-TES
Zn^{2+} BOUND (%)
Zn^{2+} OR Cu^{2+} LOAD µG

Table 6. Summary of Zn^{2+} binding in the mucosal cytosol preparation of winter flounder (values are $\bar{X} \pm SE$ for 6 experiments).

Mucosal cytosol Zn^{2+} (µg/mg protein)	% Zn^{2+} Bound at 0 load	K_1 Zn^{2+} loads 0-1.16 µg per mg protein	K_2 Zn^{2+} loads 1.16-4.4 µg per mg protein	K_3 Zn^{2+} loads 4.4-33 µg per mg protein
0.30 ±0.03	97.0 ± 1.0	2.42×10^7 $\pm 0.81 \times 10^7$	3.26×10^6 $\pm 0.96 \times 10^6$	2.41×10^5 $\pm 0.69 \times 10^5$

differences in binding using HEPES buffer was largely due to variation between fish rather than an effect of the buffers.

Section C. *In vivo* technique

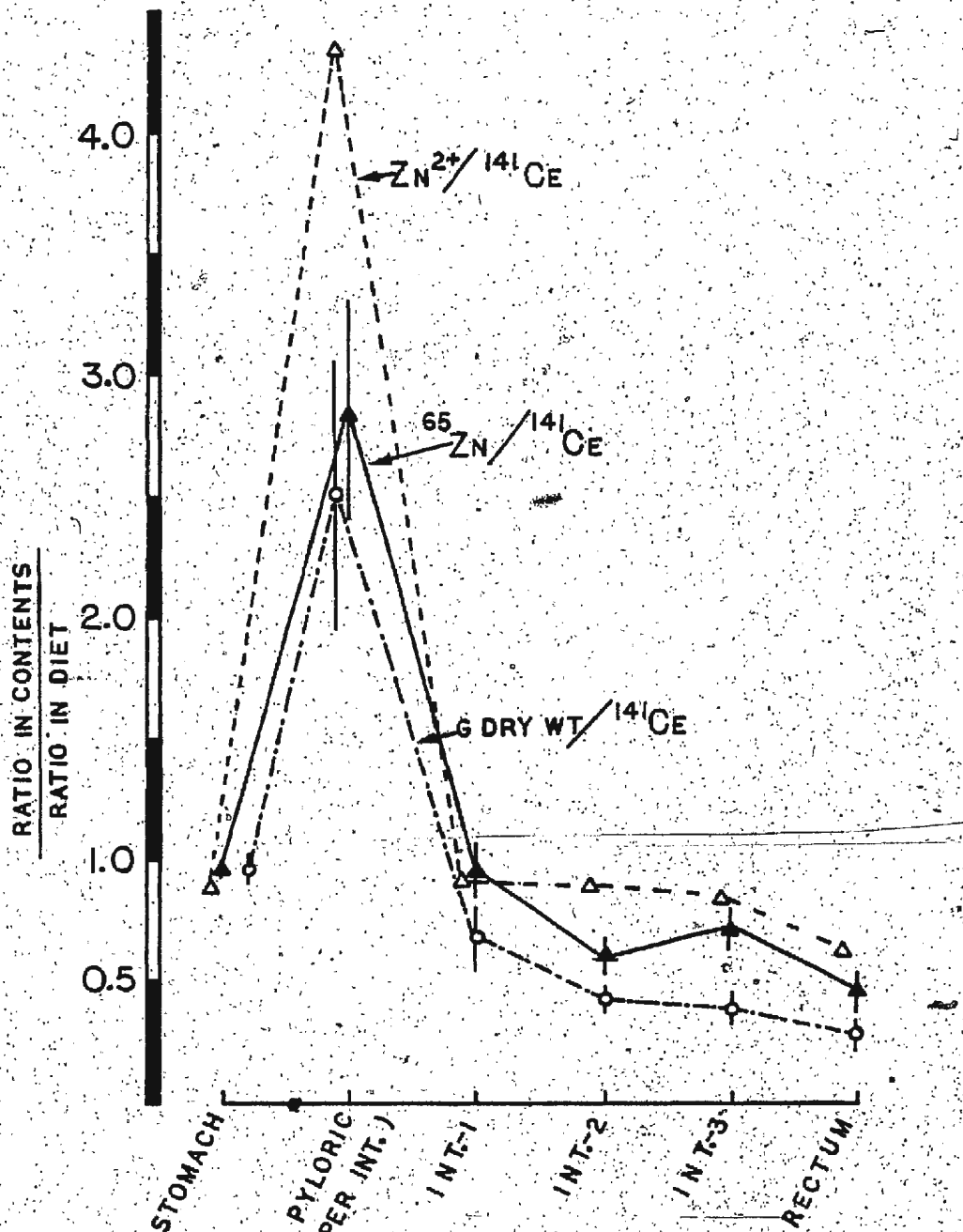
Suitability of ^{141}Ce as a non-absorbed marker

When ^{141}Ce was injected directly into the stomach of the winter flounder, the radioactivity remained associated with the gastrointestinal tract contents and the fecal material. After three days, 26 and 41% of the amount injected were found in the rectum contents and in the fecal material, respectively. By five days, less than 1% of the ^{141}Ce dose was present in the gastrointestinal contents; 53% was recovered in the fecal material. At the time points examined, less than 1% of the ^{141}Ce dose was associated with the gastrointestinal tissues and no radioactivity was detected in any of the other tissues examined (i.e. blood, kidney, gall bladder, gonad or spleen). Similar results were observed when flounder were fed ^{141}Ce-labelled pieces of capelin. The radioactivity was again associated with the gastrointestinal tract contents, not with the gastrointestinal tract tissue or any other tissue examined.

$^{65}Zn/^{141}Ce$ feeding study using radiolabelled capelin

The ratios of ^{65}Zn to ^{141}Ce in the contents of different segments of the gastrointestinal tract, relative to the ratio of ^{65}Zn to ^{141}Ce in the labelled capelin, are summarized in Fig. 15. There appeared to be little or no net absorption of ^{65}Zn in the stomach, a large net secretion of ^{65}Zn into the upper intestine and a net absorption of ^{65}Zn along the rest of the tract. The flounder's net absorption of ^{65}Zn from the labelled capelin averaged 53%.

Fig. 15. Ratios of $^{65}Zn/^{141}Ce$, $Zn^{2+}/^{141}Ce$ and dry material/^{141}Ce in the digestive tract contents of winter flounder fed radiolabelled capelin for 18-19 days. Flounder were fed radiolabelled capelin (^{65}Zn and ^{141}Ce) daily for 18-19 days (at the end of 4-5 weeks total feeding period). The day after the last feeding, the digestive tract was ligated into sections *in situ*. ^{65}Zn and ^{141}Ce were counted in the contents; the dry weights of the contents were also determined. The data are expressed as the ratio of $^{65}Zn/^{141}Ce$ in the contents divided by the ratio of $^{65}Zn/^{141}Ce$ in the capelin ($\bar{X} \pm SE$, n=9-17) and as the ratio of dry material(g)/^{141}Ce in the contents divided by the dry material(g)/^{141}Ce in the capelin ($\bar{X} \pm SE$, n=5-10). The average levels of stable Zn^{2+} concentrations in the stomach and intestinal contents of flounder fed unlabelled capelin (Table 7) were used to estimate the stable $Zn^{2+}/^{141}Ce$ ratios in the present study. Data are plotted as the ratio of $Zn^{2+}/^{141}Ce$ in the contents divided by the $Zn^{2+}/^{141}Ce$ in the capelin. The % net absorption of ^{65}Zn, Zn^{2+} and dry material can be calculated by subtracting the relevant ratio, expressed as a %, from 100%. A ratio greater than one yields a negative absorption value, which would, according to theory, represent net secretion.


RATIO IN CONTENTS / RATIO IN DIET
4.0
3.0
2.0
1.0
0.5
$Zn^{2+}/^{141}Ce$
$^{65}Zn/^{141}Ce$
G DRY WT/^{141}Ce
STOMACH
PYLORIC (UPPER INT.)
INT.-1
INT.-2
INT.-3
RECTUM

The trend of net absorption and secretion of dry material in different segments of the gastrointestinal tract was similar to the trend observed for ^{65}Zn (Fig. 15). On average, the flounder absorbed (net) 72% of the dry material.

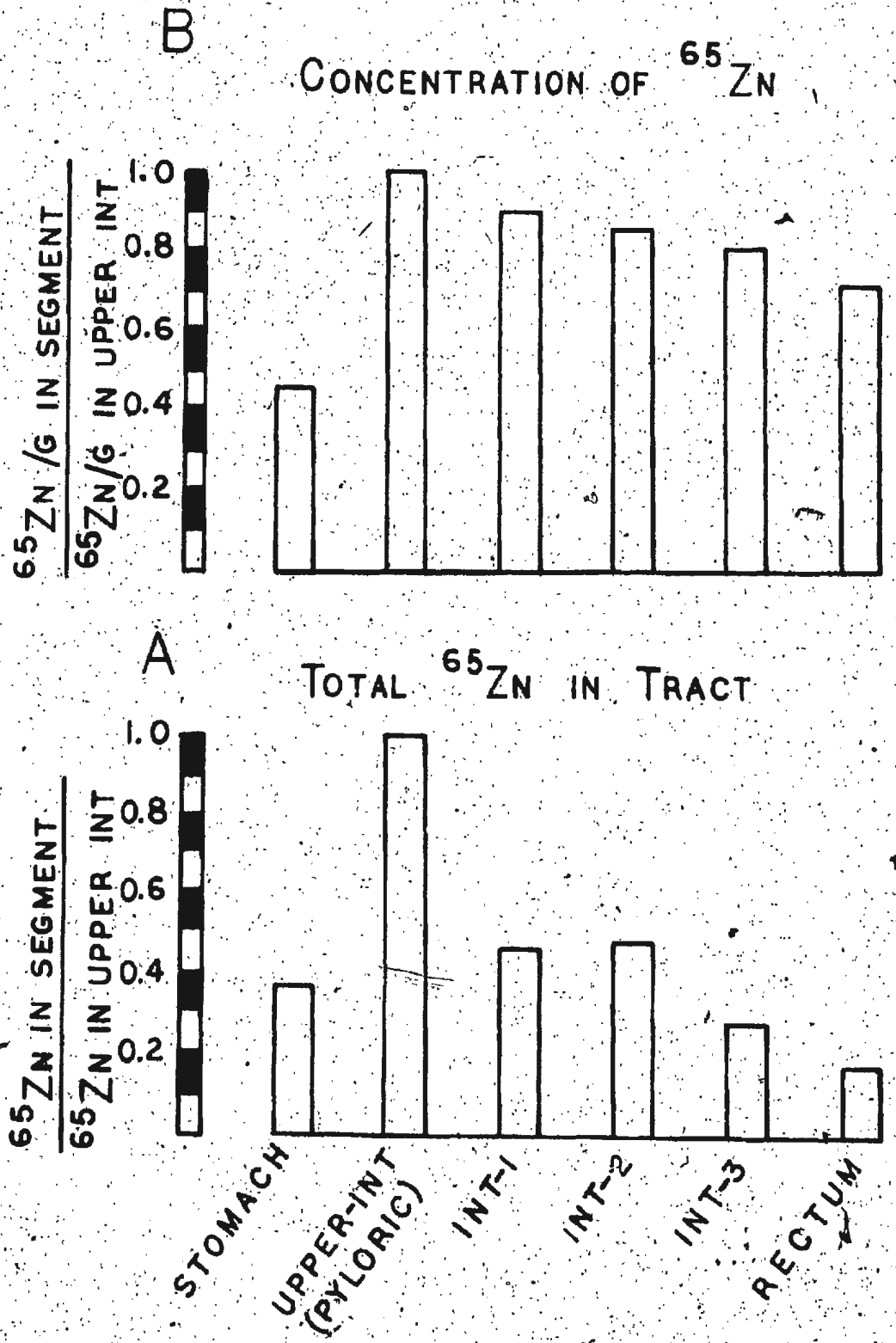
It was not possible to analyze the stable Zn^{2+} concentrations of the contents in this feeding study. However, average values for the Zn^{2+} concentration of the stomach contents (i.e. 54.7 µg Zn^{2+}/g dry weight, n=4) and the contents in the rest of the intestine (137.8 ± 15.9 µg Zn^{2+}/g dry weight, $\bar{X}$ ± SE, n=14) of capelin-fed flounder were obtained by dissecting five fish which had been fed chopped capelin for a two-month period (Table 7). Using these values for stable Zn^{2+}, the ratios of stable Zn^{2+} to ^{141}Ce in the contents were estimated and compared to the ratio of stable Zn^{2+} to ^{141}Ce in the capelin (µg Zn^{2+}/g dry weight of capelin= 57.1 ± 2.2, $\bar{X}$ ± SE of 15 samples). The trend of net absorption and secretion of stable Zn^{2+} in different regions of the gastrointestinal tract was similar to that observed for ^{65}Zn (Fig. 15). However, when net absorption was based on the ratios calculated with stable Zn^{2+}, there appeared to be a slightly greater net absorption of Zn^{2+} in the stomach and a lower net absorption in the rectum contents than indicated by the ^{65}Zn. Only 37% of the stable Zn^{2+} in the capelin was absorbed compared to 53% of the ^{65}Zn.

The level of total ^{65}Zn in the gastrointestinal tract tissues was highest in the upper intestine (which includes the pyloric appendages) but there was little difference in the concentration of ^{65}Zn (i.e. cpm ^{65}Zn/g wet weight) in the intestinal tissue from the different regions (Fig. 16).

Table 7. The stable Zn^{2+} concentrations (μg Zn^{2+}/g dry weight) of gastrointestinal tract tissue and contents of winter flounder fed capelin for a 2-month period. Flounder were fed chopped capelin for 2 months at approximately 5% of their body weight per day. The day after the last feeding the digestive tract was ligated into sections *in situ*. The stable Zn^{2+} concentrations were determined on nitric acid digests of the tissue and contents using atomic absorption spectrophometry. Values are $\bar{X} \pm SE$ where n=number of samples.

	Stomach	Upper Int.	Mid Int.	Low Int.	Rectum
Tissue (μg Zn^{2+}/g dry wt)	86.7 ±3.6 (n=5)	98.3 ±2.9 (n=5)	97.7 ±1.9 (n=5)	107.1 ± 3.4 (n=4)	101.8 ± 1.8 (n=4)
Contents (μg Zn^{2+}/g dry wt)	54.7 ±7.2 (n=4)	129.4 ±35.4 (n=5)	116.2 ±24.7 (n=2)	164.2 ±37.2 (n=4)	118.1 ±28.5 (n=3)

Fig. 16. The distribution of ^{65}Zn in the digestive tract of winter flounder fed radiolabelled capelin for an 18-19 day period. Flounder were fed radiolabelled (^{65}Zn and ^{141}Ce) capelin daily for 18-19 days (at the end of 4-5 weeks total feeding period). The day after the last feeding, the digestive tract was ligated into sections *in situ*. ^{65}Zn and ^{141}Ce were counted in the tissue. The total amount of ^{65}Zn (cpm) (A) and the concentration of ^{65}Zn (cpm ^{65}Zn/g wet weight) (B) were determined for each segment of the tract. Values plotted are the mean total amounts of ^{65}Zn (A) and the mean concentration of ^{65}Zn (B) expressed as a ratio of the mean values obtained for the upper intestine (which includes the pyloric appendages). 17 fish were examined.


B
CONCENTRATION OF ^{65}Zn
$\frac{^{65}Zn/G \text{ IN SEGMENT}}{^{65}Zn/G \text{ IN UPPER INT}}$
1.0
0.8
0.6
0.4
0.2
A
TOTAL ^{65}Zn IN TRACT
$\frac{^{65}Zn \text{ IN SEGMENT}}{^{65}Zn \text{ IN UPPER INT}}$
1.0
0.8
0.6
0.4
0.2
STOMACH
UPPER-INT (PYLORIC)
INT-1
INT-2
INT-3
RECTUM

The stable Zn^{2+} concentrations (μg Zn^{2+}/g dry weight) of the intestinal tissue, examined in flounder fed unlabelled capelin, were similar along the tract (Table 7).

$^{65}Zn/^{141}Ce$ feeding study using radiolabelled food pellets

When flounder were fed radiolabelled food pellets the net absorption of ^{65}Zn from this diet was lower than the net absorption of ^{65}Zn from the radiolabelled capelin (i.e. 14 versus 53%, compare Fig. 15 and 17). The net absorption of stable Zn^{2+} from the food pellets by the flounder averaged 17% (Fig. 17). This was lower than the % net absorption of stable Zn^{2+} from the capelin but the concentration of Zn^{2+} in the food pellets was higher than in the capelin (i.e. 92.8 and 57.1 μg Zn^{2+}/g dry weight, respectively). The net absorption of dry material from the food pellets averaged 41% compared to 72% from the capelin (Fig. 15 and 17).

The relative distribution of ^{65}Zn in the gastrointestinal tissues of flounder fed the radiolabelled pellets (Fig. 18) was similar to that observed in flounder fed radiolabelled capelin for a similar time period (Fig. 16). However, the absolute levels of radioactivity were approximately six times lower, reflecting the lower specific activity of the food pellets (48 and 330 cpm ^{65}Zn per μg stable Zn^{2+} in the food pellets and capelin, respectively).

Feeding $^{65}Zn/^{141}Ce$ labelled capelin to flounder injected (i.p.) with Zn^{2+} or saline

The net absorption of ^{65}Zn (53%), stable Zn^{2+} (51%) and dry material (76%) from capelin by saline-injected flounder was similar to that observed from non-injected fish (see Fig. 15). In flounder which had

Fig. 17. Ratios of $^{65}Zn/^{141}Ce$, $Zn^{2+}/^{141}Ce$ and dry material/^{141}Ce in the digestive tract contents of winter flounder fed radiolabelled fish food pellets for 18 days. Flounder were fed radiolabelled (^{65}Zn and ^{141}Ce) fish food pellets daily for 18 days (at the end of a 4-week total feeding period). The day after the last feeding, the digestive tract was ligated into sections *in situ*. ^{65}Zn and ^{141}Ce were counted in the contents; the dry weight of the contents (g) and the concentration of stable Zn^{2+} (µg/g dry weight) were also determined. The data are expressed as: the ratio of $^{65}Zn/^{141}Ce$ in the contents divided by the ratio of $^{65}Zn/^{141}Ce$ in the diet; the ratio of $Zn^{2+}/^{141}Ce$ in the contents divided by the ratio of $Zn^{2+}/^{141}Ce$ in the diet; and the ratio of dry material (g)/^{141}Ce in the contents divided by the ratio of dry material (g)/^{141}Ce in the diet. Values plotted are $\bar{X} \pm SE$ (n=4-5). The % net absorption of ^{65}Zn, Zn^{2+} and dry material can be calculated by subtracting the relevant ratio, expressed as a %, from 100%. A ratio greater than one yields a negative absorption value, which would, according to theory, represent net secretion.

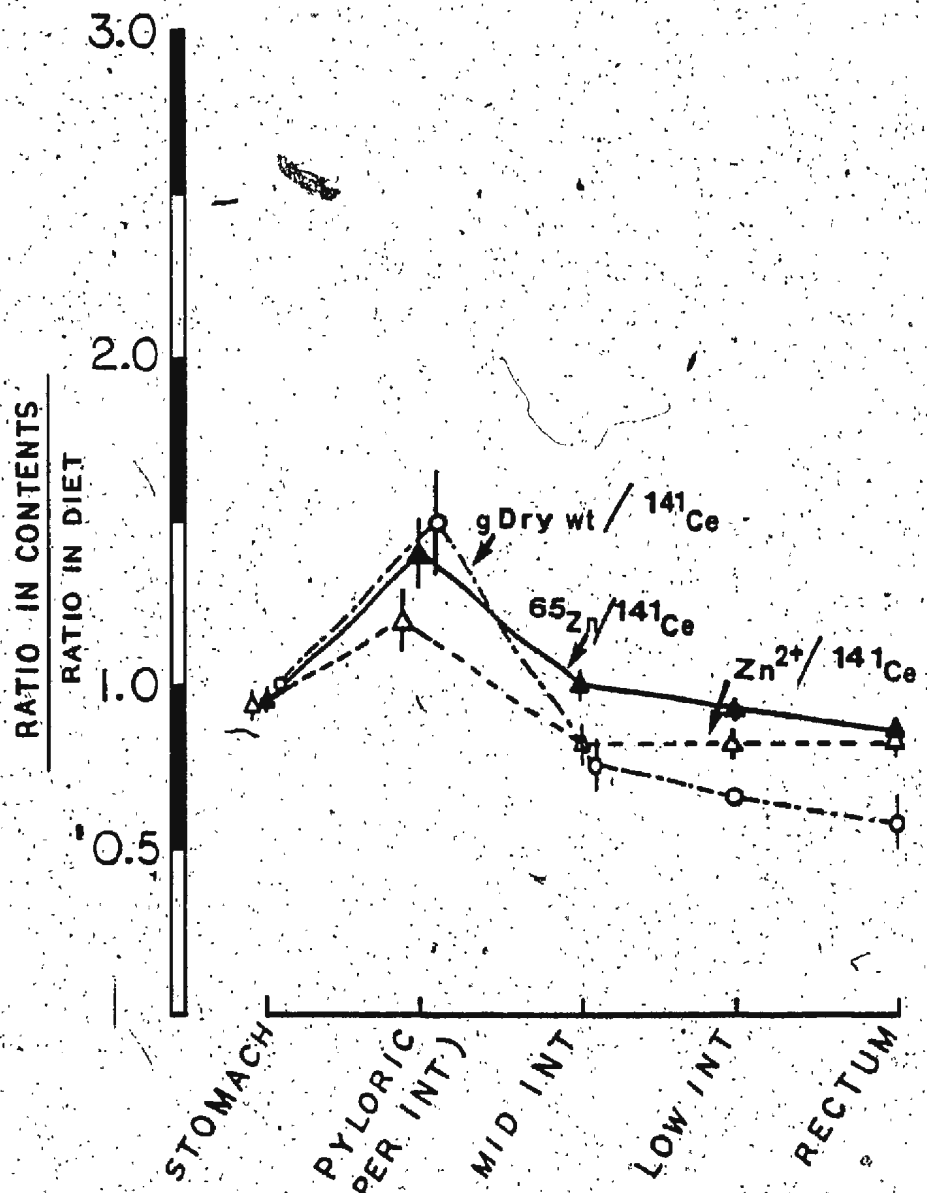
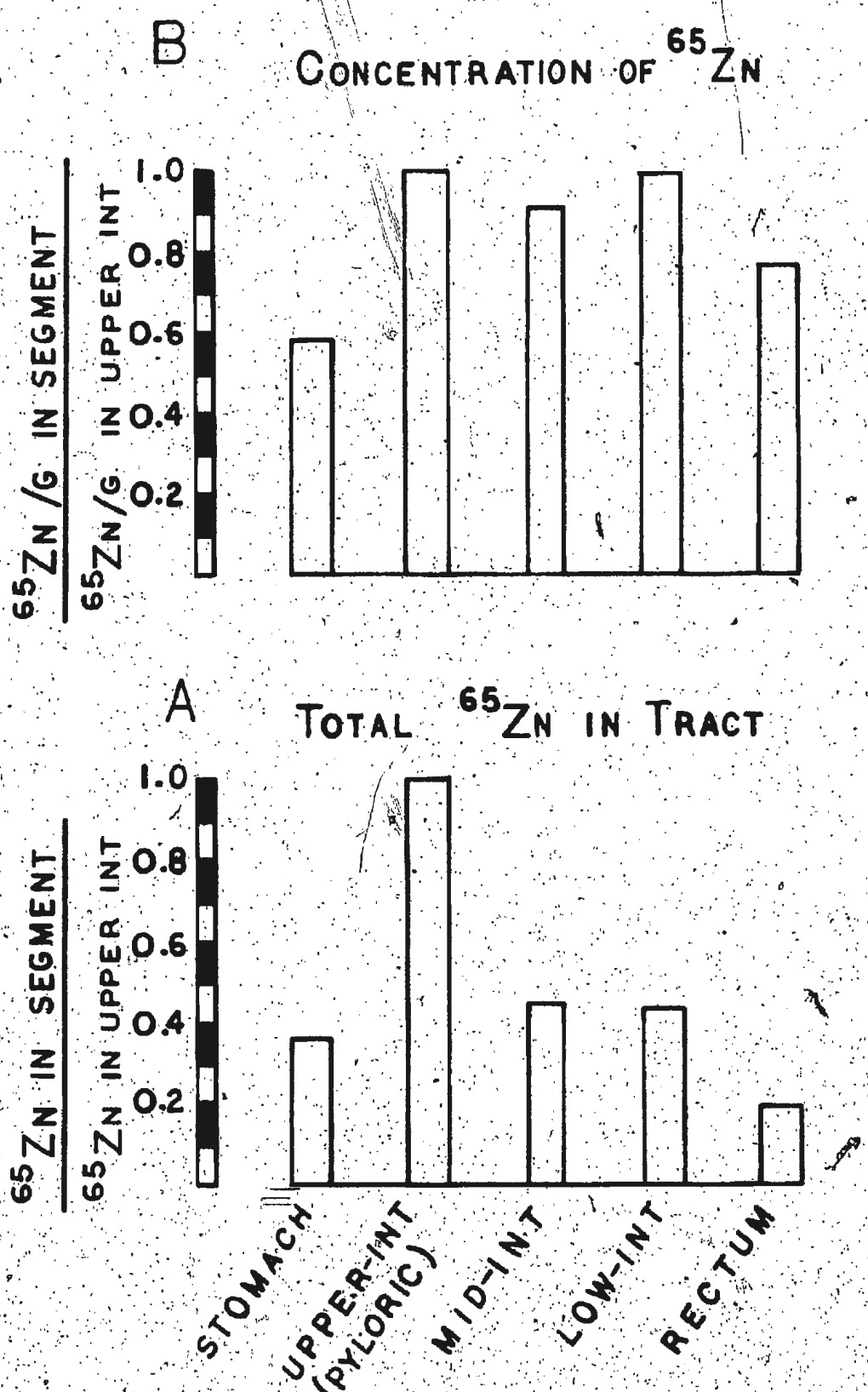

3.0
2.0
1.0
0.5
RATIO IN CONTENTS / RATIO IN DIET
g Dry wt / 141Ce
65Zn / 141Ce
Zn2+ / 141Ce
STOMACH
PYLORIC (UPPER INT.)
MID INT
LOW INT
RECTUM

Fig. 18. The distribution of ^{65}Zn in the digestive tracts of winter flounder fed radiolabelled fish food pellets for 18 days. Flounder were fed radiolabelled (^{65}Zn and ^{141}Ce) fish food pellets daily for 18 days (at the end of a total 4-week feeding period). The day after the last feeding, the digestive tract was ligated into sections *in situ*. ^{65}Zn and ^{141}Ce were counted in the tissue. The total amount of ^{65}Zn (cpm) (A) and the concentration of ^{65}Zn (cpm/g) (B) were determined for each segment of the tract. Values plotted are the mean total amount of ^{65}Zn (A) and the concentration of ^{65}Zn (B) expressed as a ratio of the mean values obtained for the upper intestine (which includes the pyloric appendages). 6 fish were examined.


B
CONCENTRATION OF ^{65}Zn
^{65}Zn /G IN SEGMENT / ^{65}Zn/G IN UPPER INT
1.0
0.8
0.6
0.4
0.2
A
TOTAL ^{65}Zn IN TRACT
^{65}Zn IN SEGMENT / ^{65}Zn IN UPPER INT
1.0
0.8
0.6
0.4
0.2
STOMACH
UPPER-INT (PYLORIC)
MID-INT
LOW-INT
RECTUM

been injected with Zn^{2+} (two weeks prior to dissection) the net absorption of ^{65}Zn from the radiolabelled capelin was significantly lower (only 5% was absorbed) and a net secretion of stable Zn^{2+} occurred (net secretion=15%) (Table 8).

Distribution of ^{65}Zn and ^{141}Ce in the lumen contents of flounder fed radiolabelled capelin

Following centrifugation of the contents of the tract (from the upper, mid and low intestine), on average, 88% of the total ^{141}Ce in the contents was associated with the precipitated material; only 51% of the ^{65}Zn was localized in the precipitate. The ^{65}Zn remaining in the supernatant was not precipitable with TCA (trichloroacetic acid).

Table 8. The ratios of $^{65}Zn/^{141}Ce$, $Zn^{2+}/^{141}Ce$ and dry material/^{141}Ce in the rectum contents of capelin-fed winter flounder injected with stable Zn^{2+} or saline. Flounder were fed radiolabelled capelin (^{65}Zn and ^{141}Ce) for 7 days (at the end of 6 weeks total feeding time). Fourteen days prior to the last day of feeding, the flounder were injected (i.p.) with Zn^{2+} (25% of their estimated total body Zn^{2+}) or saline (1% NaCl). The day after the last feeding the gastrointestinal tract was ligated *in situ*. The cpm ^{65}Zn and ^{141}Ce in the contents were counted. The dry weight and stable Zn^{2+} concentration (µg Zn^{2+}/g dry weight) of the rectum contents were determined. Data are expressed as: the ratio of $^{65}Zn/^{141}Ce$ in the rectum contents divided by the ratio of $^{65}Zn/^{141}Ce$ in the capelin; the ratio of $Zn^{2+}/^{141}Ce$ in the contents divided by the ratio of $Zn^{2+}/^{141}Ce$ in the capelin; the ratio of g dry material/^{141}Ce in the contents divided by the ratio of g dry material/^{141}Ce in the capelin. Values are $\bar{X}$ ± SE, n=number of fish.

	$^{65}Zn/^{141}Ce$	$Zn^{2+}/^{141}Ce$	dry matter (g)/^{141}Ce
Zn^{2+} injected	0.95 ± 0.18 (n=6)	1.15 ± 0.16 (n=6)	0.39 ± 0.08 (n=5)
Saline injected	0.47 ± 0.05 (n=5)	0.49 ± 0.07 (n=5)	0.24 ± 0.05 (n=5)
P	<0.05	<0.01	NS*

NS* = not significant at the 0.05 level

DISCUSSION

The gastrointestinal tract of the winter flounder is similar to that of other vertebrates in being comprised of a fore-gut (oesophagus and stomach), a mid-gut (intestine) and a hind-gut (rectum) (Barrington 1957).

Results from the *in situ* studies indicate that the uppermost end of the intestine of the flounder had the greatest capacity to absorb Zn^{2+}. These findings are in agreement with studies carried out on ligated segments of the rat gastrointestinal tract where it was found that ^{65}Zn was taken up more rapidly from the duodenum than from other sections of the intestine; very little ^{65}Zn was absorbed from the stomach (Van Campen and Mitchell 1965; Methfessel and Spencer 1973a). Although there is little information in the literature on metal uptake from the gastrointestinal tract of fish, the absorption of other substances has been found to be site dependent. For example, Bogé et al. (1979) found, using an *in situ* perfusion method, that glycine uptake in rainbow trout (*Salmo gairdneri*) was linear in the upper intestine and a saturable phenomenon in the lower intestine.

The sites of Zn^{2+} absorption were also studied in intact feeding flounder using the non-absorbed marker, cerium-141. In agreement with the *in situ* findings, the feeding studies indicated that little or no ^{65}Zn absorption occurs in the stomach (compare Fig. 1 p. 24 with Figs. 15 p. 62 and 17 p. 69). The high $^{65}Zn/^{141}Ce$ ratios (>1) in the contents of the uppermost portion of the intestine suggest a net secretion of ^{65}Zn into this area. This apparent secretion of ^{65}Zn in feeding fish masks the absorption from this area which is observed in the *in situ* studies. The $^{65}Zn/^{141}Ce$ ratios in the contents of the rest of the intestine are suggestive of a net absorption of ^{65}Zn as the contents pass down the tract

from the upper intestine. Similar net absorption and secretion trends along the tract were observed for stable Zn^{2+} and dry material. Interestingly, these results are similar to those reported for ruminants. Miller and Cragle (1965) reported an elevation of the $^{65}Zn/^{141}Ce$ ratio in the contents of the first intestinal segment, indicating a large net secretion of ^{65}Zn. Ratios representative of net absorption of ^{65}Zn were observed throughout the rest of the small intestine. The question which arises from Miller and Cragles (1965) findings and the findings in the present study is whether the net secretion of stable Zn^{2+}, ^{65}Zn and dry material into the upper intestine is real or whether it represents an artifact of the method.

In order to be useful for determining the site(s) of absorption of a substance, an indicator must move through the digestive tract at the same rate as the ingesta. Ellis and Huston (1968) evaluated the use of radiocerium as a digesta flow marker in ruminants and found that it remained in close physical association with the indigestible residues. As such, they reasoned that cerium could be used as a marker for determining the flow of the total overall array of residues in the diet but might be less valid to use as a marker for determining the flow of the more digestible residues. In flounder fed radiolabelled capelin, most of the ^{141}Ce in the ingesta was associated with the solids while ^{65}Zn was evenly distributed between soluble material and solids. If the non-digestible material in the ingesta, to which most of the ^{141}Ce adsorbs, moved through the upper intestine at a faster rate than the digestible material this could account for the apparent elevation of dry material, ^{65}Zn and stable Zn^{2+}, with respect to ^{141}Ce, in the contents of the uppermost region of the intestine (i.e. region containing the pyloric appendages). It may be that the non-absorbed marker technique is only

useful in determining the net absorption or secretion of nutrients by the entire intestine (i.e. based on the ratios in the rectum contents or fecal material) but is less useful in determining the site(s) of uptake along the tract.

Most of the information on the factors affecting gastrointestinal absorption of Zn^{2+} in the flounder was obtained using the *in situ* technique. Zn^{2+} absorption from the intestinal lumen of the flounder appears to involve at least two steps: 1. accumulation of Zn^{2+} by the intestinal tissue, followed by, 2. transfer of Zn^{2+} into the body. When Zn^{2+} absorption was examined over a 48-hour period, Zn^{2+} accumulated in the intestinal tissue within the first hour of exposure with very little being transferred into the body. Following this initial accumulation period, the amount of Zn^{2+} transferred into the body increased at a linear rate. A similar two-step absorption process, where Zn^{2+} transfer into the blood follows an obligatory accumulation of Zn^{2+} in the intestinal tissue, has been hypothesized to occur in rats (Sahagian et al. 1966; Kowarski et al. 1974; Smith et al. 1978a). In addition, there appears to be a second, more rapid process in rats, whereby Zn^{2+} is transferred into the body within minutes of contact with the mucosal tissue, i.e. without any appreciable binding to the mucosal tissue (Davies 1980; Jackson et al. 1981).

When different loads of Zn^{2+} were instilled into the intestinal lumen of the flounder, the proportion of Zn^{2+} accumulated in the intestinal tissue was greater from lower than from higher doses. This implies that the first step in Zn^{2+} absorption in the flounder involves binding to specific sites on or within the intestinal cells. *In situ* studies indicated that Zn^{2+} accumulation by the intestinal tissue of mice involved binding of Zn^{2+} to specific sites within the mucosa (Hamilton et al. 1978).

In rats, Zn^{2+} appeared to bind to specific sites at low doses (1-50 µg) but non-specific binding was apparent at higher doses (50-200 µg) (Davies 1980).

The amount of Zn^{2+} transferred into the body of the flounder appeared to be directly proportional to the amount of Zn^{2+} accumulated by the intestinal tissue. This suggests that the transfer of Zn^{2+} from the mucosal tissue into the blood may be a passive process. Such a mechanism has been proposed for the absorption of ^{65}Zn from water via the gills of the plaice (*Pleuronectes platessa*) (Pentreath 1973b). In this instance it was suggested that adsorption of ^{65}Zn to gill mucus resulted in a higher concentration of ^{65}Zn in the gill tissue than in the blood flowing through the gill lamellae. Thus the ^{65}Zn could be transferred into the blood stream down a concentration gradient. By contrast, in rats, a plot of the Zn^{2+} transferred into the body, versus the Zn^{2+} accumulated in the intestinal tissue exhibited saturation kinetics, suggesting that the release of Zn^{2+} to the blood involved a carrier mediated process which would be rate limiting at high concentrations of Zn^{2+} in the intestinal tissue (Davies 1980).

Using an *in situ* perfusion technique, Smith et al. (1978b) concluded that the rat intestine required a protein carrier in the vascular perfusate in order for Zn^{2+} transfer to take place. They suggested that albumin was the plasma protein in the portal blood which was responsible for the removal of Zn^{2+} from the mucosal cells. Albumin has been identified as the major plasma protein that Zn^{2+} is associated with in systemic transport in mammals (Henkin 1974). Under normal physiological conditions the molar ratio of Zn^{2+} to albumin in plasma is low (i.e. 0.06), leading Bremner and Mills (1981) to suggest that if albumin is the carrier protein Zn^{2+} absorption would probably not be limited by an inadequate

flux of available binding sites on the carrier, i.e. even allowing for only one Zn^{2+} binding site per albumin molecule one would expect that there would normally be an excess of sites available for Zn^{2+} binding. In addition, it has been observed in birds that the plasma proteins bind Zn^{2+} with greater strength than the intestinal mucosa proteins, presumably facilitating the movement of Zn^{2+} from the intestine into the blood (Suso and Edwards 1971).

Fletcher and Fletcher (1978) examined the binding affinity of winter flounder plasma proteins for Zn^{2+} using an equilibrium dialysis technique. More than one binding system for Zn^{2+} was detected. The highest affinity binding system appeared to be undersaturated with Zn^{2+}, i.e. they estimated that it would take twice the normal plasma Zn^{2+} concentration to saturate the highest affinity binding system. The association constant of this system, 10^7-10^8, was similar to that of the mammalian serum albumin-Zn^{2+} complex. When flounder serum was chromatographed on Sephadex G-150, Fletcher and Fletcher (1980) found that over 95% of the Zn^{2+} was associated with a protein fraction having a molecular weight (75,000) comparable to albumin-like components of carp plasma (Nagano et al. 1975; Yanagisawa et al. 1977) and to mammalian serum albumin (Peters 1975). This fraction probably represents the higher affinity Zn^{2+} binding system in the flounder plasma. Therefore, it appears that an albumin-like protein may also be involved in the systemic transport of Zn^{2+} in fish.

An examination of the affinity of the intestinal mucosa proteins for Zn^{2+} using the equilibrium dialysis technique revealed that more than one binding system is also present for Zn^{2+} in the mucosal cytosol of the flounder. The association constant of the highest affinity binding system ($K=2.42 \times 10^7$), obtained from the Scatchard-type plot of the data (Rosenthal 1967), represents the affinity of the binding proteins for Zn^{2+}

when the amount of Zn^{2+} added to the system is up to four times the amount of endogenous Zn^{2+} found in the mucosal cytosol. This suggests it would take four times the normal endogenous Zn^{2+} level (0.30 µg Zn^{2+}/mg protein) to saturate the highest affinity binding system. The association constant of this system was lower ($P < 0.05$) than that reported for the blood plasma which, if the system is involved in Zn^{2+} absorption, would facilitate the transfer of Zn^{2+} from the mucosal cells to the blood. Furthermore, since the high affinity binding system in the flounder plasma appears to be undersaturated with Zn^{2+}, one would not expect transfer and binding of Zn^{2+} to plasma proteins to be a limiting step. This is consistent with the hypothesis that Zn^{2+} bound to the intestinal tissue in the flounder may be transferred into the blood by a passive process which occurs down a concentration gradient.

Several intraluminal factors affect Zn^{2+} absorption in mammals (Becker and Hoekstra 1971) and it appears that these variables are also important determinants in the extent of Zn^{2+} absorption in the flounder. In keeping with the dependency of the Zn^{2+} transfer step in the flounder on the prior accumulation of Zn^{2+} in the intestinal tissue, the factors which altered the extent of accumulation also altered the extent of transfer into the body.

As previously discussed, the level of Zn^{2+} in the lumen seems to be an important determinant of the extent of Zn^{2+} absorption in the flounder. Although the proportion of Zn^{2+} absorbed declined as the intraluminal Zn^{2+} load increased, the amount of Zn^{2+} absorbed actually increased with increasing loads over a fairly wide range (5-250 µg). Based on these *in situ* findings the potential exists for flounder to take up increasing amounts of Zn^{2+} if the Zn^{2+} level in their diet (or in the sediments ingested with the diet) increases. The proportion of Zn^{2+} absorbed from the diet by rats also declined as the amount of Zn^{2+} increased, but Jackson et al. (1981) suggested that under normal dietary conditions the

absorptive mechanism operated in the linear portion of the curve. In other words, a rise in the Zn^{2+} content of the diet resulted in a proportionate rise in the amount of Zn^{2+} absorbed. Similarly, Miller (1970) found in ruminants that, although the percentage of dietary Zn^{2+} absorbed from the diet declined with increasing levels of Zn^{2+} in the food, the total amount of Zn^{2+} absorbed actually increased.

The extent of Zn^{2+} absorption in mammals is also influenced by dietary factors which alter its availability (O'Dell et al. 1972; Solomons 1982). When flounder were fed radiolabelled capelin (stable Zn^{2+}=57.1 µg/g dry weight) the net absorption of stable Zn^{2+} from the capelin averaged 37%. Only 17% of the stable Zn^{2+} was absorbed from a diet of fish food pellets (stable Zn^{2+}=92.8 µg/g dry weight). The differences in % absorption from the two diets may be attributable to the different Zn^{2+} levels, but the digestibility of the two diets also appeared to be very different. The digestibility of the fish food pellets was much lower than the capelin (i.e. the net absorption of dry material averaged 41 and 72%, respectively) so that less of the Zn^{2+} may have been available for absorption. Using the non-absorbed marker technique, Miller and Cragle (1965) observed a positive correlation between dry matter digestibility and Zn^{2+} absorption in ruminants. There is little information pertaining to fish on the availability of Zn^{2+} from different diets but Milner (1979, 1982) estimated that only 40% of the Zn^{2+} in a diet of *Artemia salina* nauplii was available to young plaice for absorption. The low availability of Zn^{2+} was attributed to the binding of Zn^{2+} to non-digestible cuticular material.

Several food constituents have been found to enhance the absorption of metals in mammals (Forth and Rummel 1973). Kroe et al. (1963) found that ^{59}Fe appeared more quickly, and in greater amounts, in the blood

when an amino acid (0.1 M methionine, proline, phenylalanine, serine, glutamic acid or histidine) was placed in the ligated intestine of rats with the iron. However, a 0.1 M solution of several of the same amino acids did not enhance Zn^{2+} absorption (tissue accumulation or transfer into the body) from the ligated intestine of the winter flounder. Addition of histidine (0.1 M) actually decreased Zn^{2+} absorption. In mammals, histidine appears to enhance the uptake of iron into the tissue (and its subsequent transfer) by complexing the iron in the lumen and increasing the amount that is available to binding sites on or within the mucosal cells (Forth and Rummel 1973). However, the donor effect of a chelator only takes place if the effective stability of the complex is not too high. If the stability of a complex is high the extent of absorption may depend on the extent to which the entire complex is absorbed. Perhaps the conditions employed in the present study were such that the Zn^{2+} was not released from the histidine-Zn^{2+} complex for accumulation into the tissue. Pecon and Powell (1981) found that the ratio of histidine:metal influenced the extent of Cd^{2+} uptake from the cardiac stomach of crabs (*Callinectes sapidus*). Low histidine: Cd^{2+} ratios (1:1) resulted in an increased Cd^{2+} transport over that observed without histidine; at higher histidine : Cd^{2+} ratios (10:1 to 400:1) histidine had no effect on Cd^{2+} transport. They reasoned that Cd^{2+} and histidine were co-transported. In the present study, inhibition of Zn^{2+} uptake was observed at a histidine: Zn^{2+} ratio of 125:1, a ratio at which Pecon and Powell (1981) observed no effect on Cd^{2+} uptake in crabs and lower than the ratio of histidine:metal (5000:1) found to enhance iron uptake in rats (Van Campen 1973). Recently Oestreicher and Cousins (1982) reported that several intraluminal constituents thought to be zinc chelators (i.e. citric acid, cysteine, reduced glutathione, histidine, methionine, picolinic acid and

tryptophan) did not enhance the transfer of zinc from the lumen to the portal circulation of the vascularly perfused rat intestine. At a luminal pH value of 6.6 (versus pH 4.2) they found that methionine, histidine, cysteine, tryptophan and glutathione, present in a ratio of chelator to Zn^{2+} of 14:1 decreased the lumen to plasma transfer of ^{65}Zn; at a chelator to Zn^{2+} ratio of 69:1, only tryptophan exhibited an inhibitory effect.

Fructose is another dietary constituent which has been shown to increase iron absorption in rats (Forth and Rummel 1973). Charley et al. (1963) proposed that this was due to the formation of a fructose-iron complex which was soluble at alkaline pH. They speculated that Zn^{2+}-fructose chelates were also formed in the presence of excess fructose. However, in the present study, no significant difference in Zn^{2+} accumulation in the intestinal tissue or transfer into the body was observed when an excess of fructose was injected into the ligated intestine of the flounder. It may be that an alternative explanation for the effect of fructose on iron absorption in mammals is applicable. Pollack et al. (1964) proposed that the effect of fructose on iron absorption was not due to its chelating properties but attributable to the metabolism of fructose during its absorption. The uptake of fructose was not examined in the winter flounder, but Cowey et al. (1974) reported that marine flatfish have very limited amylytic enzyme activity in their gastrointestinal tissue and as such may not be able to digest carbohydrates.

Another important variable found to influence Zn^{2+} absorption in mammals is the presence of other metals. Depending on the experimental conditions, copper, cadmium, iron and chromium have been found to exert an antagonistic effect on Zn^{2+} uptake (Van Campen 1969; Hiers et al. 1967; Hamilton et al. 1978; Hahn and Evans 1975; Hall et al. 1979).

Based on the *in situ* findings of the present study, several metals exert an inhibitory effect on the accumulation of Zn^{2+} in the intestinal tissue of the flounder and on its transfer into the body. However, in the equilibrium dialysis experiments, copper was the only metal which interferred with the binding of Zn^{2+} to the mucosal cytosol proteins. Similar antagonism has been reported in the binding of copper and zinc to rat albumin (Evans and Hahn 1974) and to the flounder plasma proteins (Fletcher and Fletcher 1978). In view of this, the inhibitory effect on the *in situ* uptake of Zn^{2+} caused by most of the other metals (i.e. Ni^{2+}, Cr^{2+}, Fe^{3+}, Cd^{2+} and Co^{2+}) may be mediated at the surface of the mucosal cells, whereas Cu^{2+} may also interfere with the transfer step. In rats it has been suggested that Cu^{2+} and Zn^{2+} compete for similar sites on mucosal binding proteins (Van Campen 1969) or on the albumin molecule (plasma transport protein) at the transfer step (Evans et al. 1975).

Under normal circumstances the diet of the winter flounder would probably contain much lower levels of cadmium, chromium, nickel and copper than zinc (i.e. the metal composition of a typical food organism, the polychaete, *Nereis diversicolor*, expressed as µg/g dry weight = cadmium 0.1; chromium 0.05; copper 18; iron 450; manganese 9; nickel 1.5; zinc 170 (Bryan 1976)). However, food organisms living in contaminated areas may contain elevated levels of these metals. For example, *N. diversicolor* from a contaminated estuary contained 1140 µg copper per g dry weight (Bryan 1976). The potential therefore exists for elevated levels of heavy metals to interfere with a fishes ability to obtain their required zinc.

When examined under ambient conditions both the accumulation of Zn^{2+} by the intestinal tissue and the transfer of Zn^{2+} into the body varied seasonally. The ability of the flounder to absorb Zn^{2+} was greatest during

the feeding period. This coincides with the growth period of the flounder (see Fig. 47 p. 230) when Zn^{2+} would be required for incorporation into new tissues. In addition, the fish spawn in June and the Zn^{2+} which was deposited in the gonads (approximately 4% of the total body Zn^{2+} in males and 25% in females) is presumably lost from the body and must be reacquired during the feeding period.

The question which arises from the observed seasonal change in Zn^{2+} absorption in the flounder is whether or not it is a response to a physiological demand for Zn^{2+} (i.e. a homeostatic response) or merely a fortuitous occurrence. The accumulation and transfer of Zn^{2+} by flounder maintained in the laboratory in heated seawater (approximately $8^{o}C$) and fed during what normally is a non-feeding period, resembled that of "summer" fish. Transfer of ambient fish (which had been maintained in the laboratory, water temperature approximately $0^{o}C$) to heated seawater ($8^{o}C$) in April resulted in no change in tissue accumulation but a four-fold increase in transfer was observed. The increase in Zn^{2+} transfer in the warm acclimated flounder during the winter may have been due to an increase in diffusion of Zn^{2+} at the higher temperature. However, this seems unlikely since the temperature coefficient for physical diffusion of a solute is approximately 1.4 per $10^{o}C$ rise in temperature (Lehninger 1975). It seems more likely that the increase in temperature may have exerted an indirect effect on Zn^{2+} absorption by altering the blood flow to the digestive tract. Since plasma proteins may play a role in the transfer of Zn^{2+} from the gut to the blood, an increase in blood flow could conceivably result in an increase in Zn^{2+} transfer. Factors other than the water temperature are probably also important since the Zn^{2+} absorption in flounder held under ambient conditions started to decline

in September when the water temperature was still high (see Fig. 10 p. 44).

There was a seasonal fluctuation in the stable Zn^{2+} concentration of the intestinal tissue but this did not appear to be a controlling factor in the extent of Zn^{2+} absorption. There was no apparent relationship between the endogenous stable Zn^{2+} content of the tissue and the amount of Zn^{2+} accumulated from the lumen *in situ*. Nor was any relationship apparent when the concentration of Zn^{2+} in the intestinal tissue was elevated by injections of Zn^{2+}.

Artificially elevating the Zn^{2+} status of the flounder by injections of stable Zn^{2+} did not alter the intestinal accumulation of ^{65}Zn (or ^{65}Zn in the presence of a Zn^{2+} load) or its transfer into the body. If the flounder maintains its whole body Zn^{2+} level by controlling the extent of absorption from the intestine one would expect to see a reduction in Zn^{2+} absorption in Zn^{2+}-injected fish. Cousins and his co-workers (Richards and Cousins 1975a; Smith et al. 1978a; Cousins 1979) found that absorption of ^{65}Zn was reduced in rats which were previously injected with a Zn^{2+} load. They attributed the decrease in transfer to the binding of ^{65}Zn to metallothionein in the intestinal tissue, and theorized that homeostatic control of Zn^{2+} absorption was modulated through changes in the metallothionein content of the intestinal tissue.

This involvement of metallothionein has been challenged by several investigators (Starcher et al. 1980; Evans et al. 1979; Jackson et al. 1981) and does not appear to apply to the flounder. Metallothionein was detected in the intestinal cytosols of Zn^{2+}-injected flounder (see Chapter III) but the presence of the protein was not associated with any enhancement or depression of Zn^{2+} uptake.

Prior injection of the flounder with a Zn^{2+} load did result in a reduction of the net absorption of ^{65}Zn from radiolabelled capelin. Based on the ratios of ^{65}Zn to ^{141}Ce in the rectum contents of flounder dissected 14 days after the injection, the net absorption of ^{65}Zn from capelin averaged 53% in saline-injected flounder and only 5% in the Zn^{2+} injected fish. If (as is suggested by the *in situ* findings of ^{65}Zn uptake from the upper intestine) there is no difference in the absorption of ^{65}Zn in the saline- and Zn^{2+}-injected fish, the observed differences in net absorption *in vivo* must be due to secretion of ^{65}Zn. Based on the ratio of stable Zn^{2+} to ^{141}Ce in the rectum contents there was a net secretion of stable Zn^{2+} in the Zn^{2+}-injected flounder.

Feeding the flounder a high level of Zn^{2+} in the diet (600 versus 60 µg Zn^{2+}/g wet weight) for five weeks did not result in any significant elevation of Zn^{2+} in the tissues examined (i.e. kidney, liver or intestine). If the Zn^{2+} in the diet was available for absorption, one would expect that more Zn^{2+} would have been absorbed from the diet with the added Zn^{2+} than from the control diet (see Fig. 5 p. 32). Since there appears to be no decrease in the Zn^{2+} absorptive capacity of the flounder fed a high-Zn^{2+} diet, it would appear that regulation of the tissue Zn^{2+} levels was achieved by elimination of the extra absorbed Zn^{2+}.

The relative importance of absorption and excretion to the homeostasis of Zn^{2+} is a difficult problem to resolve. Miller et al. (1971) proposed that high levels of Zn^{2+} in the diet had a greater effect on absorption of Zn^{2+} by ruminants than on endogenous excretion. They based this on the observation that feeding a high Zn^{2+} diet (600 µg/g) did not have a greater effect on ^{65}Zn loss from the body than did a 200 µg/g diet. Furchner and Richmond (1962) drew similar conclusions in

rats since the dietary Zn^{2+} level did not appear to have any effect on the long-lived component of the whole-body retention of orally administered ^{65}Zn (i.e. the rate of decline of the long component of the retention function was unchanged by differences in the dietary zinc concentration). As discussed previously, it has been hypothesized that the extent of Zn^{2+} absorption in the rat is controlled by alterations in the metallothionein level (Cousins 1979). However, Evans et al. (1979) have proposed that dilution of the oral dose of ^{65}Zn (used to access the extent of Zn^{2+} absorption) by endogenous secretions of stable Zn^{2+} has not been given enough consideration in estimating the retention of ^{65}Zn from different diets. Using the specific activity of the ^{65}Zn in the kidney and intestine of the rats to calculate the contribution of endogenous Zn^{2+} to the total amount of Zn^{2+} in the intestinal contents (a method developed by Wiegand and Kirchgessner 1976 a,b), they postulated that rats maintain homeostasis by secreting excess Zn^{2+} into the intestine. Similarily, since kinetic evidence on Zn^{2+} uptake in the rat indicated that under normal dietary conditions a rise in the Zn^{2+} content of the diet would result in a proportionate rise in the amount of Zn^{2+} absorbed, Jackson et al. (1981) hypothesized that to maintain homeostasis, a rise in Zn^{2+} excretion must occur. In a study on the dietary utilization of Zn^{2+}, Wiegand and Kirchgessner (1980) observed that, at high Zn^{2+} levels in the diet, rats absorbed Zn^{2+} in amounts which exceeded their net requirement for body maintenance and growth. They concluded that under these conditions Zn^{2+} balance in the rat was maintained by eliminating Zn^{2+} from endogenous pools in the body via the feces.

In a marine fish such as the winter flounder, which has only a portion of the year in which to obtain its annual requirement of Zn^{2+}, uptake of Zn^{2+} may not be limited by the Zn^{2+} status of the animal. Rather uptake of Zn^{2+} may be maximized. Such a process would be advantageous if the availability of Zn^{2+} from the diet is limited by factors such as an excess of other metals or the digestibility of the diet. In the event of exposure to elevated levels of Zn^{2+}, the present study indicates that elimination mechanisms may play a greater role in Zn^{2+} homeostasis than limitation of gastrointestinal uptake.

CHAPTER-II

DYNAMICS OF ZINC TURNOVER IN THE TISSUES AND WHOLE BODY: INVOLVEMENT OF THE GASTROINTESTINAL TRACT IN ZINC EXCRETION

INTRODUCTION

Studies with the radiotracer ^{65}Zn, either administered orally or injected directly into the animal, have demonstrated that Zn^{2+} is incorporated into different tissues at different rates. Several similarities exist in the distribution patterns of ^{65}Zn in the tissues of ruminants, dogs, mice and rats (Sheline et al. 1948; Gilbert and Taylor 1956; Rubini et al. 1961; Cotzias and Papavasiliou 1964; Miller 1969). In general, the muscle and bone accumulate ^{65}Zn very slowly in comparison to the more rapid accumulation observed in tissues such as the liver, kidney, spleen, pancreas, heart, lungs and gastrointestinal tract. The turnover of Zn^{2+} in several of the tissues also appears to change with the Zn^{2+} status of the animal. Miller et al. (1967) observed that most of the soft tissues of Zn^{2+}-deficient ruminants accumulated a higher percentage of an oral dose of ^{65}Zn than did tissues of normal animals. The tissues of the Zn^{2+}-deficient animals also retained the ^{65}Zn longer, i.e. the ^{65}Zn exhibited a longer biological half-life.

As well as resulting in changes in the extent of Zn^{2+} absorption (see Chapter I), changes in the requirements of mammalian tissues for Zn^{2+} appear to result in alterations in the elimination of Zn^{2+}. Ruminants fed a Zn^{2+}-deficient diet (6 µg/g) exhibited lower fecal losses of a single intravenous injection of ^{65}Zn than did those fed a control diet (46 µg/g) (Miller et al. 1966). The ^{65}Zn loss was further reduced when the animals developed signs of clinical Zn^{2+} deficiency (Miller 1969). The opposite result was observed when animals were fed high-Zn^{2+} diets. Fecal excretion of an intravenous injection of ^{65}Zn was increased when calves were fed high-Zn^{2+} diets, with 200 µg/g supplemental Zn^{2+} (i.e. added to the control diet which contained 38 µg/g) having as great an effect as 600 µg/g

(Miller et al. 1971). The whole-body loss of an injection of ^{65}Zn (i.p.) was accelerated in mice when the dietary Zn^{2+} level was increased (Cotzias et al. 1962). Similarly, Furchner and Richmond (1962) observed that rats maintained on diets supplemented with Zn^{2+} retained less ^{65}Zn than rats on normal diets. However, in this experiment, the ^{65}Zn was administered orally and the authors attributed the differences in ^{65}Zn retention largely to inhibition of absorption. They proposed that this was a reasonable conclusion since the rate of loss of the long-lived component of the ^{65}Zn retention function did not appear to be affected by differences in the dietary Zn^{2+} concentration.

In mammals, the general concensus is that Zn^{2+} elimination from the body occurs mainly via the digestive tract (Underwood 1977). Collection of feces and urine following oral administration or injection of ^{65}Zn have demonstrated that very little zinc is excreted in the urine (Rubini et al. 1961; Cotzias et al. 1962; Miller et al. 1966; Methfessel and Spencer 1973 b; Wiegand and Kirchgessner 1976 a,b). Birnstingl et al. (1957) proposed that pancreatic secretions accounted for most of the endogenous Zn^{2+} in the feces of dogs but this does not appear to be the predominant route in other species. For example, Pekas (1966) found that fecal excretion of endogenous Zn^{2+} was not greatly reduced in pigs when the pancreatic duct was ligated. Similarly, Stake et al. (1974) concluded that Zn^{2+} excretion via the pancreas accounted for 25% or less of the total endogenous loss in calves. Ligation of different segments of the gastrointestinal tract of rats following an injection of ^{65}Zn (i.v.) demonstrated that Zn^{2+} could be secreted into all regions of the tract, the principal site being the small intestine (Methfessel and Spencer 1973 b). Since the duodenal segment did not contain greater amounts of ^{65}Zn than the more distal portions of the small intestine, this study also appears to contradict the hypothesis that

pancreatic juices constitute the primary source of endogenous fecal Zn^{2+}. The mechanism of intestinal Zn^{2+} secretion has not been elucidated but *in vitro* studies using rat intestine suggest that the transport of Zn^{2+} across the intestinal mucosa, from the serosal to the mucosal surface, may be an active process requiring metabolic energy (Kowarski et al. 1974). Alternatively, it has been suggested that Zn^{2+} excretion may occur by desquamation of mucosal cells containing Zn^{2+} or by the secretion of Zn^{2+} along with intestinal juices (Methfessel and Spencer 1973 b).

The dynamics of ^{65}Zn turnover (accumulation and loss) in whole fish and in fish tissues has been examined in several radioecological studies designed to assess both the potential toxicity of the radionuclide to the fish and the potential health hazard of the fish (considered as a food organism) to humans (Nakatani 1966; Baptist et al. 1970; Merlini et al. 1973; Pentreath 1973 a,b, 1976). In agreement with observations in mammals, these studies indicate that Zn^{2+} follows a specific pathway through the body, i.e. different tissues accumulate and loss ^{65}Zn at different rates.

In the present study the dynamics of Zn^{2+} turnover in the winter flounder was investigated by examining the distribution of ^{65}Zn in the tissues following intramuscular injections of the radiotracer. In addition, the whole-body retention of ^{65}Zn was examined in normal flounder and in flounder in which the Zn^{2+} status was elevated by injections of stable Zn^{2+}. Studies were also carried out to determine the site(s) and extent of ^{65}Zn excretion into the gastrointestinal tract.

MATERIALS AND METHODS

Section A. Distribution of ^{65}Zn in several organs and tissues of the winter flounder following single, intramuscular injections.

The distribution of ^{65}Zn in various organs and tissues of the winter

flounder was investigated by monitoring the radioactivity in the fish following a single intramuscular injection (i.e. into the dorsal fin muscle, 20 μCi per fish). The flounder used in these studies were maintained under ambient conditions of temperature and photoperiod; they were fed chopped capelin from June to October. The fish ranged in body length from 30-39 cm and in body weight from 300-874 g.

In one study the flounder were injected in May. Five fish at each time point were then killed and dissected 1, 3, 7, 15, 29, 71, 156, 296, 392 and 528 days following the injection. Samples of several organs and tissues were weighed and placed in vials; the cpm ^{65}Zn in the tissues were then determined using a gamma scintillation counter (Packard model 578). Standards were counted at each time interval to correct for physical decay of the isotope. The concentration of ^{65}Zn in the tissues (cpm ^{65}Zn/g wet weight) was expressed as a % of the total amount of ^{65}Zn injected into each flounder. The data were plotted on semi-log paper and estimations made of the biological half-time ($TB_{1/2}$) and the rate constants (K) of ^{65}Zn decline in the tissues.

$$TB_{1/2} = \frac{0.3t}{\log (A_o/A)} \qquad \text{(Comar 1955)}$$

and $$K = \frac{0.693}{TB_{1/2}}$$

where, A_o = concentration of ^{65}Zn at $time_o$

A = concentration of ^{65}Zn at $time_t$

t = time interval (days)

The total amount of ^{65}Zn in a given organ or tissue was calculated by multiplying the cpm ^{65}Zn per g by the total weight of the tissue. The weights of the gonad, liver, spleen, gastrointestinal tract, kidney, interhaemal spine (bone) and eyes were determined for each fish by directly

weighing the tissue. The total weight of blood in each fish was estimated as 5% of its total body weight. The total weights of white muscle, gills, skin and scales for each fish were estimated using equations which relate the tissue weight to the body length of the fish (see Appendix A, equations derived by Fletcher and King, personal communication). The total amount of ^{65}Zn in each tissue was expressed as a % of the total amount of ^{65}Zn injected into each flounder.

In one flounder (a male), the total amount of ^{65}Zn remaining in the fish 528 days post-injection was estimated by completely dissecting the fish and directly counting the ^{65}Zn in all of the tissues. In this fish, in addition to the tissues mentioned above, the skeleton, fins and fin muscle were also examined.

In a second study, winter flounder (all female) were injected with ^{65}Zn (into the dorsal fin muscle) in August. The fish ranged in body length from 30-42 cm and in body weight from 299-1049 g. Groups of 10 and 9 flounder were dissected in October and April, respectively. The concentration (^{65}Zn/g wet weight) and total amount of ^{65}Zn, expressed as a % of the amount of ^{65}Zn injected into each fish, were calculated for several representative tissues. The total amount of white muscle in each fish was estimated from equations which relate the tissue weight to the body length of the fish (see Appendix A, equations derived by Fletcher and King, personal communication).

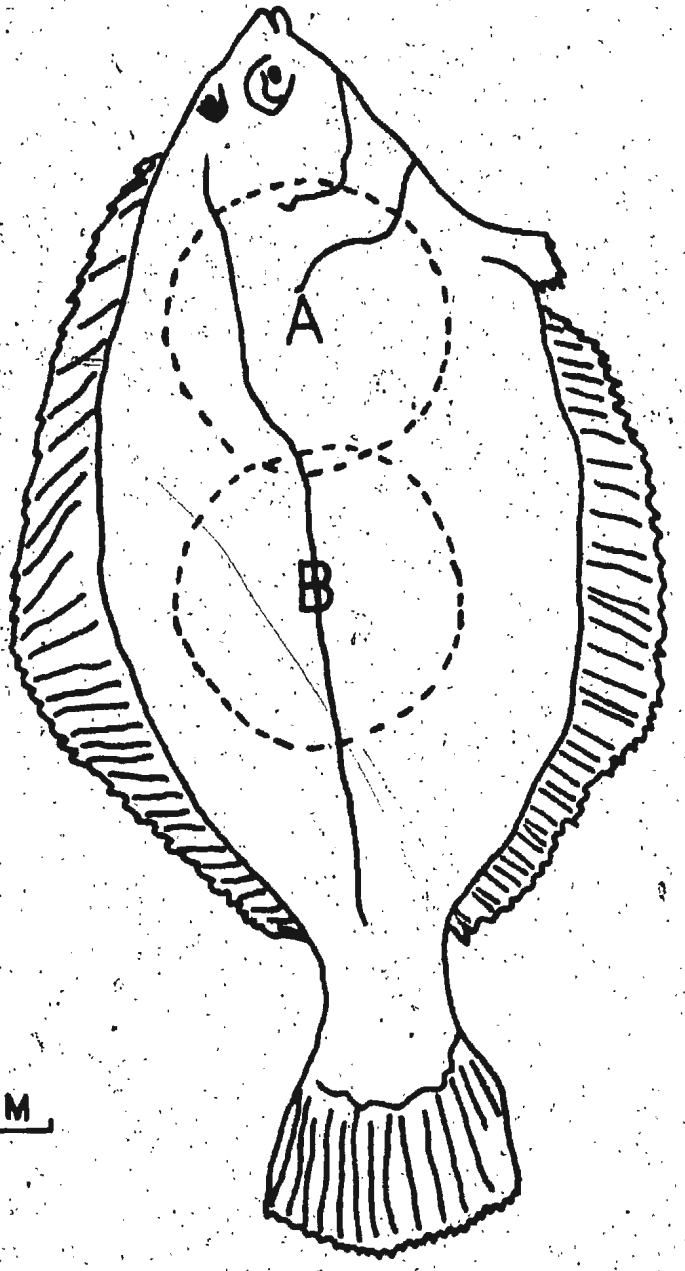
Section B. Whole-body retention of ^{65}Zn in winter flounder following single intraperitoneal injections.

Whole-body retention of zinc was followed by measuring the radionuclide content of fish injected intraperitoneally with ^{65}Zn (30-50 μCi per fish) in saline or ^{65}Zn plus a load of stable Zn^{2+} (25% of the total body load of Zn^{2+} in the flounder based on an estimated whole body con-

centration of 15 µg Zn^{2+}/g wet weight). The fish examined comprised both sexes and ranged in body weight from 300 to 700 g. One group of flounder (4 saline- and 3 Zn^{2+}-injected) was injected in late August; another group (3 saline- and 3 Zn^{2+}-injected) was injected in December. Feeding was terminated in early September. Both groups were monitored through to the following June. Four saline- and two Zn^{2+}-injected flounder were also monitored from June to August; the fish were fed chopped capelin during this interval. The flounder were maintained in individual 10 L tanks supplied with flowing seawater at ambient temperature.

Each flounder was radioanalyzed live by placing it in a plexiglass container through which seawater was passed. The fish were anesthetized (for 10 minutes in MS 222) prior to being placed in the container and held in a fixed position such that the same regions of each flounder were counted at successive time intervals. The fish in the container was counted between two 3" by 3" NaI crystals. Two regions of each fish, designated site A and B, were counted (Fig. 19). The peak of ^{65}Zn radioactivity at each site was integrated; corrections were made for physical decay of the isotope by counting a ^{65}Zn standard at each time interval. The activity at site A in each fish was expressed as a % of the initial counts per minute detected 3-5 hours after the injection. The activity at site B in each fish was expressed as a % of the maximum counts per minute detected (1-4 days after the injection). The data were plotted against time on semi-log paper and analyzed by the standard kinetic approach usually applied to first-order reactions (Comar 1955; Baptist and Price 1962). Where the curve appeared to consist of more than one component (i.e. retention at site A), the slope of the linear tail of the curve was more accurately determined by the method of least squares and extrapolated back to the y axis. The extrapolated values were subtracted from the corresponding values of the first

Fig. 19. The regions (designated as site A and site B) of the winter flounder monitored for ^{65}Zn activity at successive time intervals following intraperitoneal injections of ^{65}Zn plus stable Zn^{2+} or ^{65}Zn plus an equivalent volume of saline.


A
B
4 CM

component of the curve, yielding a straight line. The retention process may then be expressed by the form $R = a_1 e^{-k_1 t} + a_2 e^{-k_2 t}$ in which a_1, a_2 and k_1, k_2 are the intercept and rate constants, respectively of the individual components of the curve. The intercepts (a_1,a_2), represent the activity (% of ^{65}Zn) present at zero time in each component. Values of K (rate constant) were calculated by multiplying the slope of each line by 2.3, the slope being log A_0 - log A/t in which A_0 represents the activity present at zero time and A the activity present at time t. The biological half-time of each component was determined by the equation $TB_{1/2} = \frac{0.693}{K}$.

Section C. Comparison of Zn^{2+} concentrations in several tissues of winter flounder injected with Zn^{2+} or an equivalent volume of saline.

To complement the study on the whole-body retention of ^{65}Zn in saline- and Zn^{2+}-injected flounder, the stable Zn^{2+} concentrations of several tissues were analyzed. The study was conducted in January. The flounder (all males) were injected intraperitoneally with Zn^{2+} (25% of the estimated total body Zn^{2+}) or an equivalent volume of saline (1% NaCl). Fifteen days later the fish were killed, bled from a caudal blood vessel and dissected. Samples of kidney, liver and intestinal tissue (uppermost portion of the intestine, including the pyloric appendages) were dried, digested with nitric acid and the Zn^{2+} content of the digests determined by atomic absorption spectrophotometry (Varian Tectron model AA5) as described in Chapter I, p. 15). The Zn^{2+} concentrations are expressed per g of dry tissue.

Section D. The site(s) and extent of ^{65}Zn "secretion" into the gastrointestinal tract.

Winter flounder were injected intravenously with ^{65}Zn and a stable Zn^{2+} load (equivalent to 25% of the estimated body load of Zn^{2+} of a 200 g

fish, i.e. injected with 750 µg of Zn^{2+} per fish). The experiment was conducted in October; the fish were not fed during the study period. Four to five fish per time point were killed 2, 15, 24, 48, 168 and 336 hours after the injection and bled from a caudal blood vessel. An incision was made in the body wall and the gastrointestinal tract tied off *in situ* into the following segments: stomach, upper-intestine (including the pyloric appendages), mid-intestine, low-intestine and rectum. The segments were dissected out of the body and the lumen contents emptied into counting vials. The ^{65}Zn in each sample was determined using a gamma scintillation counter (Packard model 578). The values were converted to µg Zn^{2+} based on the specific activity of the injection. To facilitate comparison between tissues, the data were expressed as the ratio of µg Zn^{2+} per g tissue divided by the µg Zn^{2+} injected per g body weight.

The distribution of zinc was then compared in flounder with ligated or unligated upper intestines. The study was conducted in November. The surgical procedure described in Chapter I (p.11) was used to ligate the upper intestine (region including the pyloric appendages) of half of the test fish. The flounder were subsequently injected intravenously with ^{65}Zn plus a stable Zn^{2+} load (equivalent to 25% of the estimated body load of a 400 g fish, i.e. injected with 1500 µg Zn^{2+} per fish) or ^{65}Zn plus an equivalent volume of saline. Eighteen hours after the injection each flounder was killed and bled from a caudal blood vessel. The gastro-intestinal tract were tied off into segments *in situ* (i.e. the mid- and low-intestine were tied off in fish in which the upper intestine was previously ligated; in the remainder of the fish the upper-, mid- and low-intestine were tied off). The ^{65}Zn in the lumen contents, intestinal tissue, liver, kidney and a blood sample was determined as described in the previous study.

The distribution of ^{65}Zn was also examined in saline- and Zn^{2+}-injected

flounder in the summer feeding period (June-July). Fish used in this study were maintained under ambient conditions of temperature and photoperiod; they were fed chopped capelin during the test period. ^{65}Zn (20 μCi per fish) plus a stable Zn^{2+} load (24% of the estimated total body Zn^{2+}) or ^{65}Zn plus an equivalent volume of saline (1% NaCl) were injected intravenously. Five flounder of each test group were killed 9 and 27 days later. The fish were bled, an incision made in the body wall and the upper portion of the intestine (including the pyloric appendages) tied off *in situ*. The contents of this region were emptied into a counting vial. The cpm ^{65}Zn in the intestinal tissue, lumen contents, kidney and liver were determined as previously described and expressed as a % of the total ^{65}Zn injected into each flounder.

RESULTS

Section A. Distribution of ^{65}Zn in several tissues and organs of the winter flounder following single intramuscular injections.

The changes in tissue ^{65}Zn concentrations following a single intramuscular injection are demonstrated in Fig. 20, 21 and 22. Only the values ($\bar{X} \pm SE$) for the male flounder are plotted since they represented the majority of the fish examined at each time interval (i.e. 3-5 of the 5 fish dissected at each time point). The values for the female flounder generally fell within the same range as the males but there were too few values to determine if there were any differences in the tissue turnover of ^{65}Zn attributable to the sex of the fish.

Up to 15 days following the injection, the decline in the ^{65}Zn concentration in the blood was accompanied by a rise in the ^{65}Zn concentration in the other tissues of the body. From day 15 (May 22) to day 71 (July 17) there was an overall decline in the ^{65}Zn concentration in the liver,

Fig. 20. Concentration of ^{65}Zn in the blood, kidney, gill, spleen and liver of flounder dissected from 1 to 528 days following a single intramuscular injection (May 7). Data expressed as % of Injected Dose X 10^{-4} ($\bar{X} \pm SE$, n=3-5 males per point), where, % Injected Dose = $\frac{\text{cpm } ^{65}Zn/g \text{ tissue}}{\text{total cpm } ^{65}Zn \text{ inj.}}$ X 100.

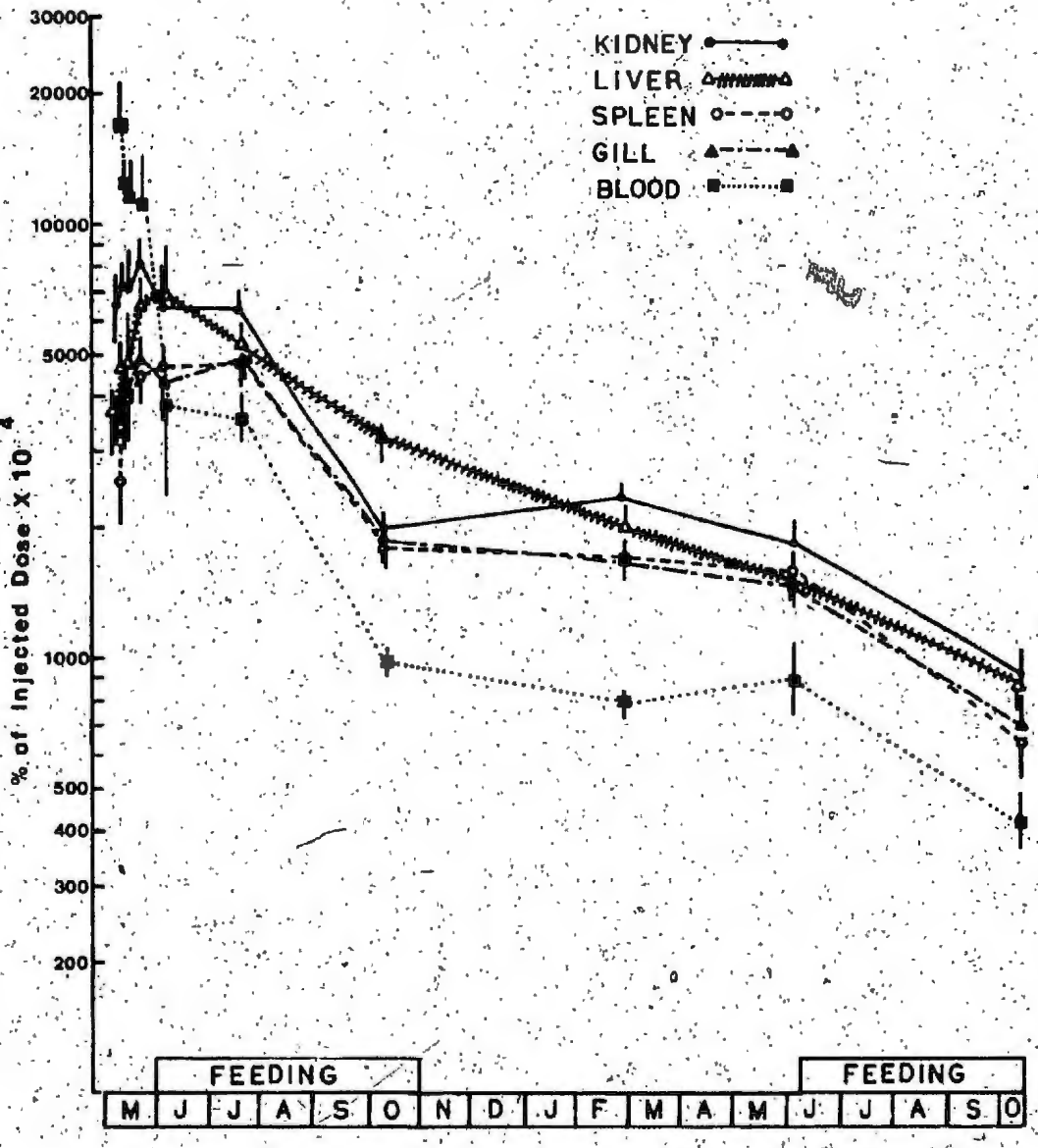

KIDNEY
LIVER
SPLEEN
GILL
BLOOD
% of Injected Dose X 10⁻⁴
30000
20000
10000
5000
1000
500
400
300
200
FEEDING
FEEDING
M J J A S O N D J F M A M J J A S O

Fig. 21. Concentration of ^{65}Zn in the gastrointestinal tract tissues of flounder dissected from 1 to 528 days following a single intramuscular injection (May 7). Data expressed as % of Injected Dose X 10^{-4} ($\bar{X} \pm SE$, n=3-5 males per point) where, % Injected Dose = $\frac{\text{cpm } ^{65}Zn/g \text{ tissue}}{\text{Total cpm } ^{65}Zn \text{ inj.}}$ X 100.

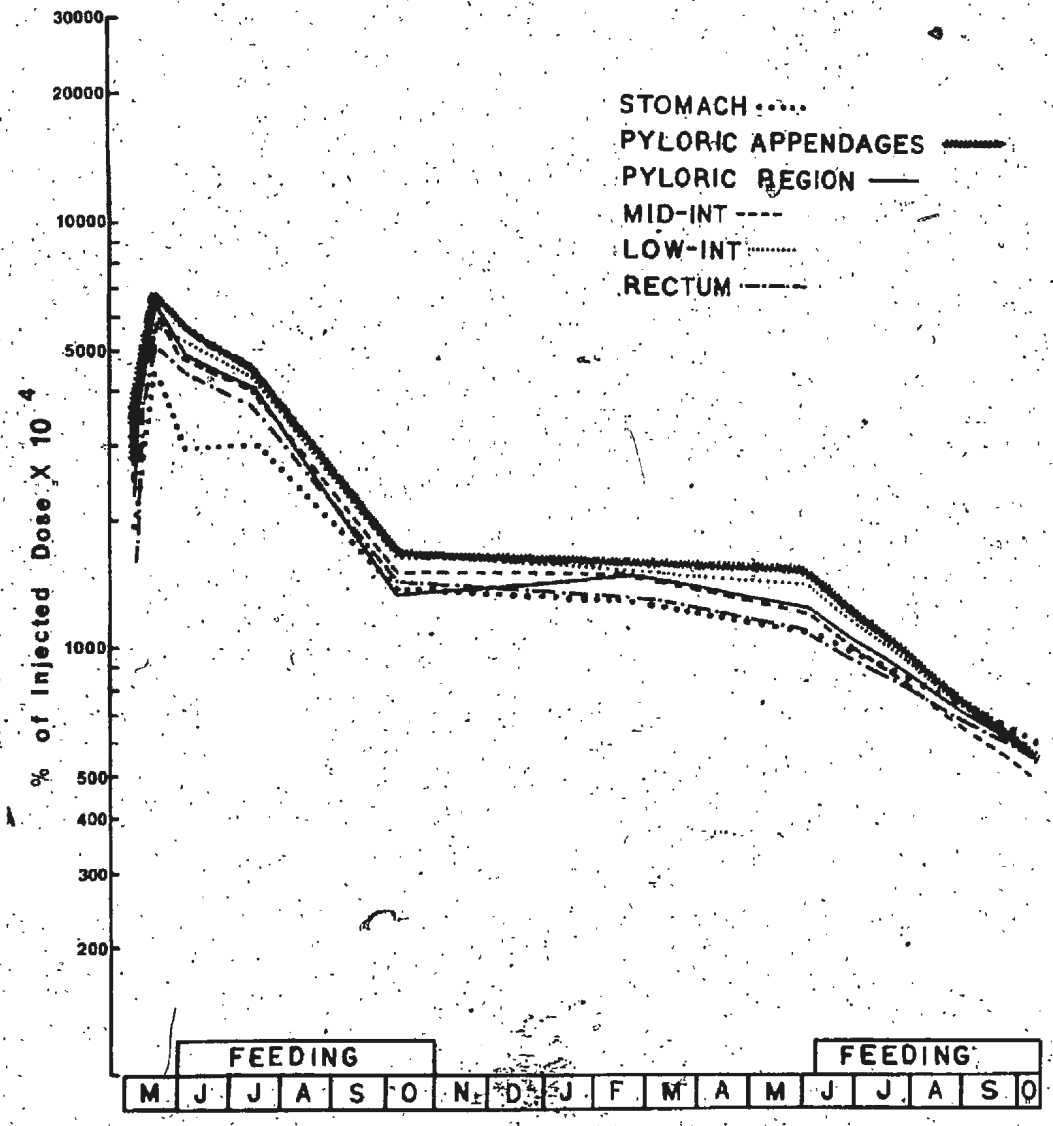
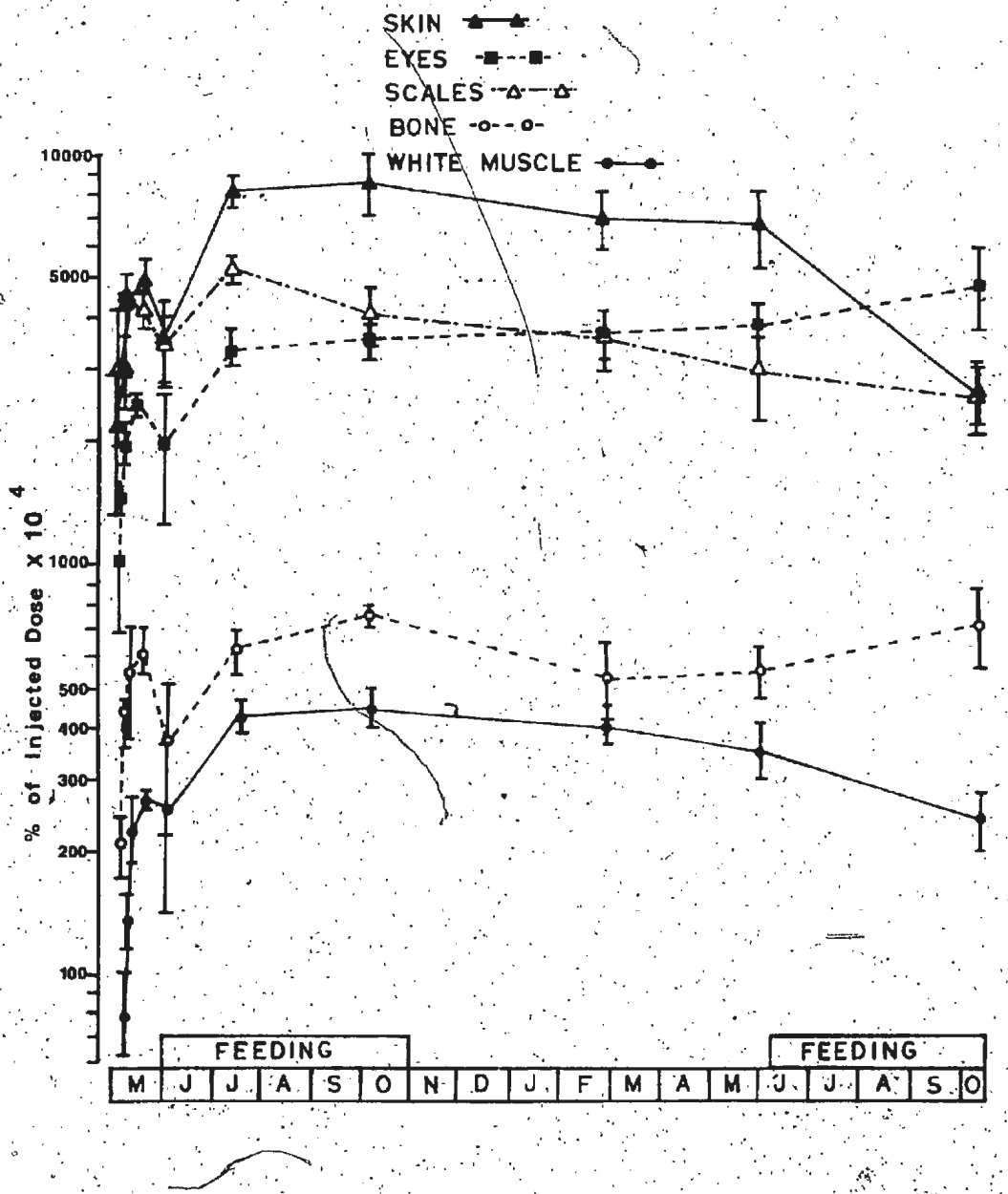

30000
20000
10000
5000
1000
500
400
300
200
% of Injected Dose X 10 4
STOMACH
PYLORIC APPENDAGES
PYLORIC REGION
MID-INT
LOW-INT
RECTUM
FEEDING
FEEDING
M J J A S O N D J F M A M J J A S O

Fig. 22. Concentration of ^{65}Zn in the skin, scales, eyes, white muscle and interhaemal spine (bone) of flounder dissected from 1 to 528 days following a single intramuscular injection (May 7). Data expressed as % of Injected Dose X 10^{4} (X ± SE, n=3-5 males per point), where, %

$$\text{Injected Dose} = \frac{\text{cpm } ^{65}Zn/g \text{ tissue}}{\text{Total cpm } ^{65}Zn \text{ inj.}} \times 100.$$


SKIN
EYES
SCALES
BONE
WHITE MUSCLE
% of Injected Dose X 10 4
10000
5000
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500
400
300
200
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FEEDING
M J J A S O N D J F M A M J J A S O

gastrointestinal tissues and kidney; there was very little change in the ^{65}Zn concentration in the gills and spleen. From day 71 (July 17) through to the end of the experiment (day 528) similar ^{65}Zn turnover patterns were observed in the gastrointestinal tissues, blood, gills, spleen and kidney (Fig. 20, 21).

The biological half-times ($TB_{1/2}$, days) and rate constants (K, $days^{-1}$), based on the ^{65}Zn concentrations in the tissues of flounder dissected 71, 156, 392 and 528 days after the initial injection, are summarized in Table 9. The ^{65}Zn concentration in the kidney, blood, gastrointestinal tissues, spleen and gills declined fairly rapidly from July to October (i.e. $TB_{1/2}$ averaged 55 days, K averaged 0.0128 day^{-1}). Very little decline in the ^{65}Zn concentration in these tissues occurred from October to June (i.e. $TB_{1/2}$ ranged from 638 to 1817 days, K ranged from 0.0004 to 0.0011 day^{-1}), which corresponded to the non-feeding period. The rate of decline in the ^{65}Zn concentration of these tissues increased again when feeding was resumed (i.e. from June to October the $TB_{1/2}$ ranged from 87 to 135 days, K ranged from 0.0051 to 0.0080 day^{-1}). The rate of loss of ^{65}Zn from the liver did not differ greatly between the feeding and non-feeding periods.

The ^{65}Zn concentration in white muscle, skin and interhaemal spine (bone) tended to increase or remain constant in the period from July to October when the level in most tissues was declining (compare Fig. 22 with Fig. 20 and 21). In one tissue, the eyes, the ^{65}Zn concentration continued to rise over the duration of the study (Fig. 22).

Twenty-four hours following the injection, 52.5 ± 6.92% ($\bar{X}$ ± SE, n=5 fish) of the total amount of ^{65}Zn injected per fish was estimated to be recovered in the tissues which were sampled (i.e. blood, gonad, liver, spleen, gastrointestinal tissues, kidney, gill, white muscle, skin, scales, interhaemal spine and eyes) (Table 10). These tissues together accounted

Table 9. Biological half-times ($TB_{1/2}$) and rate constants (K) of ^{65}Zn decline in tissues of the winter flounder (♂) based on the concentration of ^{65}Zn in the tissues of fish dissected 71, 156, 392 and 528 days after the initial injection on May 7. (used $\bar{X}$ of 3-5 fish per time point).

Tissue	July→October (71- and 156-days post-inj.)		October→June (156- and 392-days post-inj.)		June→October (392- and 528-days post-inj.)	
	$TB_{1/2}$ (days)	K ($days^{-1}$)	$TB_{1/2}$ (days)	K ($days^{-1}$)	$TB_{1/2}$ (days)	K ($days^{-1}$)
Spleen	60	0.0116	1251	0.0006	103	0.0067
Liver	118	0.0059	211	0.0033	164	0.0042
Gill	62	0.0112	638	0.0011	126	0.0055
Kidney	50	0.0137	1817	0.0004	135	0.0051
Gastrointestine (pyloric appendages)	58	0.0119	1692	0.0004	87	0.0080
Blood	45	0.0154	1716	0.0004	127	0.0055
White muscle	-	-	633	0.0011	233	0.0030
Interhaemal spine (bone)	-	-	476	0.0015	-	-

where, $TB_{1/2} = \frac{0.3 \times t}{\log \left(\frac{A_o}{A}\right)}$ (Comar 1955)

and

$$K = \frac{0.693}{TB_{1/2}}$$

A_o = concentration of ^{65}Zn at $time_o$

A = concentration of ^{65}Zn at $time_t$

t = time interval (days)

$$\text{concentration of } ^{65}Zn \text{ in tissue} = \left[\frac{\text{cpm } ^{65}Zn/g}{\text{cpm } ^{65}Zn \text{ inj. per fish}} \times 100\right] \times 10^{-4}$$

Table 10. Estimates of the total amount of ^{65}Zn[a] (expressed as a % of the amount of ^{65}Zn injected per fish) in several organs and tissues[b] of the winter flounder following a single intramuscular injection. Values expressed as $\bar{X} \pm SE$, n=number of fish dissected.

Days Post-inj. / Date / Tissue	1- May 8 (n=5)	29- June 6 (n=3)	71- July 17 (n=5)	156- Oct 10 (n=3)	296- Feb 27 (n=4)	392- June 4 (n=5)	528- Oct 17 (n=4)
Blood	36.08± 4.29	8.82± 2.75	7.96± 0.46	2.24± 0.14	1.66± 0.25	1.87± 0.43	1.33± 0.15
Gonad	1.23± 0.14	3.68± 0.69	5.66± 1.15	0.67± 0.12	2.67± 0.44	2.49± 0.52	3.67± 0.18
Liver	1.49± 0.15	4.02± 0.28	2.07± 0.20	1.46± 0.08	1.02± 0.16	0.52± 0.06	0.88± 0.11
Spleen	0.10± 0.01	0.24± 0.05	0.22± 0.03	0.09± 0.01	0.08± 0.01	0.06± 0.01	0.04± 0.01
Gastroint. tract	1.75± 0.25	5.38± 1.08	3.88± 0.33	1.04± 0.11	0.93± 0.13	0.93± 0.11	0.61± 0.12
Kidney	1.08± 0.09	1.10± 0.02	1.07± 0.09	0.24± 0.03	0.36± 0.08	0.29± 0.04	0.27± 0.01
Gill	1.83± 0.26	2.13± 0.28	2.25± 0.11	0.85± 0.08	0.93± 0.12	0.77± 0.11	0.46± 0.08
White muscle	1.30± 0.23	4.16± 1.71	8.42± 0.84	7.18± 0.99	6.38± 0.86	4.12± 0.77	4.23± 0.61
Skin	6.13± 1.63	8.97± 2.04	15.95± 0.79	12.48± 2.38	13.00± 1.81	11.48± 3.00	4.50± 0.16
Scales	1.38± 0.31	2.20± 0.40	2.54± 0.15	1.64± 0.34	3.13± 0.62	1.95± 0.43	2.00± 0.43
Interhaemal spine (bone)	0.02± 0.003	0.04± 0.01	0.05± 0.01	0.06± 0.01	0.06± 0.01	0.06± 0.01	0.08± 0.02
Eyes	0.11± 0.02	0.25± 0.07	0.34± 0.02	0.24± 0.02	0.41± 0.06	0.41± 0.06	0.55± 0.14
Total	52.5 ± 6.92	41.0 ± 7.37	50.1 ± 1.76	28.2 ± 4.21	30.6 ± 3.84	26.1 ± 5.29	18.6 ± 1.63

[a] $\text{Total } ^{65}Zn\ (\%) = \frac{\text{cpm } ^{65}Zn/g \times \text{weight (g)}}{\text{cpm } ^{65}Zn \text{ inj. per fish}} \times 100$

[b] The weights of the gonad, liver, spleen, gastrointestinal tract, kidney, interhaemal spine (bone) and eyes were determined by directly weighing. Blood weight was estimated to be 5% of the total body weight. Weights of the gill, white muscle, skin and scales were estimated from equations relating tissue weight to body length (see Appendix A).

for 60-70% of the total body weight of the flounder (Table 11). The % of ^{65}Zn recovered in these tissues declined to 18.6 ± 1.63% ($\bar{X}$ ± SE, n=4 fish) in flounder dissected 528 days following the injection (Table 10). By comparison, 38.8% of the total amount of ^{65}Zn injected was recovered in one fish which was completely dissected and counted after the same time interval (Table 12). The difference was largely attributable to the ^{65}Zn recovered in the skeleton, fins (and skin covering the fins) and other muscle (i.e. fin and belly muscle).

The distribution of ^{65}Zn in the bile, urine and intestinal tract contents is summarized in Fig. 23. Very little ^{65}Zn was detected in the bile (i.e. 0.01% of the total ^{65}Zn injected per fish). Somewhat higher levels of ^{65}Zn were detected in the urine; up to 21 days following the injection approximately 0.1% of the ^{65}Zn injected per fish was detected in the urine sample of a few of the flounder. The maximum ^{65}Zn values in the intestinal lumen contents were observed in the fish dissected in June and July (29 and 71 days post-injection). At these times, approximately 0.1-0.2% of the total ^{65}Zn injected per fish was detected in the lumen contents of a few of the flounder. The level of radioactivity was similar in the lumen contents of the upper and lower intestine (i.e. divided the intestine into two parts before draining the contents).

The % of ^{65}Zn in the liver, white muscle, kidney, gill filaments and gastrointestinal tract of the female flounder declined significantly ($P < 0.05$) from October to April (63- and 236-days after a single intramuscular injection) (Fig. 24 A). There was a large increase in the % of ^{65}Zn in the gonads over the same period. The concentration of ^{65}Zn in the tissues was not significantly different between flounder dissected in October and April but there was a significant decline in the weights ($P<0.05$) (expressed as a % of the total body weight) of several of the tissues (i.e.

Table 11. Weights[a] of tissues and organs expressed as a % of the body weight. Values are $\bar{X} \pm SE$ (n=number of fish dissected).

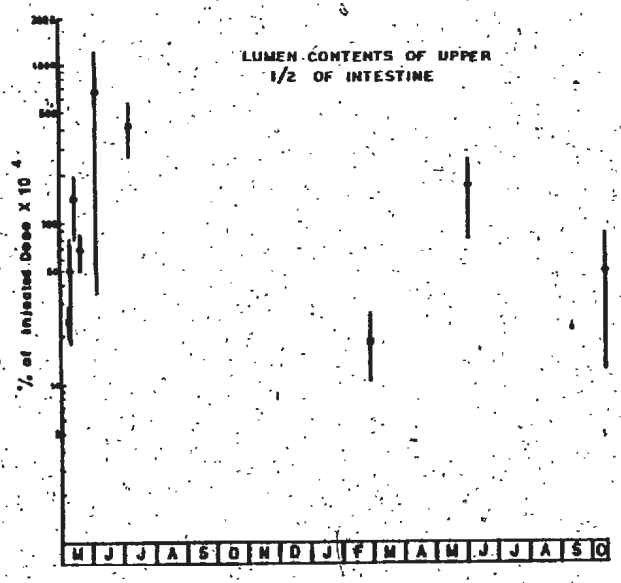
Date	May 8 (n=5)	June 6 (n=3)	July 17 (n=5)	Oct 10 (n=3)	Feb 27 (n=4)	June 4 (n=5)	Oct 17 (n=4)
Gonad	9.98± 0.41	8.45± 1.92	13.18± 1.19	0.57± 0.01	6.69± 0.90	9.21± 1.50	13.46± 1.36
Liver	0.96± 0.04	1.30± 0.14	0.88± 0.06	1.00± 0.05	1.20± 0.05	0.80± 0.04	1.80± 0.25
Spleen	0.09± 0.01	0.11± 0.02	0.10± 0.01	0.10± 0.01	0.11± 0.02	0.08± 0.01	0.12± 0.02
Gastroint. tract	2.12± 0.13	2.55± 0.45	2.36± 0.21	1.53± 0.05	1.65± 0.21	1.79± 0.09	1.89± 0.25
Kidney	0.41± 0.03	0.37± 0.04	0.37± 0.02	0.26± 0.01	0.38± 0.04	0.36± 0.02	0.52± 0.08
Gill	1.23± 0.06	1.17± 0.08	1.20± 0.05	1.00± 0.05	1.40± 0.08	1.22± 0.03	0.90± 0.03
White muscle	36.31± 0.97	34.72± 2.17	43.61± 2.54	37.89± 1.70	40.52± 2.10	36.15± 0.73	36.55± 3.38
Skin	7.39± 0.14	6.84± 0.38	7.07± 0.30	5.91± 0.29	8.22± 0.40	7.06± 0.16	5.30± 0.16
Scales	2.45± 0.05	2.33± 0.13	2.06± 0.08	1.69± 0.09	2.73± 0.13	2.41± 0.06	1.56± 0.07
Interhaemal spine (bone)	0.24± 0.01	0.24± 0.01	0.19± 0.02	0.18± 0.00	0.26± 0.02	0.24± 0.01	0.19± 0.01
Eyes	0.20± 0.04	0.28± 0.01	0.24± 0.01	0.16± 0.04	0.29± 0.08	0.25± 0.02	0.20± 0.01

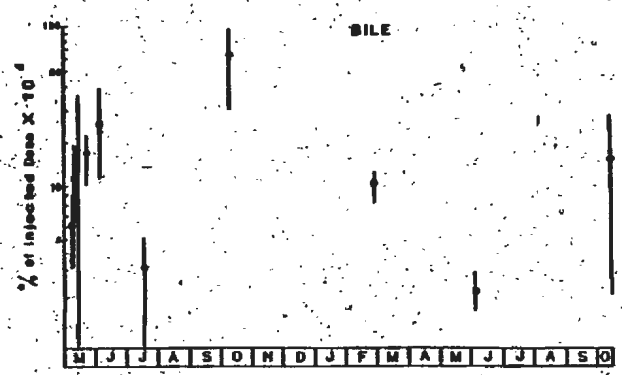
[a] Weights of gonad, liver, spleen, gastrointestinal tract, kidney, interhaemal spine (bone), and eyes were determined by directly weighing. The total weights of white muscle, gills, skin, and scales for each fish were estimated using equations which relate the tissue weight to the body length of the fish (see Appendix A).

Table 12. Total amount of ^{65}Zn (expressed as a % of the amount injected) in several organs and tissues of a male winter flounder totally dissected 528 days following a single intramuscular injection.

Tissue	% of Inj.
Blood	1.24
Gonad	3.35
Liver	0.59
Spleen	0.04
Gastrointestinal tract	0.44
Kidney	0.27
Gill	0.31
White muscle	4.91
Skin	4.72
Scales	1.31
Interhaemal spine (bone)	0.07
Eyes	0.54
Other skin (on fins, head and belly)	4.67
Other muscle (on fins and belly)	4.70
Fins	3.60
Other bones (operculum, atlas, centras, skeleton)	5.52

Fig. 23. Total amount of ^{65}Zn (expressed as a % of the amount of ^{65}Zn injected per fish) in the bile, urine, and lumen contents of the upper one half of the intestine, in flounder dissected from 1 to 528 days following a single intramuscular injection (May 7). Data expressed as % of Injected Dose X 10^{4} (X ± SE, n=2-5 fish per point), where, % Injected Dose = $\frac{\text{total cpm } ^{65}Zn}{\text{cpm } ^{65}Zn \text{ inj.}}$ X 100.


LUMEN CONTENTS OF UPPER
1/2 OF INTESTINE
% of Injected Dose X 10⁻⁴
M J J A S O N D J F M A M J J A S O


BILE
% of Injected Dose X 10⁻⁴
M J J A S O N D J F M A M J J A S O

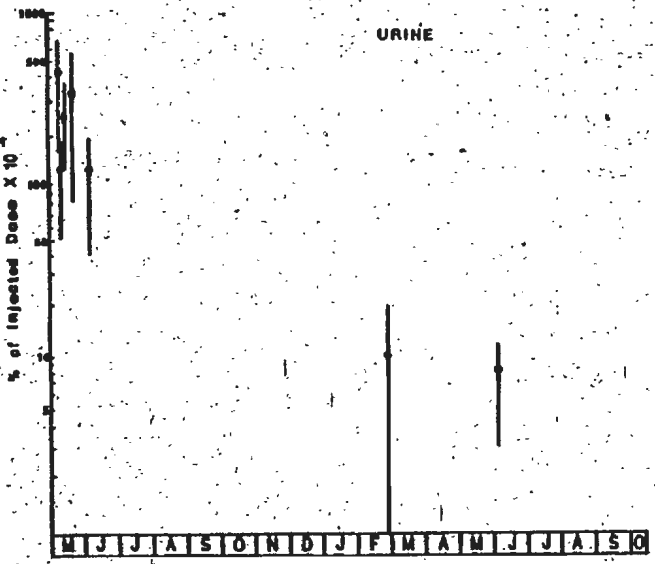
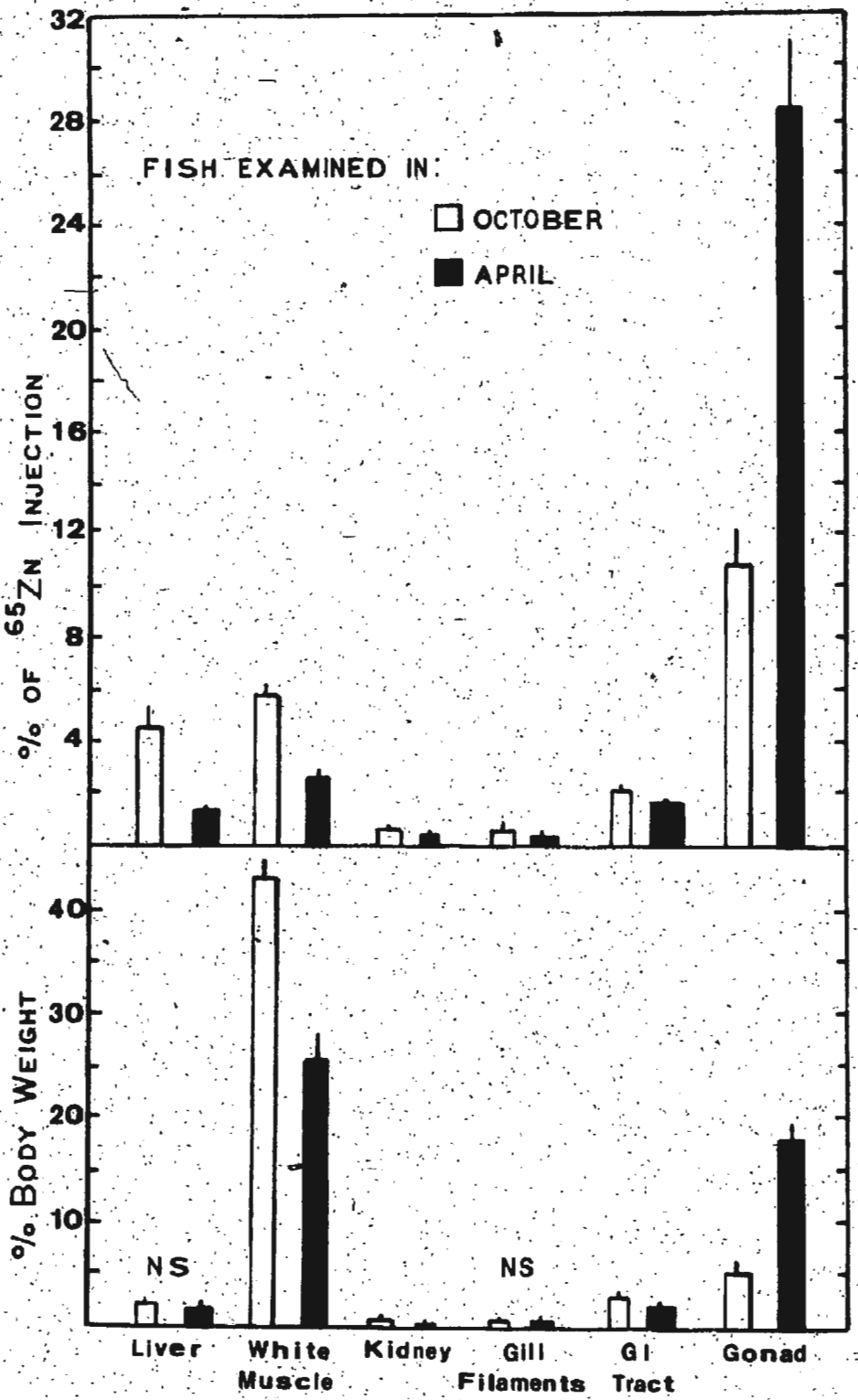

URINE
% of Injected Dose X 10⁻⁴
M J J A S O N D J F M A M J J A S O

Fig. 24 A. Total amount of ^{65}Zn (expressed as a % of the amount of ^{65}Zn injected per fish) in several organs and tissues of female winter flounder dissected 63 (i.e. in October □) and 236 days (i.e. in April ■) following a single intramuscular injection. % of ^{65}Zn inj. = $\frac{\text{cpm } ^{65}Zn/g \text{ X Weight (g)}}{\text{cpm } ^{65}Zn \text{ inj.}}$ X 100.

Fig. 24 B. Tissue weights (expressed as a % of body weight) of female flounder dissected in October (□) (n=10) and April (■) (n=9) (see Fig. 24 A). The weights of liver, kidney, gastrointestinal tissue and gonad were determined by directly weighing the tissue. The total amount of white muscle and gill filaments in each fish was estimated from equations which relate tissue weight to body length (see Appendix A).


FISH EXAMINED IN:
OCTOBER
APRIL
% OF ^{65}Zn INJECTION
% BODY WEIGHT
32
28
24
20
16
12
8
4
40
30
20
10
NS
NS
Liver
White Muscle
Kidney
Gill Filaments
GI Tract
Gonad

white muscle, kidney and gastrointestinal tract) and a significant increase in the weight of the gonads (Fig. 24 B).

Section B. Whole-body retention of ^{65}Zn in winter flounder following single intraperitoneal injections.

Similar ^{65}Zn retention profiles were observed in the flounder injected in August or December. In addition, there were no apparent differences in whole body retention of ^{65}Zn attributable to the sex of the fish. However, fewer females than males were examined--only two of the seven saline- and two of the six Zn^{2+}-injected flounder were females. Representative ^{65}Zn retention profiles for site A of Zn^{2+}- and saline-injected flounder, monitored from August or December through to June, are depicted in Fig. 25 A. The retention curve was composed of two exponential rate functions (Fig. 25 B). The biological half-times ($TB_{1/2}$) and rate constants (K) of the two components of the curve are summarized in Table 13. A long-lived component with a $TB_{1/2}$ of 1510 ± 225 days ($\bar{X}$ ± SE of 7 fish) in the saline- and 1200 ± 266 days ($\bar{X}$ ± SE of 6 fish) in the Zn^{2+}-injected flounder accounted for the major portion of the total ^{65}Zn activity (72 and 65% in the saline- and Zn^{2+}-injected fish, respectively).

In three of the six Zn^{2+}- and five of the seven saline-injected flounder, the % of ^{65}Zn retained at site B remained unchanged or increased slightly over the examination period. In the remainder of the fish the $TB_{1/2}$ of the long-lived component was similar to that determined for site A (i.e. $TB_{1/2}$ of 2081 days ($\bar{X}$ of 3 fish) for Zn^{2+}- and 2109 ($\bar{X}$ of 2 fish) for saline-injected flounder) (Fig. 25 C).

The rate of ^{65}Zn loss increased in flounder monitored from June to August (Fig. 26 A and B). The $TB_{1/2}$, when monitored at site A, was 223 ± 74 days ($\bar{X}$ ± SE, n=4) and 219 ± 50 days ($\bar{X}$ ± SE, n=2) in saline- and Zn^{2+}-

Fig. 25. Whole-body retention of ^{65}Zn in flounder injected with saline or a Zn^{2+} load.

A. Representative profiles of flounder monitored at "site A" from August or December through to June.

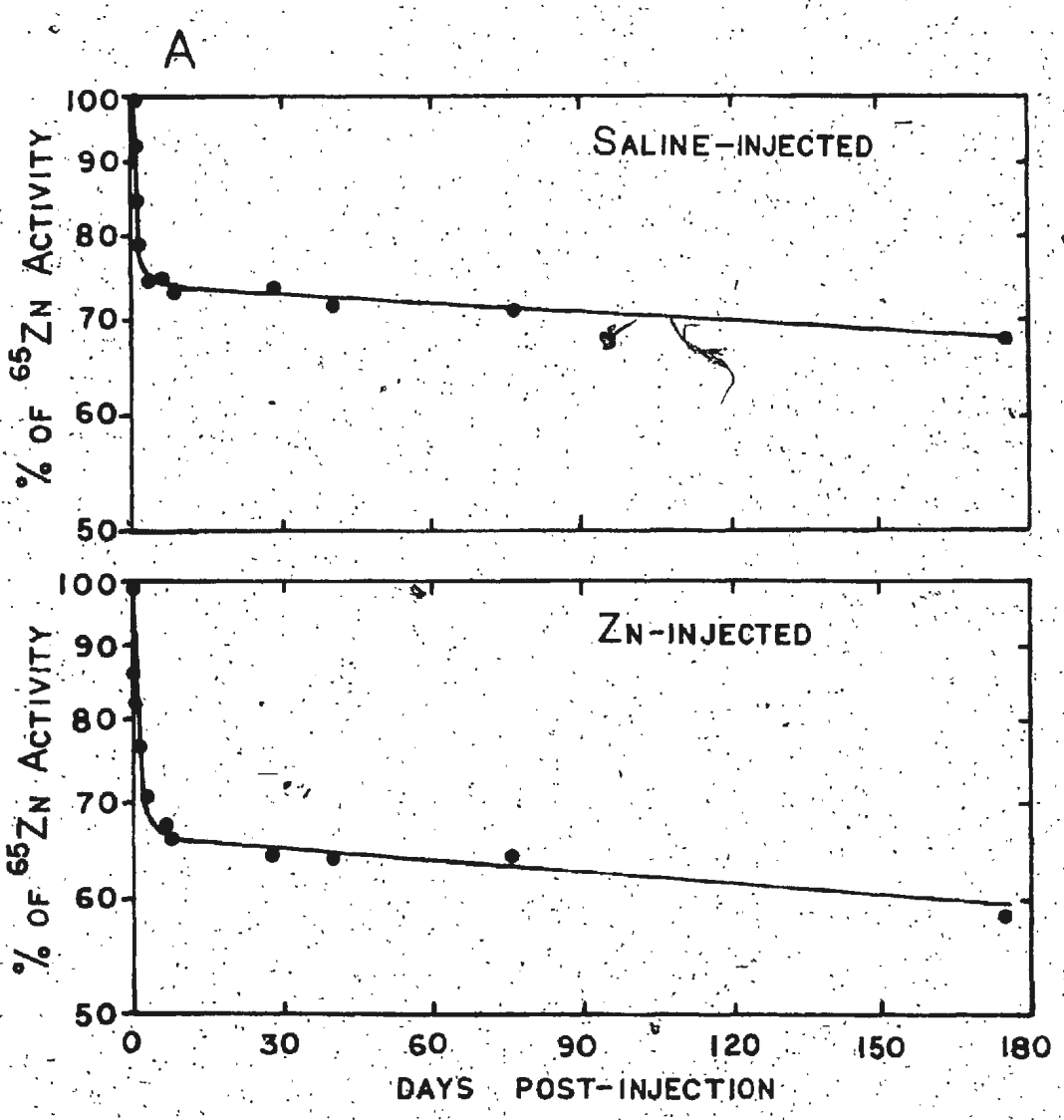

A
SALINE-INJECTED
ZN-INJECTED
% OF 65ZN ACTIVITY
100
90
80
70
60
50
0
30
60
90
120
150
180
DAYS POST-INJECTION

Fig. 25. Whole-body retention of ^{65}Zn in flounder injected with saline or a Zn^{2+} load.

B. Resolution of composite curve shown in Fig. 25 A into two rate functions. The broken line represents the disappearance curve for the first component which has been corrected for the effect of the slope of the second component.

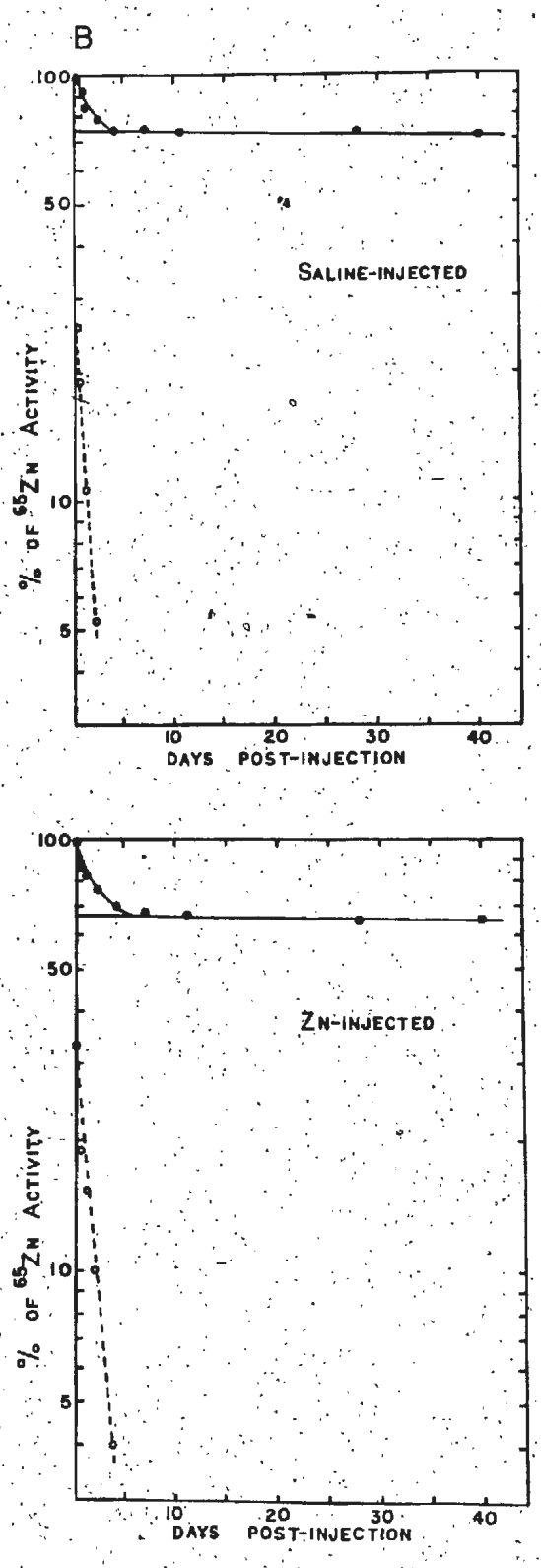

B
100
50
10
5
% OF ^{65}Zn ACTIVITY
SALINE-INJECTED
10
20
30
40
DAYS POST-INJECTION
100
50
10
5
% OF ^{65}Zn ACTIVITY
Zn-INJECTED
10
20
30
40
DAYS POST-INJECTION

Fig. 25. Whole-body retention of ^{65}Zn in flounder injected with saline or a Zn^{2+} load.

C. Representative profile of flounder monitored at "site B" from August or December through to June.

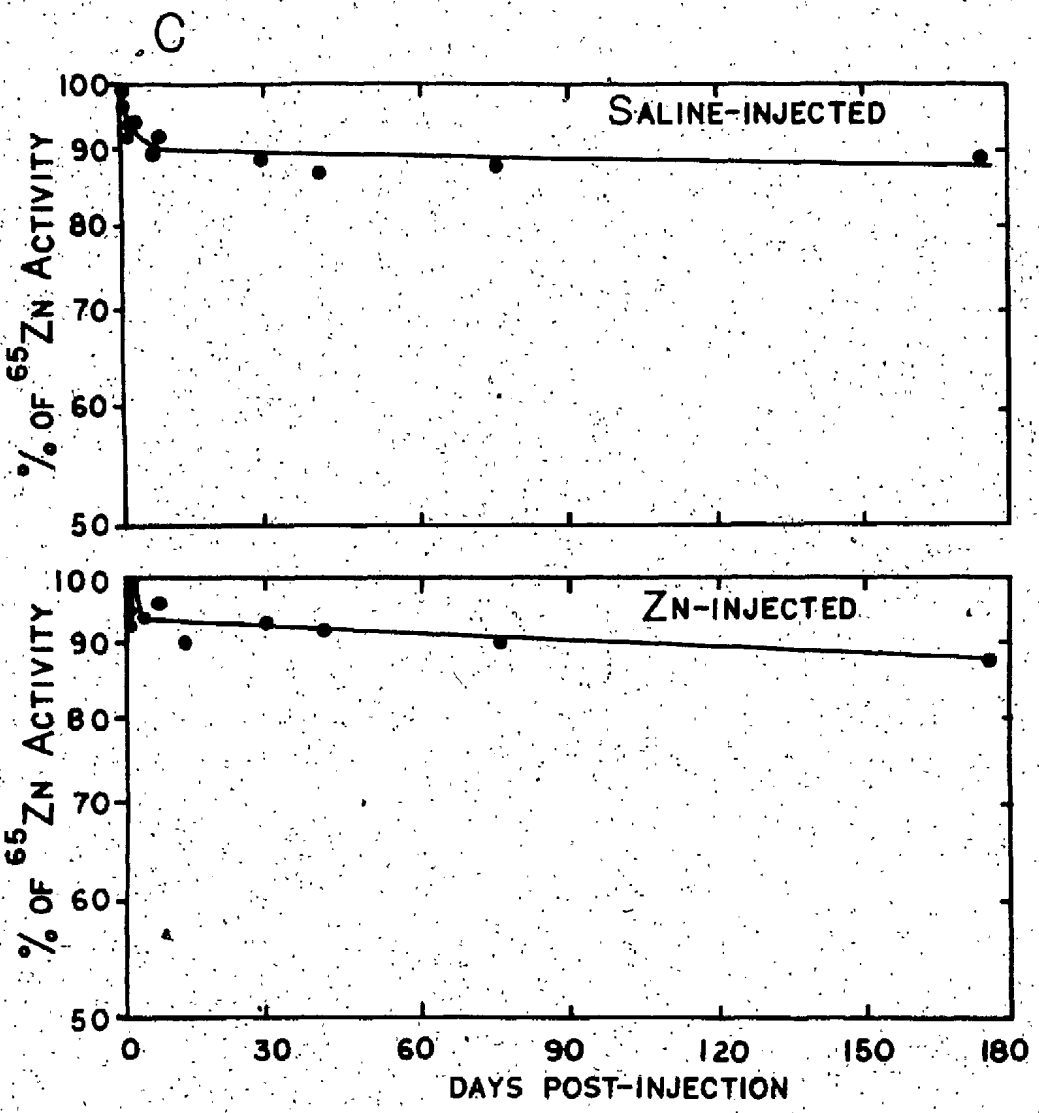

C
SALINE-INJECTED
ZN-INJECTED
% OF ^{65}ZN ACTIVITY
100
90
80
70
60
50
0
30
60
90
120
150
180
DAYS POST-INJECTION

Table 13. Summary of the components of the whole-body retention of ^{65}Zn in winter flounder injected with ^{65}Zn plus a stable Zn^{2+} load or ^{65}Zn plus an equivalent volume of saline. Data obtained from fish monitored at site A from August or December through to June ($\bar{X}$ ± SE, n=number of fish).

	Component I			Component II		
	Initial amount (%)	$TB_{1/2}$ (days)	K ($days^{-1}$)	Initial amount (%)	$TB_{1/2}$ (days)	K ($days^{-1}$)
Saline-injected (n=7, 2♀, 5♂)	28 ± 2	1.6 ± 0.3	0.5577 ±0.1110	72 ± 2	1510 ± 225	0.0005 ±0.0001
Zn^{2+}-injected (n=6, 2♀, 4♂)	35 ± 3	2.2 ± 0.4	0.3709 ±0.0620	65 ± 3	1200 ± 266	0.0007 ±0.0002

injected flounder, respectively. When flounder were monitored at site B, the $TB_{1/2}$ was 181 ± 27 days and 207 ± 20 days in the saline- and Zn^{2+}-injected flounder, respectively (Fig. 26 A,B).

Section C. Comparison of Zn^{2+} concentrations in several tissues of winter flounder injected with Zn^{2+} or an equivalent volume of saline.

Fifteen days following the injections, the stable Zn^{2+} concentration in the kidney, liver and intestinal tissue of Zn^{2+}-injected flounder was elevated 70, 21 and 26%, respectively, above that observed in the saline-injected fish (Table 14).

Section D. Site(s) and extent of Zn^{2+} "secretion" in the gastrointestinal tract.

The distribution of zinc in several tissues of winter flounder, examined from 2 hours to 14 days following intravenous injections of ^{65}Zn plus a Zn^{2+} load, is summarized in Fig. 27. The concentration of Zn^{2+} in the blood reached a plateau by 15 hours post-injection; concentrations in the kidney and liver tended to rise over the period from 2 to 14 days. The Zn^{2+} concentration in the gastrointestinal tissues peaked at day 7, declining slightly by day 14; the concentrations were similar in all regions of the tract. The μg Zn^{2+} present in the contents of the gastrointestinal tract was greatest in the flounder examined 24 hours post-injection (Fig. 28). The level was highest in the lumen contents of the upper intestine (region including the pyloric appendages); at 24 hours post-injection, approximately 1.0% of the total Zn^{2+}-injected per fish was detected in the lumen contents of the upper intestine.

Similar levels of ^{65}Zn were detected in the lumen contents of flounder injected (i.v.) with ^{65}Zn plus stable Zn^{2+} or ^{65}Zn plus an equivalent

Fig. 26. Whole-body retention of ^{65}Zn in flounder injected with saline or a Zn^{2+} load.

A. Representative profile of flounder monitored at "site A" from August to August.

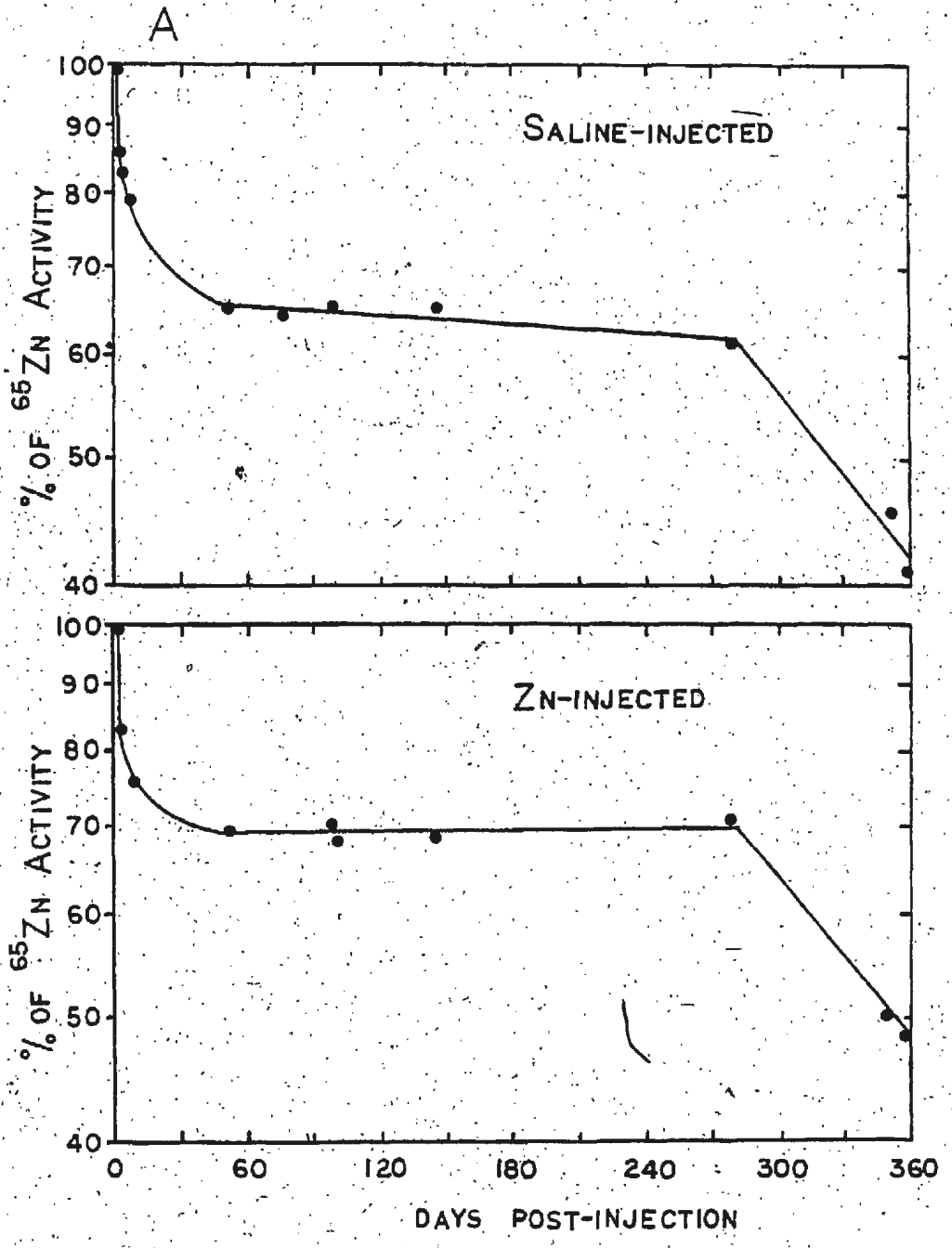

A
SALINE-INJECTED
ZN-INJECTED
% OF 65ZN ACTIVITY
100
90
80
70
60
50
40
0
60
120
180
240
300
360
DAYS POST-INJECTION

Fig. 26. Whole-body retention of ^{65}Zn in flounder injected with saline or a Zn^{2+} load.

B. Representative profiles of flounder monitored at "site B" from August to August.

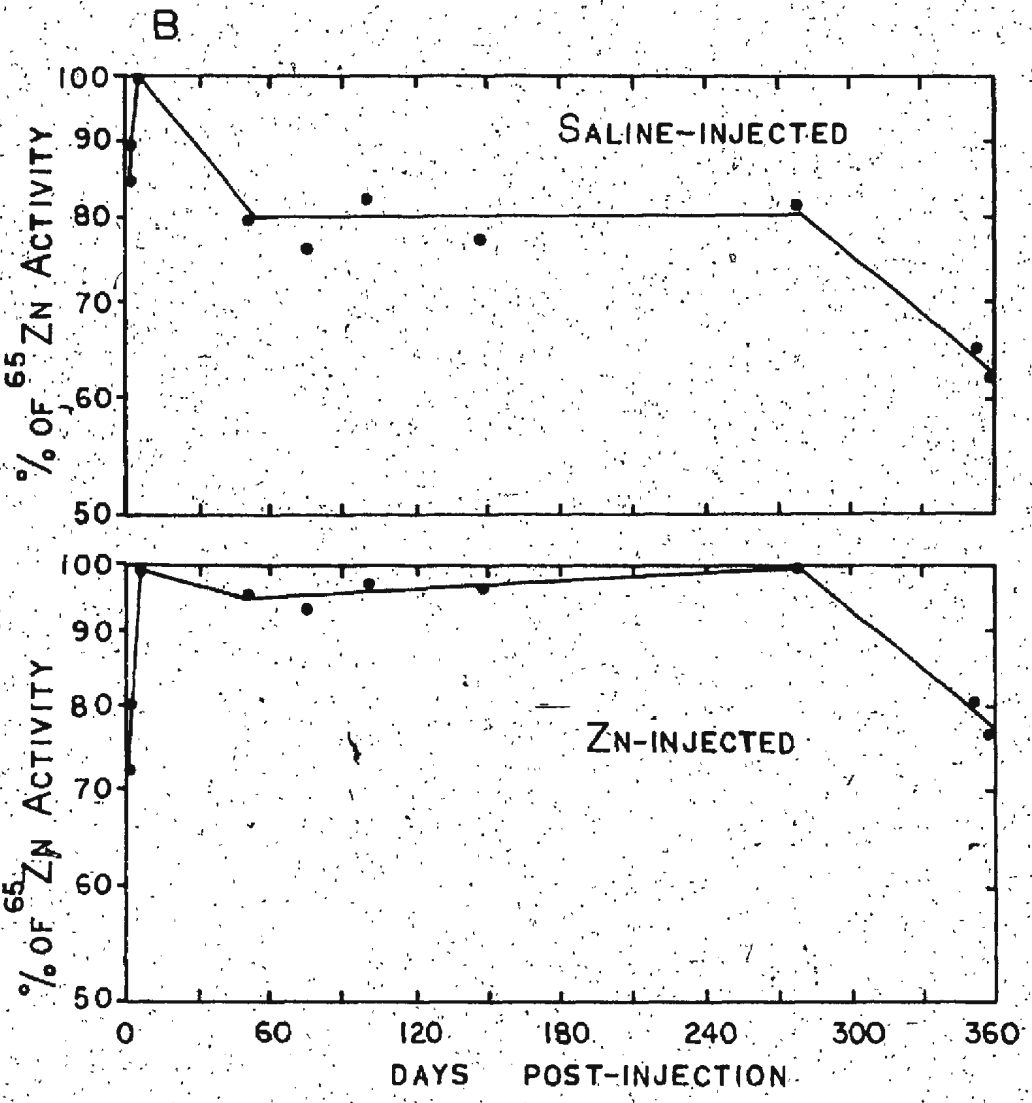

B
SALINE-INJECTED
ZN-INJECTED
% OF 65ZN ACTIVITY
100
90
80
70
60
50
0
60
120
180
240
300
360
DAYS POST-INJECTION

Table 14. Stable Zn^{2+} concentrations (μg Zn^{2+}/g dry weight) of tissues of winter flounder (♂) dissected 15 days following injection (i.p.) with Zn^{2+} (25% of estimated whole body Zn^{2+}) or an equivalent volume of saline. (n=number of fish examined).

	Kidney	Liver	Upper Int.
Zn^{2+}-inj.	319.41 ± 20.03 (n=12)	129.97 ± 4.34 (n=12)	141.36 ± 4.30 (n=10)
Saline-inj.	187.47 ± 14.33 (n=10)	107.59 ± 4.16 (n=10)	112.20 ± 3.29 (n=6)
P	<0.01	<0.01	<0.01

Fig. 27. The distribution of zinc in the blood, kidney, liver and upper intestinal tissue of winter flounder dissected from 2 to 336 hours following a single intravenous injection of ^{65}Zn plus a Zn^{2+} load. The $\mu g\ Zn^{2+}/g$ tissue was calculated from the cpm ^{65}Zn in each tissue and the specific activity of the injection. The data are expressed as the $\mu g\ Zn^{2+}/g$ tissue divided by $\mu g\ Zn^{2+}$ injected per g body weight. Values are $\bar{X} \pm SE$ of 4-5 fish per time point.

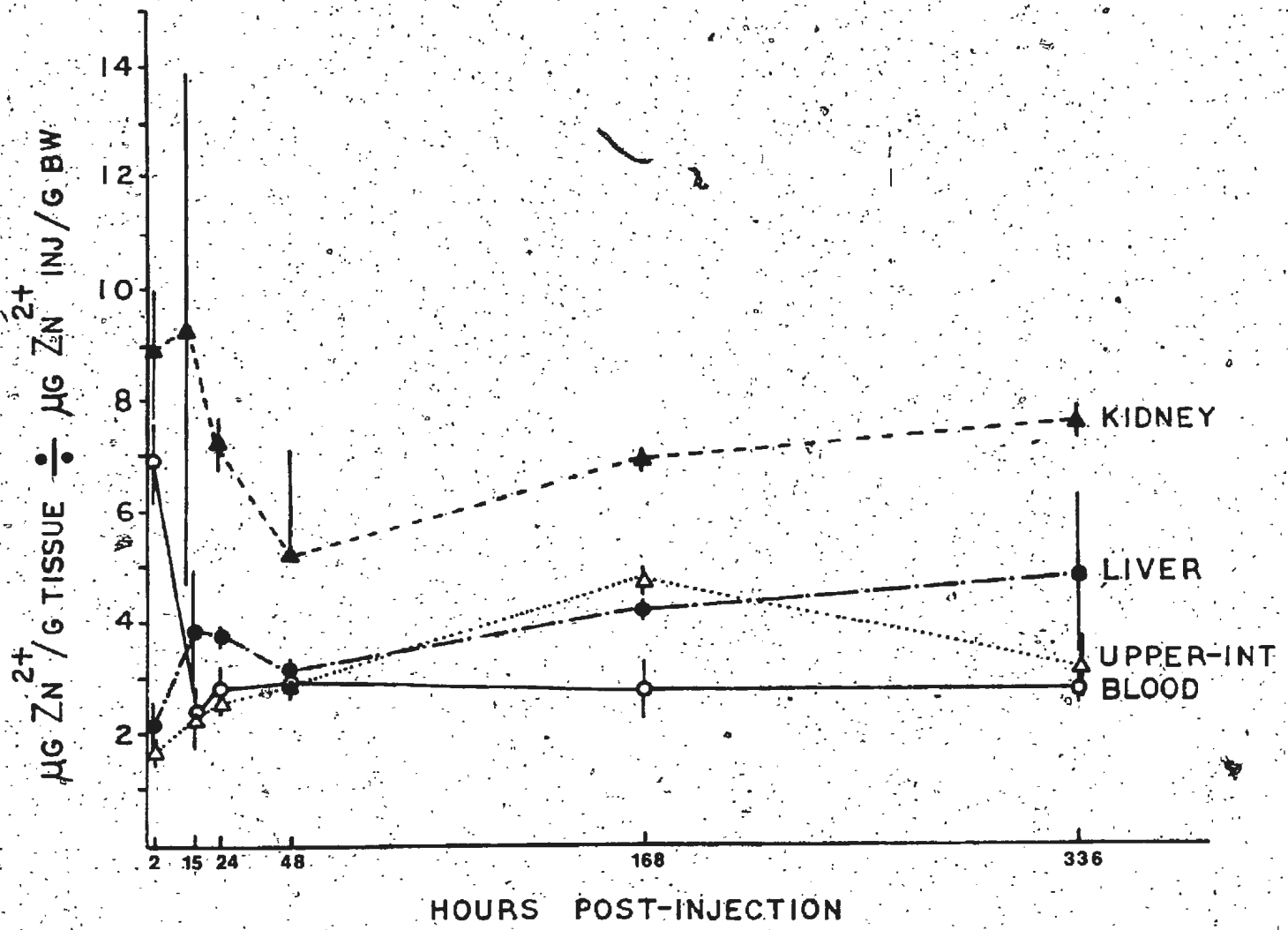
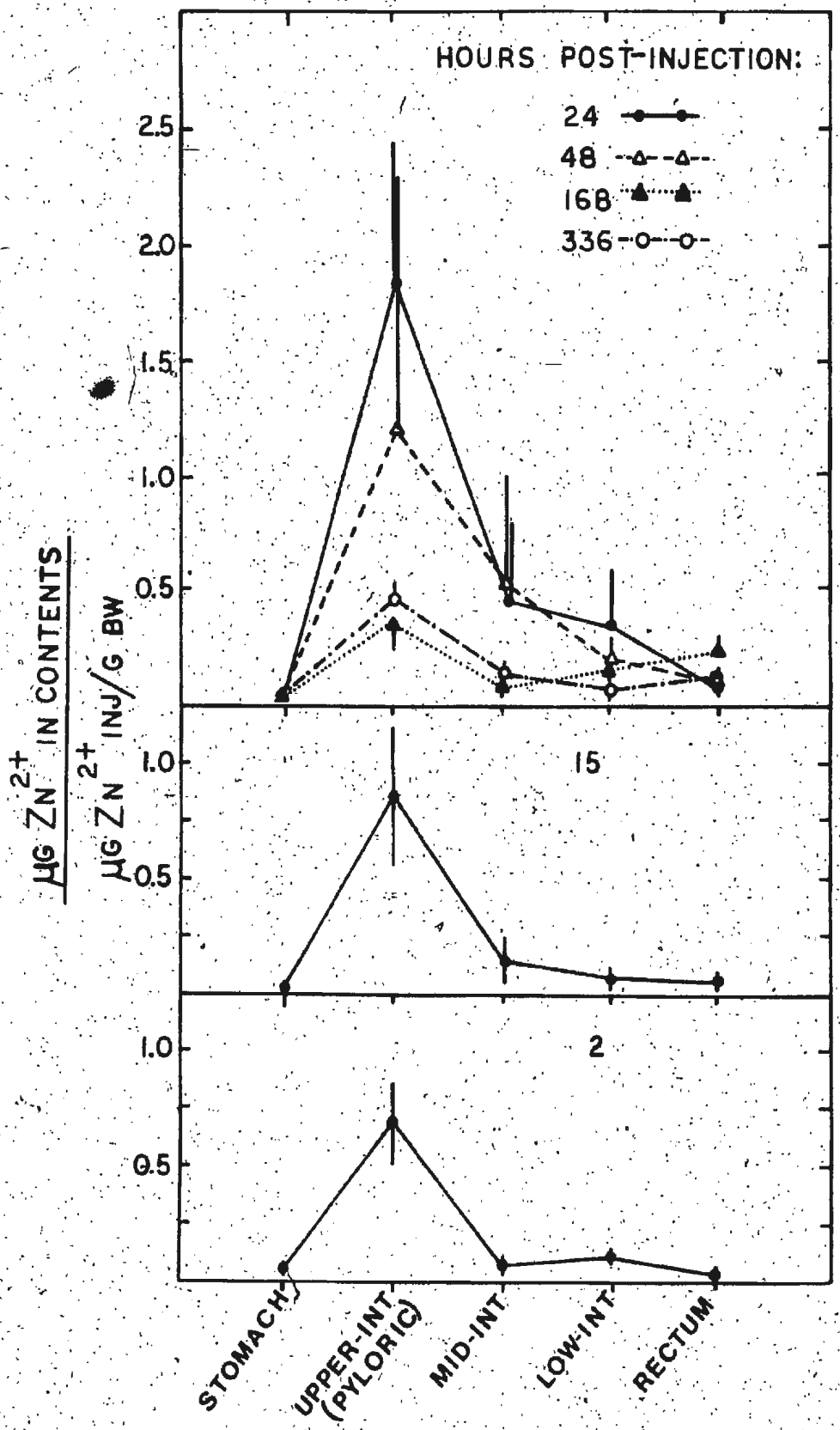

μG Zn2+/G TISSUE ÷ μG Zn2+ INJ/G BW
14
12
10
8
6
4
2
KIDNEY
LIVER
UPPER-INT
BLOOD
2
15
24
48
168
336
HOURS POST-INJECTION

Fig. 28. The distribution of zinc in the lumen contents of different segments of the gastrointestinal tract of winter flounder dissected from 2 to 336 hours following intravenous injections of ^{65}Zn plus a Zn^{2+} load. The μg Zn^{2+} in the lumen contents was calculated from the cpm ^{65}Zn in the contents and the specific activity of the injection. The data are expressed as the μg Zn^{2+} in the contents divided by μg Zn^{2+} injected per g body weight. Values are $\bar{X} \pm SE$ of 4-5 fish per time point.


HOURS POST-INJECTION:
24
48
168
336
2.5
2.0
1.5
1.0
0.5
15
2
STOMACH
UPPER-INT (PYLORIC)
MID-INT
LOW-INT
RECTUM
µG Zn2+ IN CONTENTS / µG Zn2+ INJ/G BW

volume of saline (Fig. 29). As observed in the preceeding study, the level of ^{65}Zn was highest in the contents of the upper intestine. Ligation of the upper intestine prior to the i.v. injections resulted in an elevation of the ^{65}Zn concentration in the upper intestinal tissue. The levels were elevated in both the saline- and Zn^{2+}-injected fish. The ^{65}Zn concentration in the rest of the intestinal tissue was similar to that observed when the upper intestine was not ligated. The level of ^{65}Zn in the lumen contents of flounder in which the upper intestine was ligated was 2-4 times higher than that observed in fish in which the intestine was left open. This elevation of ^{65}Zn in the lumen contents was observed at all locations in the tract in both the Zn^{2+}- and saline-injected flounder. In other words, ^{65}Zn was "secreted" into all regions of the tract, not just into the upper intestine.

The above studies were conducted in October and November. A study was also conducted to determine the distribution of ^{65}Zn in saline- and Zn^{2+}-injected flounder in the summer feeding period (June-July). The distribution of ^{65}Zn was similar in the Zn^{2+}- and saline-injected flounder; the ^{65}Zn concentrations in the tissues of both groups declined over the interval from 9 to 27 days (Fig. 30). Very little of the total ^{65}Zn injected per fish was detected in the lumen contents of the upper intestine (i.e. ^{65}Zn recovered in the contents generally accounted for less than 0.1% of the total ^{65}Zn injected per fish). There was no significant difference in the amount of ^{65}Zn detected in the lumen contents of flounder injected with Zn^{2+}- or saline.

Fig. 29. Distribution of ^{65}Zn in lumen contents of different segments of the intestine of flounder dissected 18 hours following injection (i.v.) of ^{65}Zn plus a stable Zn^{2+} load □ or ^{65}Zn plus an equivalent volume of saline ■ . Data are expressed as the cpm ^{65}Zn in the contents divided by the cpm ^{65}Zn injected per g body weight. Values are $\bar{X} \pm SE$ of 7-8 fish per treatment group.

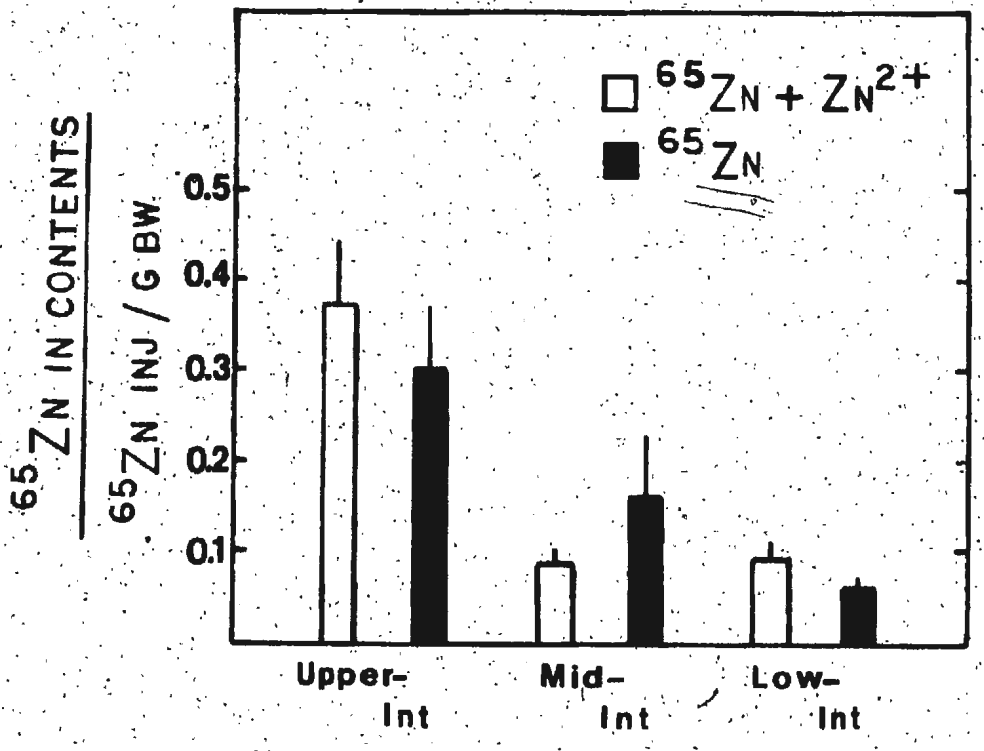
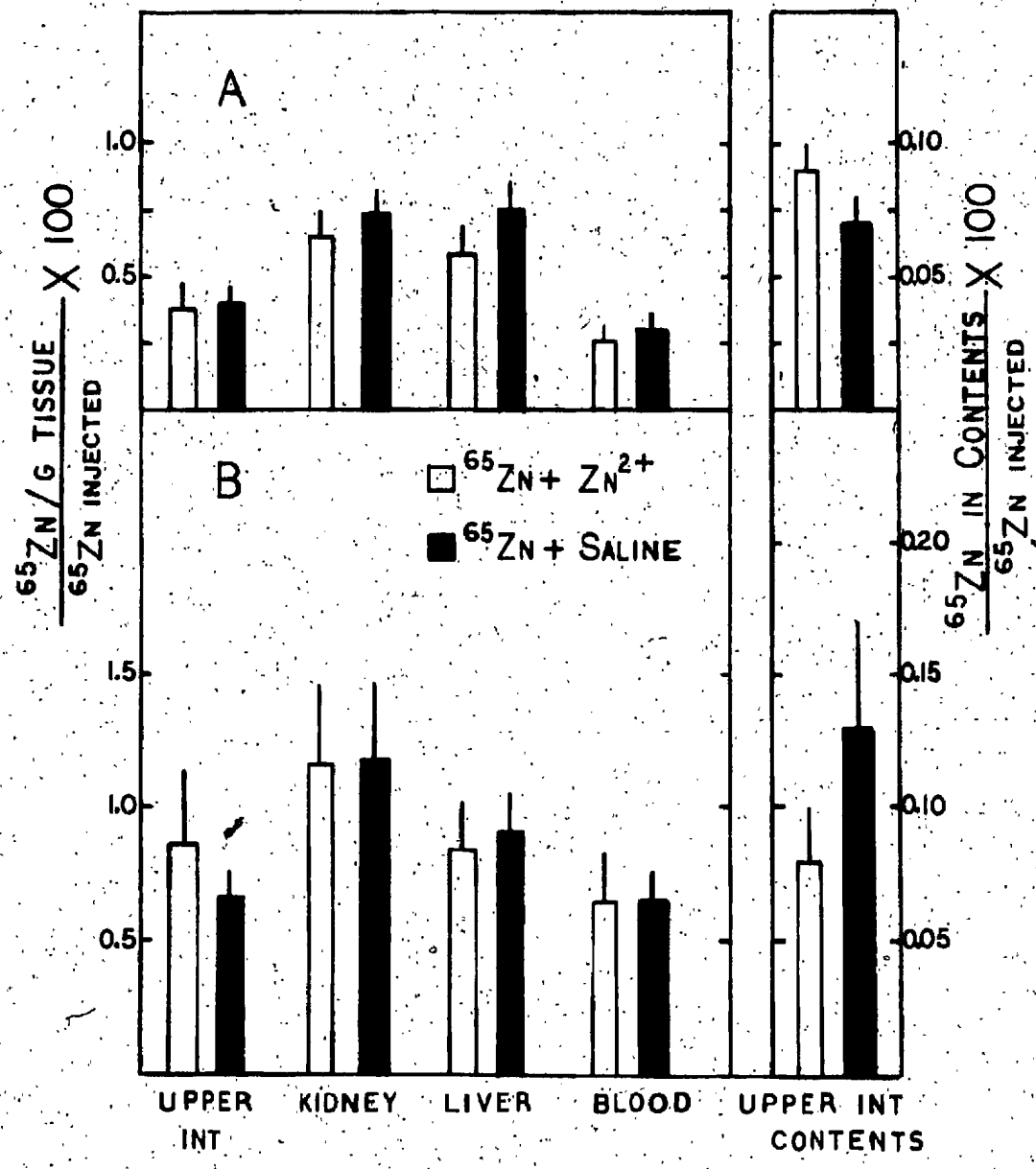

$^{65}Zn + Zn^{2+}$
^{65}Zn
^{65}Zn IN CONTENTS / ^{65}Zn INJ / G BW
0.5
0.4
0.3
0.2
0.1
Upper-Int
Mid-Int
Low-Int

Fig. 30 A. Distribution of ^{65}Zn in flounder dissected 27 days following injection (i.v.) of ^{65}Zn plus a stable Zn^{2+} load (24% of the estimated body Zn^{2+}) □ or ^{65}Zn plus an equivalent volume of saline. ■ $\bar{X} \pm SE$; 5 fish per treatment group. Data are expressed as (cpm ^{65}Zn per g tissue, or cpm ^{65}Zn in the lumen contents, divided by the cpm ^{65}Zn injected per fish) X 100.

Fig. 30 B. Distribution of ^{65}Zn in flounder dissected 9 days following injection (i.v.) of ^{65}Zn plus a stable Zn^{2+} load (24% of the estimated body Zn^{2+}) □ or ^{65}Zn plus an equivalent volume of saline ■ $\bar{X} \pm SE$; 5 fish per treatment group. Data are expressed as (cpm ^{65}Zn per g tissue, or cpm ^{65}Zn in the lumen contents, divided by the cpm ^{65}Zn injected per fish) X 100.


A
B
1.0
0.5
1.5
0.10
0.05
0.20
0.15
$^{65}Zn/G$ TISSUE / ^{65}Zn INJECTED X 100
^{65}Zn IN CONTENTS / ^{65}Zn INJECTED X 100
$^{65}Zn + Zn^{2+}$
^{65}Zn + SALINE
UPPER INT
KIDNEY
LIVER
BLOOD
UPPER INT CONTENTS

DISCUSSION

Mammalian studies with the radiotracer ^{65}Zn have shown that different tissues in the body vary in their affinity for zinc (Sheline et al. 1943; Gilbert and Taylor 1956; Rubini et al. 1961; Cotzias and Papavasiliou 1964; Miller 1969). Apparently, the rate of exchange of zinc from the blood into the tissues and vice versa is much more rapid for the metabolically active tissues such as liver and kidney than for tissues such as muscle and bone. In agreement with these studies, different tissues in the flounder also exhibit different rates of accumulation and release of ^{65}Zn following an intramuscular injection of the radiotracer. For a substantial time period (71 days) following the injection, the ^{65}Zn concentrations were an order of magnitude higher in tissues such as the kidney, liver, gill and gastrointestinal tract than in bone or muscle. In addition, during the period when the ^{65}Zn concentrations in these tissues were declining rapidly, the concentration of ^{65}Zn in the muscle and bone remained constant or increased. This implies that a fraction of the stable Zn^{2+} in several tissues of the flounder may not be readily exchangeable with Zn^{2+} absorbed from the environment. If one compares the distribution of ^{65}Zn (expressed as a % of the total amount in the tissues) with that of stable Zn^{2+}, even up to 528 days post-injection there was relatively less ^{65}Zn than stable Zn^{2+} in some tissues (notably bone and scales) and more ^{65}Zn than stable Zn^{2+} in others (notably skin) (see Table 15 A and B).

The tissue distribution of ^{65}Zn in other species of fish has been examined in several radioecological and pollution studies. The routes of labelling the fish varied greatly in these studies but, in agreement with the present findings, the ^{65}Zn concentrations tended to be lowest in the

Table 15 A. Relative distribution of ^{65}Zn[a] and stable Zn^{2+}[b] in several tissues and organs of winter flounder (male)

	% ^{65}Zn							% Zn^{2+}
Days Post inj. Date	1-May 8	29-June 6	71-July 17	156-Oct 10	296-Feb 27	392-June 4	528-Oct 17	
Liver	9.8	14.1	5.6	5.8	3.9	2.5	6.5	3.4
Spleen	0.7	0.8	0.6	0.4	0.3	0.3	0.3	0.3
Gastroint. tract	11.5	18.9	10.6	4.1	3.5	4.5	4.5	3.6
Kidney	7.1	3.9	2.9	1.0	1.4	1.4	2.0	1.4
Gill	12.1	7.5	6.1	3.4	3.5	3.7	3.4	3.3
White muscle	8.6	14.6	22.9	28.4	24.3	20.3	31.1	39.5
Skin	40.4	31.5	43.4	49.4	49.4	55.8	33.0	19.5
Scales	9.1	7.7	6.9	6.5	11.9	9.5	14.7	24.1
Interhaemal spine (bone)	0.1	0.1	0.1	0.2	0.2	0.3	0.6	1.5
Eyes	0.7	0.9	0.9	1.0	1.6	2.0	4.0	3.4

[a] Based on data in Table 10, p. 110. $\% \, ^{65}Zn = \left[\frac{^{65}Zn \text{ per tissue or organ (estimated)}}{\text{total } ^{65}Zn \text{ in tissues examined}} \right] \times 100$

[b] Based on estimation of stable Zn^{2+} in tissues of a 35 cm male flounder (Fletcher and King, personal commun., see Appendix B).

Table 15 B. Comparison of distribution of ^{65}Zn[a] and stable Zn^{2+}[b] in tissues of one male flounder which was completely dissected 528 days post injection.

Tissue	% ^{65}Zn	% Zn^{2+}
Liver	2.1	2.3
Spleen	0.1	0.1
Gastroint. tract	1.6	1.4
Kidney	1.0	0.5
Gills	1.1	1.4
White muscle	15.3	16.9
Other muscle (i.e, fin and belly)	16.7	19.7
Skin (plus underlying tissue)	22.7	8.0
Scales	4.7	9.5
Fins	12.8	10.6
Interhaemal spine (bone)	0.3	0.6
Other bones (i.e. skeleton and head)	19.7	27.1
Heart	0.1	0.1
Eyes	1.9	1.7

[a] Based on data in Table 12, p. 113. % ^{65}Zn = $\left[\frac{^{65}Zn \text{ per tissue or organ (actual)}}{\text{total } ^{65}Zn \text{ in tissues examined}}\right]$ X 100

[b] Based on estimation of stable Zn^{2+} in tissues of a similar size (32 cm) male flounder (Fletcher and King, personal commun., see Appendix A and B).

muscle and bone. For example, the muscle and bone exhibited the lowest ^{65}Zn concentrations in goldfish (*Carassius auratus*) following injection of ^{65}Zn directly into the air bladder (Hibiya and Oguri 1961), in brown bullhead (*Ictalurus nebulosus*) following exposure to ^{65}Zn-labelled water (Joyner 1961) and in pumpkinseed sunfish (*Lepomis gibbosus*) following ingestion of radiolabelled food (Merlini et al. 1973). The uptake and decline of ^{65}Zn in the tissues of the sunfish (exposed to labelled water for 1-24 days) was most rapid in the blood, gill and kidney; intermediate in the gastrointestinal tissues; slowest in the muscle and bone (Merlini et al. 1973). Similar trends of ^{65}Zn retention were observed in the tissues of young plaice (*Pleuronectes platessa*) exposed to ^{65}Zn in the water for 180 days (Pentreath 1973b). The flux of ^{65}Zn was highest in the gills, followed in descending order by the kidney, gastrointestinal tract tissues, liver, bone and muscle. The slowest rates of ^{65}Zn accumulation were also observed in the bone and muscle of rainbow trout (*Salmo gairdneri*) following ingestion of a single dose of ^{65}Zn (Nakatani 1966). The concentration of ^{65}Zn was highest in the gill tissue of the trout for short time periods (i.e. 120-168 hours post-administration) but, over extended time periods (8-182 days), the highest concentrations were observed in the gastrointestinal tract tissues. The ^{65}Zn concentrations in the blood, liver, gill filaments and kidney declined rapidly over the same time period.

The concentration of stable Zn^{2+} in most somatic tissues of the winter flounder remain relatively constant throughout the year (Fletcher and King personal commun.). However, based on the distribution of ^{65}Zn, there does appear to be a seasonal change in the turnover of Zn^{2+} in several of the tissues. For example, the ^{65}Zn concentrations in the kidney, gill and gastrointestinal tissues declined rapidly during the summer months, whereas

very little change in the ^{65}Zn concentrations occurred during the winter. During the non-feeding period several tissues decline in weight (see Fig. 24 B, p. 116) and Zn^{2+} "lost" from these tissues appears to be redistributed within the body. As shown by the distribution of ^{65}Zn in the tissues following a single injection (i.m.) (Fig. 24 A) and by stable Zn^{2+} analysis of the tissues (Fletcher and King 1978), the female flounder continues to incorporate a substantial amount of Zn^{2+} into the ovaries during the post-feeding period. It is unlikely that the ovarian requirements can be met by accumulation of Zn^{2+} from the water but they could be met by Zn^{2+} released from the tissues which are metabolized. The males have finished gonadal development by the time feeding is terminated so there is no apparent post-feeding requirement for Zn^{2+} by the male gonads. However, the Zn^{2+} released during the metabolism of tissues which lose weight may also be transferred to other tissues in the male. For example, the dry weight and amount of Zn^{2+} in the scales increases over the winter (i.e. from October to April-May) (see Appendix B). There is also a tendency for the Zn^{2+} concentrations in most tissues of the adult male to be higher than in a female of the same age (see Appendix B).

In agreement with the observed changes in ^{65}Zn retention in several of the tissues, the loss of ^{65}Zn from whole flounder also appears to change seasonally. When the activity in the area of the peritoneal cavity (referred to as "site A", see Fig. 19, p. 97) of live flounder injected in late August or December, was monitored through to June, the plot of ^{65}Zn retention resolved into two components. The first component, accounting for 28% of the initial amount, had a very short half-time, approximately 2 days, and probably represents movement of ^{65}Zn from the body cavity (i.e. the injection site) into the tissues. However, since only localized areas

of the flounder could be counted, it is not possible to say whether this represented ^{65}Zn loss from the body. For example, when a second area comprising mainly muscle was examined, the activity at this site (i.e. "site B", see Fig. 19, p. 97) actually increased over the study period in many of the fish. The second component of the ^{65}Zn retention plot had by far the longest half-time, 1510 days. It accounted for the major portion of the activity (72%) and is probably most representative of the actual rate of Zn^{2+} turnover in the flounder during the non-feeding period. Plots of whole-body retention of ^{65}Zn consist of one or more components in other species of fish examined (Baptist et al. 1970; Pentreath 1973 a,b; Willis and Jones 1977). The species used in these studies tended to be small in size or were restricted to earlier age classes. Therefore, the components presumably represent the actual loss of ^{65}Zn from the fish. However, as observed in the present study, the long-lived component usually accounted for most of the activity and is probably most representative of the exchange of Zn^{2+} in the fish with the Zn^{2+} in its environment. The size and half-time of the short-lived component(s) appears to be influenced by how the ^{65}Zn is administered (Baptist et al. 1970). Unless the tissues are uniformly labelled with ^{65}Zn, the compartments of ^{65}Zn in the fish, as determined by whole-body ^{65}Zn loss, may not actually represent compartments of stable Zn^{2+} (Willis and Jones 1977).

When the activity in the flounder was monitored from June to August (feeding was resumed in June), the rate of ^{65}Zn loss was increased from that observed over the winter (see Fig. 26, p. 127). The biological half-time of ^{65}Zn loss was similar when monitored at "site A" or "site B", averaging 223 and 181 days, respectively. Interestingly, this rate of ^{65}Zn loss is in the same order as the theoretical half-time of Zn^{2+} turnover

calculated for flounder fed a diet of capelin. Flounder maintained in the laboratory on a diet of chopped capelin (11 μg Zn^{2+}/g wet weight) consume on average, 2% of their body weight per day, over the summer feeding period. This represents a dietary intake of 109 μg Zn^{2+} per day in a 35 cm male flounder (estimated body weight 496 g). At a retention value of 37% (based on the non-absorbed marker technique) this represents a daily input of 40 μg Zn^{2+}. The flounder undergoes somatic growth and gonadal development during the feeding period so a fraction of the absorbed Zn^{2+} is presumably incorporated into new tissues. A summation of the total element levels in the tissues of a 35 cm flounder gives a total body burden of 10475 μg Zn^{2+} at the beginning of the feeding period (see Appendix B). At the end of the feeding period, due to increases in somatic growth and gonad development, the total body burden increases to 12204 μg Zn^{2+}. Over a 20-week feeding period this represents a requirement of 12 μg Zn^{2+} per day; the remainder of the Zn^{2+} which is retained from the capelin (i.e. 28 μg Zn^{2+} per day) presumably is available for exchange with the Zn^{2+} in the tissues. For the concentration of Zn^{2+} in the somatic tissues to remain constant, it also represents the amount of Zn^{2+} which must be eliminated from the fish. Using this value of Zn^{2+} input, the theoretical half-time for exchange of the total body burden of Zn^{2+} in a 35 cm male flounder would be 259 days, which is comparable to the half-time observed using whole-body retention of ^{65}Zn (i.e. approx. 200 days). It should be noted that such an estimation of biological half-time is predicated on the assumption that the flounder is exchanging Zn^{2+} with its environment as if it were a single compartment with regard to Zn^{2+} dynamics. Since different tissues appear to exchange Zn^{2+} at different rates (based on distribution of ^{65}Zn following an injection), it may be that the flounder excretes Zn^{2+} not as if it were a single compartment

but rather as a sum of compartments.

During the non-feeding period the only input of Zn^{2+} into the flounder would be from seawater. Pentreath (1973 a) estimated that plaice accumulated Zn^{2+} from seawater at a rate of 0.867 $ng^{-1}g^{-1}day^{-1}$. Using this retention value, this represents an input directly from water into a 35 cm male flounder of 0.43 µg Zn^{2+} per day, 100 times lower than the estimated dietary input from capelin.

In view of the slow rate of ^{65}Zn loss observed in flounder during the non-feeding period compared to the increased rate of ^{65}Zn loss during the feeding period (i.e. when Zn^{2+} input increases), one could speculate that the rate of Zn^{2+} intake influences the rate of Zn^{2+} elimination in the flounder. However, examination of ^{65}Zn loss in flounder injected with a load of stable Zn^{2+} tends not to support this hypothesis. The whole-body ^{65}Zn retention patterns were similar in flounder injected with saline or with a load of stable Zn^{2+}, i.e. the rate of ^{65}Zn loss did not appear to be affected by an excess of stable Zn^{2+}. Based on the relationship between the whole body concentration of Zn^{2+} in the fish and the concentration of Zn^{2+} in the water, several investigators have proposed that an active excretory process is triggered in fish when a critical "threshold" concentration is reached (Matthiessen and Brafield 1977; Chernoff and Dooley 1979; Pierson 1981). However, in the present study, flounder do not seem to have detoxified the excess Zn^{2+} by rapid excretion; 15 days following an injection of stable Zn^{2+} (25% of the total body Zn^{2+}) the concentration of Zn^{2+} was elevated 70, 21 and 26% in the kidney, liver and intestinal tissue, respectively. From this, and the whole-body retention data for ^{65}Zn, one may conclude that the mechanism of excretion of stable Zn^{2+} and ^{65}Zn in the flounder is similar; the excess Zn^{2+} does not appear

to have triggered an active excretion process.

The flounder examined in the ^{65}Zn retention and tissue distribution studies were maintained under ambient conditions; as such they were subject to changes in water temperature as well as feeding. Therefore, an alternative explanation for the seasonal change in ^{65}Zn retention is that it is related to differences in the metabolism of the fish associated with these changes. However, from the available literature it is difficult to predict what effect variables which seem to influence the metabolism of the fish (i.e. extent of feeding, rate of oxygen consumption, temperature of the water) would have on the retention of ^{65}Zn. Edwards (1967) concluded that ^{65}Zn loss in young plaice (*Pleuronectes platessa*) (labelled by 2-day exposure to ^{65}Zn in the water) was positively correlated with the respiratory rate of the fish; the loss of ^{65}Zn was greater in plaice fed at an excess rate than in those fed at a maintenance level or starved. In two other species of fish, Shulman et al. (1961) found that the ^{65}Zn loss was fastest in the species with the highest rate of oxygen consumption (i.e. $TB_{1/2}$ following ingestion of radiolabelled food was 58 and 13 days in mummichog (*Fundulus heteroclitus*) and Atlantic silverside (*Menidia menidia*), respectively; the oxygen consumption of the latter was twice that of the former). However, the $TB_{1/2}$ of ^{65}Zn in another species which they examined (cunner, *Tautogolabrus adspersus*) did not differ significantly in a starved fish or in fish fed from 58.1 to 153.7 cal/g/day. The rate of ^{65}Zn loss from black sea bass (*Centropristis striata*) (labelled by 4-7 days exposure to ^{65}Zn in water) was higher in fed fish than in unfed ones, but the variability between individuals was high and the relationship was not statistically significant (Hoss et al. 1978). Several investigators have reported a relationship between water temper-

ature and ^{65}Zn loss. The biological half-time of ^{65}Zn in mummichogs maintained at 10°C was 75 days compared to a half-time of 35 days in fish maintained at 30°C (Shulman et al. 1961). In contrast, Hoss et al. (1978) found that the rate of loss of ^{65}Zn in the pinfish (*Lagodon rhomboides*) was not greater at 25°C than at 12°C. These authors were unable to demonstrate a clear relationship between ^{65}Zn loss and metabolism of the fish in the species which they examined (i.e. pinfish and black sea bass).

While it has been demonstrated that fish can eliminate accumulated Zn^{2+} (Joyner 1961; Holcombe et al. 1979), little is known about the routes or mechanism of Zn^{2+} elimination. Based on the concentrations and rates of accumulation and loss of ^{65}Zn in the tissues following exposure of the fish to ^{65}Zn, the involvement of the gills and/or the kidney and/or the gastrointestinal tract have been suggested. Nakatani (1966) hypothesized that the gills might be a site of Zn^{2+} excretion since the gill filaments of rainbow trout (*Salmo gairdneri*) contained the highest concentration of ^{65}Zn during ingestion of ^{65}Zn. He also examined the distribution of ^{65}Zn in trout which were cannulated for urine collection and had the anus sutured. Over a 7-day period less than 1% of an oral dose was detected in the urine, 15.6% was detected in the gastrointestinal tract and 19.5% was detected in the remainder of the body. It was reportedly not possible to measure the ^{65}Zn in the large volume of water required to keep the fish alive in the metabolism tube but the 64.9% of the dose unaccounted for in the fish tissues was presumed to have been excreted by the gills. Matthiessen and Brafield (1973) found that the density and secretory activity of the "chloride" cells in the gills were increased in sticklebacks

(*Gasterosteus aculeatus*) exposed to sub-lethal levels of Zn^{2+} in the water. From this, they speculated that these cells might play an excretory role. However, Pentreath (1973b) did not observe any difference in the ^{65}Zn distribution in autoradiographs of gill tissue of plaice (*Pleuronectes platessa*) exposed to ^{65}Zn in the water or injected with ^{65}Zn to prevent direct water uptake; there was no evidence of accumulation of ^{65}Zn in the "chloride" cells. Pentreath (1973b) thought that the high flux of ^{65}Zn through the kidney of the plaice might be indicative of a route of excretion (i.e. in plaice exposed to radiolabelled water, the flux of ^{65}Zn through the kidney was second highest to that in the gill filaments). The gastrointestinal tract is the main route of Zn^{2+} excretion in mammals (Underwood 1977) and may also play a role in fish. Hibiya and Oguri (1961) speculated that the intestine was the main route of Zn^{2+} excretion in the goldfish (*Carassius auratus*) since it was the most active tissue in fish dissected 7 days following an injection of ^{65}Zn into the air bladder. Joyner (1961) also observed a high concentration of ^{65}Zn in the gastrointestinal tissues of brown bullheads (*Ictalarus nebulosus*) dissected 7 days after a 96-hour exposure to radiolabelled water. The activity in the gastrointestinal tract tissues was not due to drinking the radiolabelled water; ^{65}Zn was also detected in the intestinal tissues of fish in which the oesophagus was plugged. The gastrointestinal tract tissues have also been found to retain high activity over an extended period of time following oral doses of ^{65}Zn. The gastrointestinal tract of rainbow trout accounted for 27, 55, 65, 58 and 60% of the total radioactivity in fish dissected 8, 85, 116, 141 and 182 days after a single oral dose of ^{65}Zn (Nakatani 1966). Whether or not this represented Zn^{2+} in the process of excretion is difficult to resolve.

Following injection of ^{65}Zn into the winter flounder, the concentration of ^{65}Zn was relatively high in all three of the tissues thought to be possible routes of excretion: kidney, gill and gastrointestinal tract. In addition a similar ^{65}Zn retention pattern was observed in these tissues over the duration of the long-term study (528 days) (see Fig. 20, 21, p.102,104). However, without direct evidence, it is not possible to say whether the ^{65}Zn decline in these tissues represented any ^{65}Zn loss from the body at these sites. ^{65}Zn was detected in both the urine and intestinal lumen contents but the amounts were low (0.1% of the total ^{65}Zn injected) and highly variable between individuals (see Fig. 23, p.114). It is possible that zinc loss also occurs via the body surface. The concentration of ^{65}Zn was high in the skin; relatively more ^{65}Zn than stable Zn^{2+} was found in the skin up to 528 days post-injection.

Experiments conducted to determine the possible site(s) of Zn^{2+} excretion into the digestive tract, following an intravenous injection of ^{65}Zn, indicated that while the greatest amount of radioactivity was detected in the lumen contents of the upper intestine, ^{65}Zn was "secreted" into the lumen contents all along the digestive tract.

The level of ^{65}Zn in the lumen contents was similar in flounder injected with ^{65}Zn plus a Zn^{2+} load or an equivalent volume of saline. However, due to the differences in specific activity, the ^{65}Zn in the lumen contents of the Zn^{2+}-injected flounder presumably represents a greater amount of stable Zn^{2+} than in the saline-injected fish. It is difficult to determine what the level of radioactivity represents in terms of loss of stable Zn^{2+}. Wiegand and Kirchgessner (1976 a,b) concluded that following parenteral administration of ^{65}Zn in rats, the specific radioactivity in the plasma, kidney, small intestine or pancreas could

validly be used to estimate the amount of stable Zn^{2+} that the ^{65}Zn in the feces represented. In the flounder injected with ^{65}Zn plus stable Zn^{2+} (25% of the total body Zn^{2+}), if one uses the specific activity of the injection solution to estimate the stable Zn^{2+} "secreted" into the lumen of the upper intestine, it amounts to approximately 1-2 µg of stable Zn^{2+} in fish dissected 9 and 27 days after the injection. If one uses the specific activity of the intestinal tissue (i.e. ^{65}Zn/g divided by stable Zn^{2+}/g, determined by atomic absorption spectrophotometry) it amounts to 2-10 µg of Zn^{2+}. Therefore, while the rate of Zn^{2+} loss (based on the retention of ^{65}Zn) appears not to be affected by excess Zn^{2+} in the body, the absolute quantity of Zn^{2+} eliminated via the gastrointestinal tract is probably greater than in the saline-injected fish. As shown in a feeding study using the non-absorbed marker ^{141}Ce (see Chapter I, p. 68), prior injection of flounder with a Zn^{2+} load did result in a net secretion of stable Zn^{2+}.

CHAPTER III

INVESTIGATION OF ZINC-BINDING PROTEINS IN SELECTED TISSUES OF THE WINTER FLOUNDER

INTRODUCTION

Although it has been demonstrated that mammals maintain Zn^{2+} homeostasis by a combination of the processes of absorption, excretion and storage, little is known about the mechanisms involved in Zn^{2+} regulation at the cellular level. During the past decade research on this subject has concentrated on the involvement of metal-binding proteins, both in the absorption process and in the uptake and storage of Zn^{2+} in organs such as the liver, which play a role in Zn^{2+} metabolism. Specifically, attention has focused on the involvement of a low molecular weight, cysteine-rich protein, metallothionein.

Metallothioneins were first purified from equine kidney by Kägi and Vallee (1960, 1961). It was subsequently found that metallothioneins occurred in the tissues of a wide variety of vertebrates, invertebrates and microorganisms and that the concentrations could be dramatically elevated by exposing these organisms to heavy metals (i.e. Cd^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+}). The major contributions made to this area of research over the two decades following the initial identification of metallothionein are reviewed by Kägi and Nordberg (1979). Complete amino acid sequences have been determined for metallothioneins purified from various vertebrate tissues [i.e. equine kidney (Kojima et al. 1976), equine liver (Kojima et al. 1979), human liver (Kissling and Kägi 1977), mouse liver (Huang et al. 1977, 1981)], from invertebrate tissues (i.e. the crab, *Scylla serrata*, Lerch et al. 1982) and from the fungi, *Neurospora crassa* (Lerch 1980). A high degree of sequence homology exists in the metallothioneins isolated from these very diverse species which suggests a conservation of function during evolution. However, the physiological function of metallothionein is not yet clear (Kägi et al. 1981; Brady 1982; Webb

and Cain 1982). In recent years measurement of metallothionein mRNA levels using cDNA hybridization techniques has demonstrated quite conclusively that heavy metals regulate the expression of the metallothionein gene at the level of transcription (Durnam et al. 1980; Durnam and Palmiter 1981). Since exposure to Cd^{2+} and Hg^{2+} increases the tissue concentrations of metallothionein, there has been much speculation that the protein serves to detoxify harmful heavy metals (Webb 1979). Consistent with this hypothesis is the observation that cell lines (i.e. mice and hamster) selected for Cd^{2+}-resistance exhibit an increased capacity for metallothionein synthesis when exposed to the metal compared to that of normal cells (Beach and Palmiter 1981; Gick and McCarty 1982). However, it has also been observed that Zn^{2+} is present in metallothionein even when the protein is induced by other metals (Winge et al. 1978). Low levels of Zn^{2+}-metallothionein also occur naturally in the tissues of several organisms (Bremner and Marshall 1974 a,b) and they are particularly elevated in the hepatic and intestinal tissues of fetal and newborn animals (Wong and Klassen 1979; Johnson and Evans 1980; Bakka and Webb 1981; Charles-Shannon et al. 1981). This information, coupled with the observation that several physiological stress conditions which alter the Zn^{2+} status of the animal also result in an increase in metallothionein synthesis (Bremner and Davis-1975; Oh et al. 1978), has led to the concensus that these proteins may play a role in the homeostasis of Zn^{2+}.

Richards and Cousins (1975a, 1976, 1977) were the first to demonstrate that injections of stable Zn^{2+} and feeding high Zn^{2+} diets result in the synthesis of metallothionein in the intestinal mucosa of rats. They concluded that the efflux of Zn^{2+} from the mucosal cell into the blood was inversely related to the level of metallothionein in the mucosal cytosol. Cousins (1979) proposed that control of Zn^{2+} absorption, in response to the

body's needs, was mediated through changes in the production of metallothionein. According to this model, when the Zn^{2+} status of an animal is elevated, metallothionein synthesis is induced in the intestinal mucosa. The metallothionein then competes for newly absorbed Zn^{2+} with the normal "carrier" protein in the cell, thereby reducing the amount of Zn^{2+} that is available for transfer into the plasma. Zn^{2+} bound to metallothionein would then be eliminated during the desquamation of the mucosal cells. Other investigators have confirmed that metallothionein is present in the intestinal tissue but opinion differs on its involvement in Zn^{2+} absorption and Zn^{2+} homeostasis. For example, Starcher et al. (1980) concluded that Zn^{2+} absorption, rather than being inversely related to the metallothionein content of mice intestine, was directly proportional to it. They suggested that metallothionein was directly involved in the absorption process.

Chen et al. (1977) examined the accumulation of Zn^{2+} in the liver and kidney of rats fed several levels of dietary Zn^{2+}. When diets containing 1000 µg/g or higher supplemental Zn^{2+} were fed to the rats, most of the excess Zn^{2+} in the tissue cytosols was associated with metallothionein. However, the turnover of Zn^{2+}-metallothionein was rapid and the accumulated Zn^{2+} was depleted to presumplemental levels within three days of feeding the rats a Zn^{2+}-deficient diet. Although a small portion of the Zn^{2+} in the metallothionein fraction appeared to be transferred to the large molecular weight proteins most of it was excreted in the feces and urine. This seems to indicate that the function of metallothionein in Zn^{2+} metabolism could be to temporarily accumulate excess levels of the metal during periods of elevated exposure and release it for excretion when feeding of high Zn^{2+} is terminated. The rapid turnover of metallothionein in the rat seems to preclude its having a significant role as a storage protein. However, Zn^{2+} does not appear to be as readily depleted from the

metallothionein fraction in ovine or bovine tissues (Whanger et al. 1981 a,b). In both lambs and cattle, excess Zn^{2+} accumulated with the metallothionein fraction in liver, kidney, pancreas, and small and large intestinal epithelia. The biological half-life of Zn^{2+}-metallothionein in the liver of lambs and cattle was 22.6 and 24.1 days, respectively, compared to 1.7 days in rats. The slower turnover of ovine and bovine metallothionein makes the proposal that this protein serves as a storage protein in certain mammalian species more feasible. Zn^{2+} stored in this form could possibly serve as a source of metal ions for Zn^{2+}-requiring metalloenzymes. It has been demonstrated *in vitro* that metallothionein can transfer Zn^{2+} to apoenzymes (Li et al. 1980; Udom and Brady 1980).

Metallothioneins, with characteristics similar to those observed in mammals, have been identified in gill, kidney and liver of both marine and freshwater fishes exposed to elevated levels of cadmium, methylmercury, copper and zinc (Olson et al. 1978; Overnell and Coombs 1979; Pierson 1980; McCarter et al. 1982; Kito et al. 1982a). It has usually been postulated that the protein has a detoxification function in fish (Bouquegneau 1979; McCarter et al. 1982; Kito et al. 1982b) but a metallothionein-like fraction has also been detected in the livers of non-exposed fish (Noel-Lambot et al. 1978). Therefore, as suggested for mammals, metallothionein may play a role in the normal metabolism of essential metals, such as Zn^{2+}, in fish.

In the present study chromatographic techniques were used to examine the presence of Zn^{2+}-binding proteins in the cytosols of several tissues of the winter flounder. Most of the emphasis was placed on the intestine and liver but the kidney and gill tissue were also examined. Both normal fish and fish which had their metal status elevated by injections of Zn^{2+} (and Cd^{2+}) were investigated. Procedures similar to those used in mammalian

studies to purify metallothionein were followed to isolate the low molecular weight binding proteins in the intestinal and liver cytosols. In addition, the incorporation of ^{35}S-cystine, used as a measure of metallothionein synthesis (Richards and Cousins 1975b), was compared in saline- and Zn^{2+}-injected flounder. Attempts were made to assess the involvement of the low molecular weight protein fraction in Zn^{2+} homeostasis in the flounder, particularly its possible role in the absorption process. Towards this end, Zn^{2+}-binding profiles in the intestinal cytosol were examined seasonally in conjunction with an examination of Zn^{2+} uptake from the *in situ* ligated upper intestine. The relationship of the low molecular weight Zn^{2+}-binding fraction and *in situ* uptake of Zn^{2+} from the intestine was also examined in saline-injected flounder and in flounder which had their Zn^{2+} status elevated by injections of Zn^{2+}.

MATERIALS AND METHODS

Preparation of tissue cytosols

To prepare the mucosal cytosol the flounder was killed by a blow on the head and the intestine immediately dissected out of the body. The intestinal contents were squeezed out and the lumen flushed with ice-cold saline (1% NaCl). The tissue was kept ice-cold throughout the isolation procedure. The intestine was cut open lengthwise and the mucosa scraped from the underlying tissue with a glass slide (Van Campen and Kowalski 1971). The tissue was homogenized in 20% weight/volume of buffer (same buffer as that later used to elute the sample from the Sephadex column) using a motor-driven, glass-Teflon, Potter Elvejhem homogenizer (8-10 passes). The homogenate was spun for 10 minutes at 12,000 g (Sorval centrifuge SM-24 rotor). The pellet was discarded and the mucosal cytosol (high speed supernatant) obtained by spinning the supernatant at 65,000 g for one

hour (International Equipment Company, centrifuge model B-60).

Similar homogenization and centrifugation steps were followed to isolate the liver, kidney and gill cytosols.

The protein concentration of the tissue cytosols was determined by the Biuret method (Layne 1957).

When the aim of the study was to isolate the low molecular weight (L.M.W.) Zn^{2+}-binding proteins in the tissue cytosols, the high speed supernatant was further treated using a modification of a procedure developed to partially purify metallothionein from rat liver (Cherian 1974). The supernatant was heated in a water bath to 70°C and maintained at that temperature for one minute. The sample was then chilled and kept ice-cold during subsequent purification steps. Following centrifugation (12,000 g for 10 minutes, Sorval centrifuge, SM-24 rotor) the pellet was discarded and ammonium sulfate added slowly, with stirring, to the supernatant to a concentration of 40% weight/volume. The centrifugation was repeated, the pellet again discarded and ammonium sulfate added to the supernatant to attain a saturated solution (100% weight/volume). This solution was centrifuged and the resulting pellet dissolved in a minimum volume of buffer (0.1 M ammonium bicarbonate, 2 mM 2-mercaptoethanol, pH 8.5). The sample was dialyzed overnight against 2 L of the same buffer to remove the ammonium sulfate.

Chromatographic techniques

The tissue cytosol (as obtained by centrifugation following heat treatment and salt fractionation) was labelled with ^{65}Zn (or ^{109}Cd) according to the procedure outlined in the individual experiments. A sample was applied to a pre-equilibrated Sephadex G-75 or G-100 column (column dimensions 2.5 X 90 cm); unless otherwise indicated it was eluted

from the column with 0.1 M ammonium bicarbonate, 2 mM 2-mercaptoethanol buffer, pH 8.5. Fractions of known volume (usually 4 mL) were collected using Gilson or LKβ fraction collectors. The total cpm ^{65}Zn (or ^{109}Cd) in each fraction was determined using a gamma scintillation counter (Packard model 578). The stable Zn^{2+} (or Cd^{2+}) concentration of the fraction was determined (by directly aspirating the eluant) using an atomic absorption spectrophotometer (AA 5 Varian Tectron). An estimate of the relative protein concentration in each fraction was determined by examining the absorbance (A 280, 250 and 230 nm) (Unicam SP 500 Series 2).

The molecular weights of the major Zn^{2+}-binding fractions were estimated by comparing the elution profiles of the samples with those obtained for proteins of known molecular weights (i.e. bovine serum albumin MW 67,000; ovalbumin MW 43,000; chymotrypsinogen MW 25,000; myoglobin MW 17,000; ribonuclease MW 13,700).

To isolate the low molecular weight (L.M.W.) Zn^{2+}-binding proteins, fractions eluting from the Sephadex G-75 column were applied to ion-exchange (DEAE-cellulose) and Bio-Gel P-30 columns. The L.M.W. Zn^{2+}-binding proteins (i.e. the peak fraction plus those fractions where the ^{65}Zn/fraction was greater than one half of the ^{65}Zn in the peak fraction) eluting from the Sephadex G-75 column were pooled and concentrated to approximately 4 mL by ultrafiltration under N_2 pressure using UM 2 Diaflo ultrafilters (Amicon). The concentrated sample was dialyzed overnight against a 0.05 M Tris-HCl buffer (pH 8.6); it was then applied to a pre-equilibrated ion exchange column of DE-52 cellulose (Whatman) (column dimensions 1 X 30 cm). The column was eluted with a linear gradient from 0.05 to 0.3 M Tris-HCl buffer (pH 8.6) (total volume = 400 mL).

Fractions of known volume were collected and again monitored for cpm ^{65}Zn, μg Zn^{2+}/mL and absorbance at 280, 230 nm. The major Zn^{2+}-binding

fractions (i.e. peak fraction plus those with ^{65}Zn greater than one half of the peak) were pooled and concentrated to approximately ten mL by ultrafiltration (UM 2 filter). The concentrated sample was dialyzed against 0.1-M ammonium bicarbonate, 2 mM 2-mercaptoethanol buffer (pH 8.5). The dialyzed sample was applied to a pre-equilibrated BioGel P-30 column (Bio-Rad) (column dimensions 2 X 80 cm) and eluted with the 0.1 M buffer. The peak L.M.W. Zn^{2+}-binding fractions were pooled, dilayzed against dilute (1:20) buffer and lyophilized (Labconco Freeze Dry-3).

The L.M.W. Zn^{2+}-binding fraction isolated by the above procedure was, in some instances, further examined using high pressure liquid chromatography (HPLC). A sample of the protein was applied to a TSK-125 BioRad gel filtration column and eluted with 0.1 M Tris-HCl, 0.1 M sodium sulfate buffer, pH 7.4 (flow rate = 1.0 mL per min). The eluant was monitored at absorbance 230 nm. An estimation of the molecular weight of the protein was made by comparison with the elution profiles of proteins of known molecular weights.

The L.M.W. Zn^{2+}-binding protein fraction was also examined using Slab Gel Electrophoresis (BioRad model 220) according to the system of Laemmli (1970). Samples were applied to 10% acrylamide gels (Tris Glycine gels with and without urea). The gels were eluted with Tris-Glycine buffer (pH 9.2), fixed in 5% trichloroacetic acid (TCA) 5% sulfosalicyclic acid, stained with Coomassie Blue and destained with 10% TCA.

The amino acid composition of selected protein fractions was determined by quantitative analysis of hydrolyzed protein (24 hr, 6 N HCl hydrolysis at 110°C). The analyses were performed on a Beckman Spinco 121 Amino Acid Analyzer. Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid (Leggett-Bailey 1967). The recovery of cysteic acid following this pro-

cedure was estimated to be 90% (Schram et al. 1954).

Section A. Zinc binding proteins in the mucosal cytosol of winter flounder.

In the initial studies on Zn^{2+}-binding proteins, the mucosa was labelled using a surgical procedure to inject ^{65}Zn directly into the intestinal lumen. Fish were placed in anaesthetic (0.5 g MS 222, tricaine methanesulfonate, in 4 L seawater) for approximately 10 minutes and a (1:2) dilution of the same solution or clean seawater passed over the gills during surgery. An incision was made in the body wall to expose the gastro-intestinal tract. One mL of saline solution (1% NaCl) containing ^{65}Zn was injected into the upper intestine. The incision was sutured and the fish returned to the aquarium. Three to five hours later, the fish was killed by a blow on the head and the intestine dissected out of the body. The intestinal cytosol was then obtained by the homogenization and centrifugation procedures outlined above. The total time which lapsed from removal of the intestine to application of the sample to the column averaged 3 hours.

i) Chromatographic separation of Zn^{2+}-binding proteins in the mucosal cytosols of normal and Zn^{2+}-injected winter flounder.

Mucosal cytosols (high speed supernatants) were obtained from normal flounder and flounder which had been previously injected with a Zn^{2+} load (25% of their estimated total body Zn^{2+}). The fish examined in this study were maintained in the laboratory in 40,000 L tanks, under ambient conditions of temperature and photoperiod. They were fed chopped capelin during the feeding period.

A sample of the mucosal cytosol from each fish was applied to a Sephadex G-100 column which had been pre-equilibrated with a TES buffer (i.e. 10 mM TES (tris(hydroxymethyl) methyl-2-amino-ethanesulfonic acid),

175 mM NaCl, pH 7.4). The elution profiles were plotted and the relative amounts of ^{65}Zn and Zn^{2+} in the major peaks determined by integration of the areas under the curve (Technicon-model AAG Integrator/Calculator).

ii) Chromatographic separation of Zn^{2+}-binding proteins in the mucosal cytosols of winter flounder examined at monthly intervals.

The chromatographic separation of Zn^{2+}-binding proteins present in the mucosal cytosol was also investigated in conjunction with an examination of the seasonal *in situ* uptake of Zn^{2+} (see Chapter I, Section A, p. 14). Flounder used in this study were collected in Conception Bay, Newfoundland and were held in the laboratory for approximately one week before being tested. They were not fed during this time.

A sample of mucosal cytosol (containing 50 mg of protein) from each fish was applied to a Sephadex G-75 or G-100 column.

iii) Chromatographic separation of Zn^{2+}-binding proteins in the mucosal cytosols of winter flounder fed diets containing different concentrations of Zn^{2+}.

The chromatographic separation of Zn^{2+}-binding proteins present in the mucosal cytosol was also investigated in flounder fed diets containing different concentrations of Zn^{2+}. This was carried out in conjunction with an *in situ* study on Zn^{2+} uptake from the upper intestine (see Chapter I, Section A, p. 15). The fish were fed food pellets containing 60 µg Zn^{2+}/g wet weight (control) or pellets with $ZnCl_2$ added (600 µg Zn^{2+}/g wet weight). Both groups were fed the control diet for two weeks, followed by the control or high Zn^{2+} diet for five weeks. The flounder were examined in September; for comparison two fish brought in from the field one week earlier were also examined. The mucosal cytosol from each fish was applied to a Sephadex G-75 column.

Section B. Isolation and purification of low molecular weight (L.M.W.) Zn^{2+}-binding proteins in the mucosal cytosol of winter flounder.

L.M.W. Zn^{2+}-binding proteins were isolated from the mucosal cytosols of Zn^{2+}-injected and normal winter flounder according to the purification procedure outlined on p. 159. ^{65}Zn was added to the samples before they were applied to the Sephadex G-75 column. In a representative study, flounder (N=9) were injected intraperitoneally with a total body load of 1.25 mg Zn^{2+}/100 g body weight. The Zn^{2+} (as $ZnCl_2$ in 1% NaCl) was administered in four injections over a 12 day period (in August). Two days after the last injection the fish were bled and killed, the intestines removed and the mucosal scrape of the fish pooled (yield = 45 g). Pooled samples of mucosal scrape (approximately 50 g) were also obtained from normal (untreated, recently brought in from the field) flounder at various times throughout the year.

Section C. The incorporation of ^{35}S-cystine into the low molecular weight (L.M.W.)-Zn^{2+} binding proteins in the mucosal cytosol of the winter flounder.

Chromatographic techniques were used to investigate the incorporation of ^{35}S-cystine into mucosal cytosol proteins of winter flounder. In a representative study, flounder were injected intraperitoneally with Zn^{2+} (as $ZnCl_2$, 25% of their estimated total body Zn^{2+}) or saline (1% NaCl). Six days after the initial injections, two of the Zn^{2+}- and two of the saline-treated fish were injected intravenously with ^{35}S-cystine (approximately 20 µCi per fish which ranged in body weight from 250-350 g). Two days later the fish were killed and the mucosal cytosol of each fish isolated. At the same time, a pooled sample of mucosal tissue was also obtained from flounder injected with Zn^{2+} (n=20 fish, yield=54 g) or saline (n=22 fish, yield=63 g) in order to obtain enough material to

characterize the L.M.W. Zn^{2+}-binding proteins.

The mucosal cytosols from the ^{35}S-cystine injected flounder were applied to Sephadex G-75 columns following heat treatment and fractionation with ammonium sulfate (see p. 160). Four mL fractions were collected; 0.2 mL of the fractions were added to 10 mL of Riafluor (New England Nuclear). The radioactivity was determined in a Beckman Scintillation Spectrophotometer. The absorbance at 230 nm and μg Zn^{2+}/mL of each fraction was also determined.

Section D. The relationship of low molecular weight (L.M.W.) Zn^{2+}-binding proteins in the mucosal cytosol of winter flounder to *in situ* uptake of Zn^{2+} from the upper intestine.

The presence of the L.M.W. Zn^{2+}-binding proteins in the mucosal cytosol was examined in conjunction with an investigation of the effects of intravenous injections of Zn^{2+} (25% of the estimated total body Zn^{2+}) or saline (1% NaCl) on *in situ* uptake of Zn^{2+} from the ligated upper intestine (see Chapter I, Section A, p. 15). This study was conducted in August; Zn^{2+} uptake was examined one week following the injections. A portion of the intestine of each flounder was used to analyze the stable Zn^{2+} concentrations of the tissue; the mucosa was scraped from the rest of the intestines of these fish. Pooled samples of 12 and 14 g were obtained from the Zn^{2+}- and saline-injected flounder, respectively. The mucosal cytosols were heat treated and fractionated with ammonium sulfate; ^{65}Zn was added to the samples before they were applied to the Sephadex G-75 column.

Section E. Chromatographic separation of Zn^{2+}-binding proteins in the liver cytosols of normal and Zn^{2+}-injected winter flounder.

Chromatographic separation of Zn^{2+}-binding proteins was carried out

on liver cytosols (high speed supernatants) obtained from normal flounder and flounder which had been injected with Zn^{2+} (0.5-1.0 mg Zn^{2+}/100 g body weight). ^{65}Zn was added to the cytosol obtained from each fish and a sample, containing 50-60 mg of protein, was applied to a Sephadex G-100 column.

Section F. Isolation and purification of low molecular weight (L.M.W.) Zn^{2+}- (and Cd^{2+}-) binding proteins in the liver cytosol of winter flounder.

i)...in the liver cytosol of Cd^{2+}-injected winter flounder.

L.M.W. proteins were isolated from winter flounder which had been injected with a total body load of 0.25 mg Cd^{2+}/100 g body weight. The Cd^{2+} (as $CdCl_2$) was administered in three injections over a 12 day period. One week after the last injection the fish were killed and the livers removed, pooled (yield from 9 fish=82 g) and frozen.

The cytosol obtained by homogenization and centrifugation of half of the pooled liver was applied directly to a Sephadex G-75 column (applied in 6 lots); the cytosol obtained from the rest of the liver was heat treated and fractionated with ammonium sulfate before application to the Sephadex G-75 column (applied in two lots). The subsequent purification steps (as outlined on p. 160) were similar for the two batches of liver.

ii)...in the liver cytosol of Zn^{2+}-injected winter flounder.

In a representative study, the presence of L.M.W. Zn^{2+}-binding proteins was examined in flounder which had been injected (i.p.) with a total body load of 1.25 mg Zn^{2+}/g body weight. The L.M.W. Zn^{2+}-binding proteins were also isolated from the intestinal cytosols of these flounder (see Section B, p.165 for details of the injection format). A pooled sample of 150 g of liver was obtained from the flounder. ^{65}Zn was added to the

liver cytosol (which had been heat treated and fractionated with ammonium sulfate) before it was applied to the Sephadex column.

iii)...in the liver cytosol of normal winter flounder.

The purification procedure used to isolate L.M.W. Zn^{2+}-binding proteins from liver cytosols of Zn^{2+}-injected flounder was followed using the liver cytosols of normal (untreated) fish. In a representative study, two hundred g of liver was pooled from winter flounder brought in from the field at the end of July.

Section G. The incorporation of ^{35}S-cystine into the low molecular weight (L.M.W.) Zn^{2+}-binding proteins in the liver cytosol of the winter flounder.

Chromatographic techniques were used to investigate the incorporation of ^{35}S-cystine into the liver cytosol proteins of winter flounder.

In a representative study, winter flounder were injected (i.v.) with ^{35}S-cystine (approx. 100 µCi per fish) 20 to 30 hours after receiving an injection (i.p.) of Zn^{2+} (1 mg/100 g body weight) or saline. On the day following the ^{35}S-cystine injection, the flounder were bled, killed and the livers removed. The liver cytosols were heat treated and fractionated with ammonium sulfate before application to a Sephadex G-75 column; 4 mL fractions were collected. 0.2 mL of the fractions were added to 10 mL of Riafluor (New England Nuclear). The radioactivity was determined in a Beckman Scintillation Spectrophotometer. The absorbance at 230 nm and µg Zn^{2+}/mL of each fraction were also determined.

Section H. Comparison of chromatographic elution profiles of Zn^{2+}-binding proteins in kidney, liver, gill and intestine of normal and Zn^{2+}-injected winter flounder.

Chromatographic separation of Zn^{2+}-binding proteins present in gill

and kidney cytosol was carried out on several normal (non-treated) and Zn^{2+}-injected winter flounder. The elution profiles of the liver and mucosal cytosols were examined in the same fish.

The intestinal mucosa was labelled by injecting ^{65}Zn directly into the lumen of the intestine; ^{65}Zn was added to the liver, gill and kidney supernatants. The samples were applied to a Sephadex G-75 column.

RESULTS

Section A. Zn^{2+}-binding proteins in the mucosal cytosol of the winter flounder.

i) Chromatographic separation of Zn^{2+}-binding proteins in the mucosal cytosols of normal and Zn^{2+}-injected winter flounder.

The elution profiles obtained by chromatographing mucosal cytosols from normal (n=3) and Zn^{2+}-injected (n=3) winter flounder on Sephadex G-100 columns are presented in Fig. 31 A, B, and C.

In both Zn^{2+}-injected and normal flounder, the majority of the absorbance at 280 nm eluted at the void volume of the column (Fig. 31 A). This comprises proteins with molecular weights greater than 100,000.

In the mucosal cytosols from the Zn^{2+}-injected flounder, ^{65}Zn was associated with protein fractions having estimated molecular weights of >150,000 (Fraction I), 80-150,000 (Fraction II), 30-40,000 (Fraction III), and 10-15,000 (Fraction IV) (Fig. 31 B). There was relatively more ^{65}Zn associated with the 10-15,000 MW proteins (Fraction IV) in the intestinal cytosol isolated from flounder one week after the Zn^{2+} injection than in intestinal cytosols isolated three days or three weeks after the injection (Table 16).

The ^{65}Zn profiles of mucosal cytosol proteins isolated from normal flounder were similar to the profiles observed for Zn^{2+}-injected fish.

Fig. 31. Separation of Zn^{2+}-binding proteins in the mucosal cytosol of normal (n=3) and Zn^{2+}-injected (n=3) winter flounder using Sephadex G-100 (column dimensions = 2.5 X 90 cm). Fraction size collected = 4 mL.

A. Absorbance 280 nm

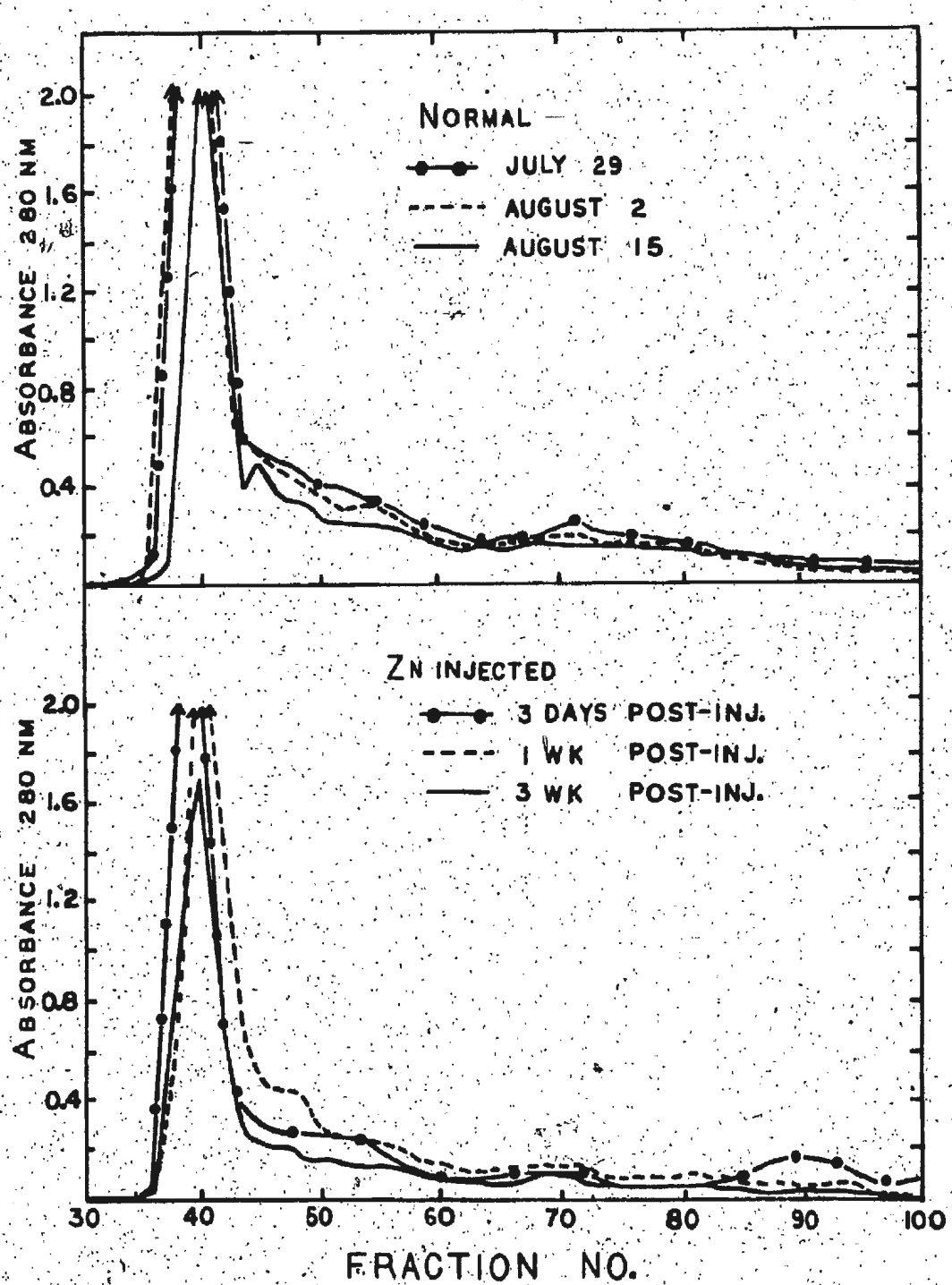
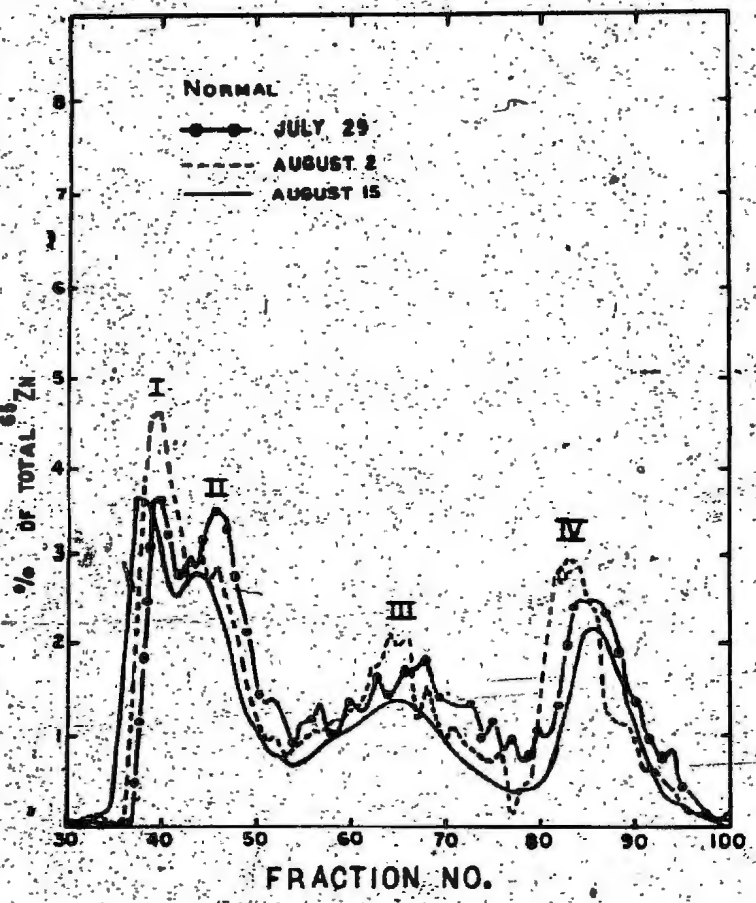

NORMAL
JULY 29
AUGUST 2
AUGUST 15
ABSORBANCE 280 NM
2.0
1.6
1.2
0.8
0.4
ZN INJECTED
3 DAYS POST-INJ.
1 WK POST-INJ.
3 WK POST-INJ.
30
40
50
60
70
80
90
100
FRACTION NO.

Fig. 31. Separation of Zn^{2+}-binding proteins in the mucosal cytosol of normal (n=3) and Zn^{2+}-injected (n=3) winter flounder using Sephadex G-100 (column dimensions = 2.5 X 90 cm). Fraction size collected = 4 mL.

B. ^{65}Zn per fraction expressed as a % of the total ^{65}Zn


NORMAL
JULY 29
AUGUST 2
AUGUST 15
% OF TOTAL ^{65}ZN
I
II
III
IV
FRACTION NO.

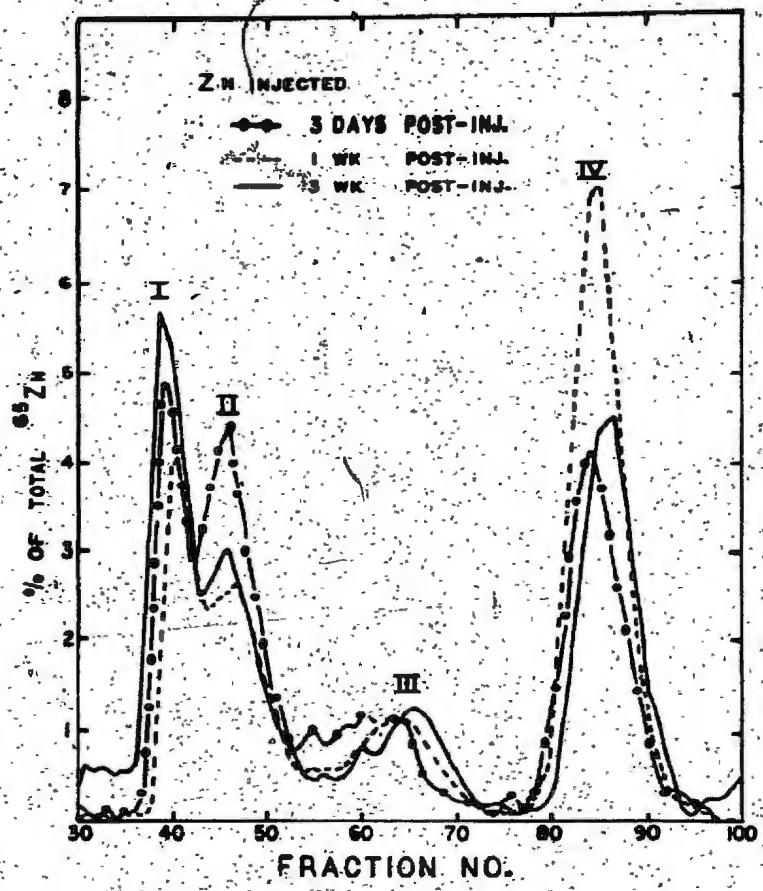

Z-N INJECTED
3 DAYS POST-INJ.
1 WK POST-INJ.
3 WK POST-INJ.
% OF TOTAL ^{65}ZN
I
II
III
IV
FRACTION NO.

Fig. 31. Separation of Zn^{2+}-binding proteins in the mucosal cytosol of normal (n=3) and Zn^{2+}-injected (n=3) winter flounder using Sephadex G-100 (column dimensions = 2.5 X 90 cm). Fraction size collected = 4 mL.

C. $\mu g\ Zn^{2+}$ per mL

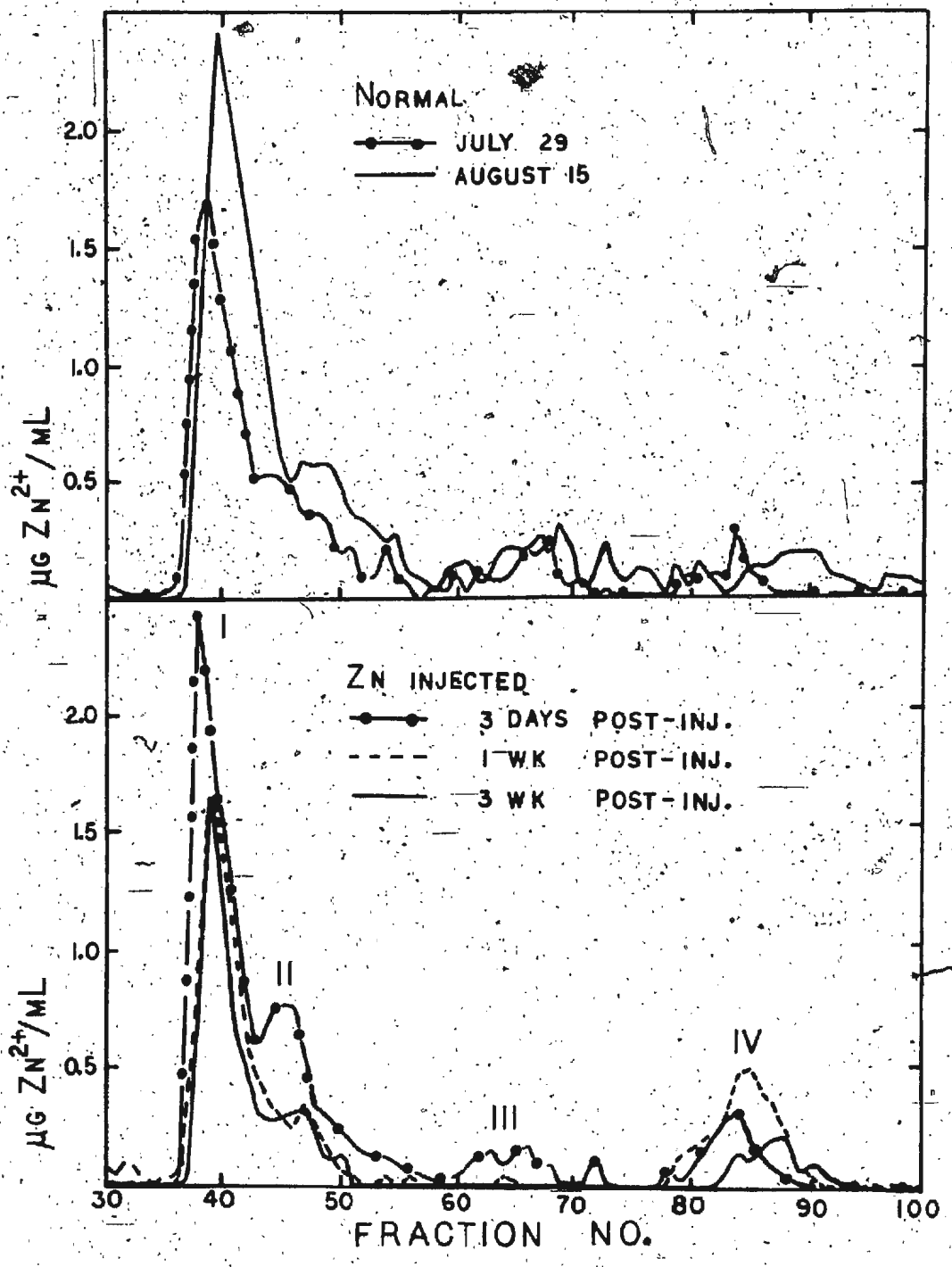

NORMAL
JULY 29
AUGUST 15
2.0
1.5
1.0
0.5
μG Zn2+/ML
ZN INJECTED
3 DAYS POST-INJ.
1 WK POST-INJ.
3 WK POST-INJ.
I
II
III
IV
30
40
50
60
70
80
90
100
FRACTION NO.

Table 16. The distribution (%) of ^{65}Zn in the major fractions eluting from a Sephadex G-100 column (see Fig. 31 B); mucosal cytosols obtained from normal flounder and flounder injected with a Zn^{2+} load (examined 3 days, 1 week and 3 weeks after the injection).

Fraction	Estimated molecular weight	% ^{65}Zn[a]					
		Zn^{2+}-injected			Normal		
		3 day (July 24-27)	1 week (July 24-31)	3 week (July 26-Aug 15)	July 29	Aug 2	Aug 15
I	>150,000	21	18	25	18	24	25
II	80-150,000	36	26	32	29	27	35
III	30-40,000	15	12	12	32	28	22
IV	10-15,000	29	44	30	21	22	18

[a] % ^{65}Zn in Fraction = $\frac{\text{Area of peak outlined by } ^{65}Zn}{\text{total Area}} \times 100$

However, less of the total ^{65}Zn applied to the column was associated with the 10-15,000 MW proteins (Fraction IV) (Fig. 31 B). Based on integration of the area of the peaks (outlined by the cpm ^{65}Zn per fraction), in Zn^{2+}-injected winter flounder 29-44% of the total ^{65}Zn was associated with proteins in the low molecular weight fraction (IV) compared to 18-22% in the normal fish (Table 16).

The distribution of stable Zn^{2+} in the column eluants differed somewhat from that of ^{65}Zn (Fig. 31 C). In the mucosal cytosols of Zn^{2+}-injected flounder, relatively more stable Zn^{2+} (i.e. 48-68% of the total Zn^{2+}) than ^{65}Zn (18-25%) was associated with Fraction I (compare Table 16 and 17). Less stable Zn^{2+} (6-23% of the total) than ^{65}Zn (29-44%) was associated with Fraction IV (compare Tables 16 and 17).

The amino acid composition of the peak of Fraction IV is shown in Table 18. In both the normal and Zn^{2+}-injected flounder, cysteine accounted for the greatest % of the amino acids (17-38.6% of the total).

The elution profiles discussed above represent those of fish examined in July and August. Several normal and Zn^{2+}-injected flounder, held at ambient conditions, were examined from February to June. Zinc-binding Fractions I, II, and III were present but Fraction IV was not detected (total number of fish examined = 16).

ii) Chromatographic separation of Zn^{2+}-binding proteins in the mucosal cytosols of winter flounder examined at monthly intervals.

The chromatographic separation of Zn^{2+}-binding proteins present in the mucosal cytosol was also investigated seasonally. The ^{65}Zn elution profiles obtained by chromatographing mucosal cytosols on Sephadex G-75 columns indicated that two Zn^{2+}-binding fractions were present throughout the year--a fraction representing proteins with molecular weights $\geq$

Table 17. The distribution (%) of stable Zn^{2+} in the major fractions eluting from a Sephadex G-100 column (see Fig. 31 C); mucosal cytosols obtained from normal flounder and flounder injected with a Zn^{2+} load (examined 3 days, 1 week and 3 weeks after the injection).

Fraction	Estimated molecular weight	% stable Zn^{2+}[a]			
		Zn^{2+}-injected			Normal
		3-day (July 24-27)	1 week (July 24-31)	3 week (July 26-Aug 15)	July 29
I	>150,000	48	50	68	54
II	80-150,000	41	27	22	33
III	30-40,000	5	-	-	7
IV	10-15,000	6	23	10	6

[a] % Zn^{2+} in Fraction = $\frac{\text{Area of peak outlined by } \mu g\ Zn^{2+}/mL}{\text{total Area}} \times 100$

Table 18. % Amino acid composition of Fraction IV obtained by separation of mucosal cytosols (from normal and Zn^{2+}-injected winter flounder) on Sephadex G-100.

Amino Acid	% Total Residues			
	Zn^{2+}-injected			Normal
	3-day	1 week	3 weeks	Aug. 2
Aspartic acid	13.3	9.1	7.7	8.6
Threonine	6.9	6.3	7.5	7.1
Serine	9.4	9.3	9.6	8.7
Proline	trace	1.7	trace	trace
Glutamic acid	7.4	5.1	4.0	4.8
Glycine	13.6	14.3	9.0	11.2
Alanine	8.6	9.0	5.2	5.5
Cysteine*	17.0	21.3	38.6	28.2
Valine	2.8	4.6	7.2	6.6
Methionine+	1.6	0.4	1.0	1.5
Isoleucine	7.1	4.3	3.2	5.0
Leucine	8.0	6.8	4.5	6.6
Tyrosine	0	0	0	0
Phenylalanine	0	0	0	trace
Lysine	1.1	7.2	5.1	5.6
Histidine	0	0	0	0
Arginine	4.8	2.6	1.3	3.1

* determined as cysteic acid

\+ determined as methionine sulfone

70,000 (corresponds to Fractions I and II on a Sephadex G-100 column) and a fraction representing proteins with molecular weights of 30-40,000 (Fraction III). Fraction IV, comprising proteins of low molecular weights (10-15,000), was not always detected. Of 39 fish examined, a ^{65}Zn peak corresponding to Fraction IV was only present in 10 individuals. It was not detected in flounder examined from January to May; it was detected in some, not all, of the fish examined from June to December. During the period when Fraction IV was present, similar elution patterns (^{65}Zn, stable Zn^{2+} and A 280 nm) were observed in the different months (Fig. 32).

iii). Chromatographic separation of Zn^{2+}-binding proteins in the mucosal cytosols of winter flounder fed diets containing different amounts of stable Zn^{2+}.

The ^{65}Zn elution profiles obtained by chromatographing mucosal cytosols of winter flounder fed a control diet (60 µg Zn^{2+}/g wet weight), a high Zn^{2+} diet (600 µg Zn^{2+}/g wet weight), and those recently brought in from the field (feeding on a "natural" diet) were similar (Fig. 33). Three ^{65}Zn-binding fractions were present: a fraction representing proteins with molecular weights >70,000; a fraction representing proteins with molecular weights of 30-40,000; a fraction comprising proteins of smaller molecular weights (10-15,000).

Fig. 32. Separation of Zn^{2+}-binding proteins in the mucosal cytosol of flounder examined in July, September and December using Sephadex G-75 (column dimensions = 2.5 X 90 cm). Fraction size collected = 4.4 mL.

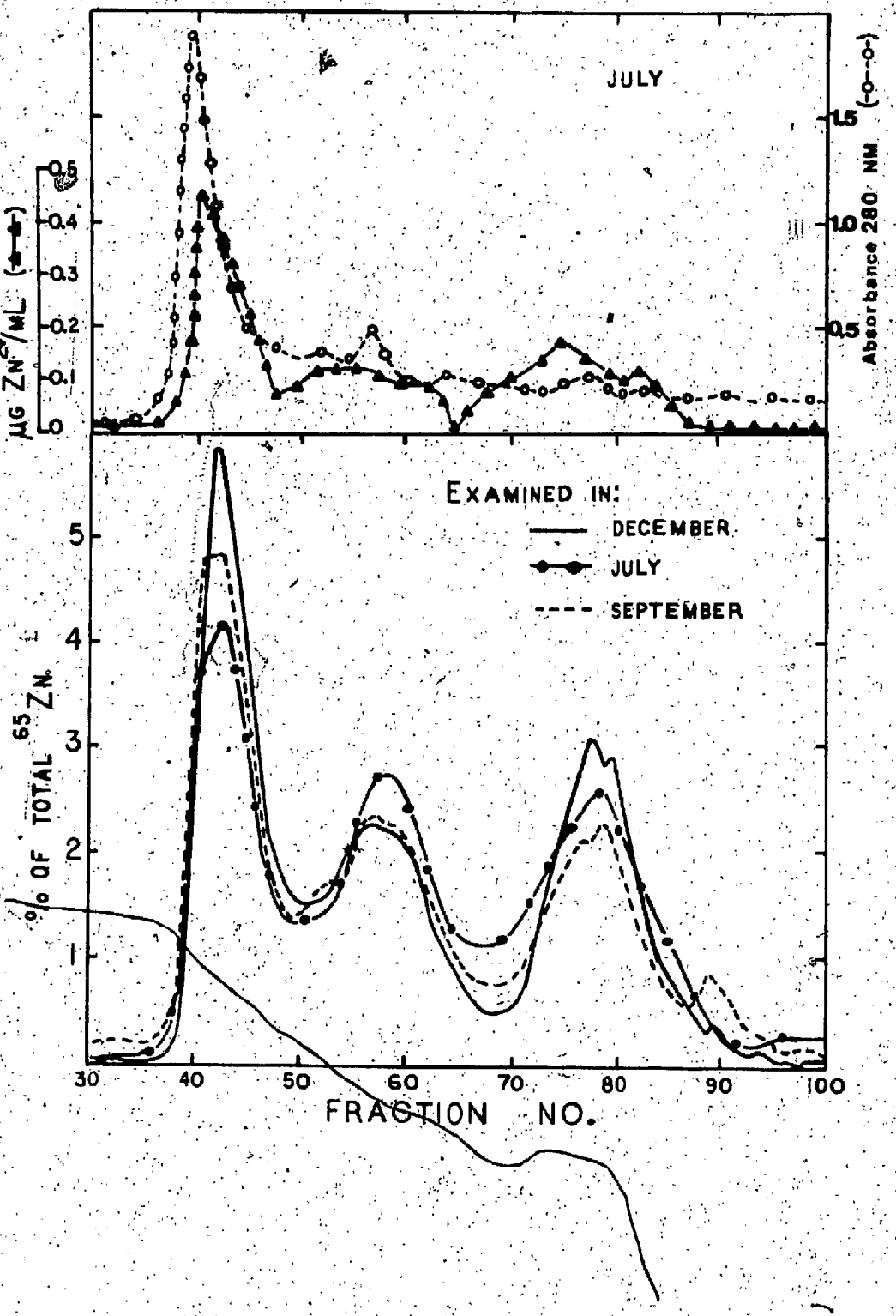
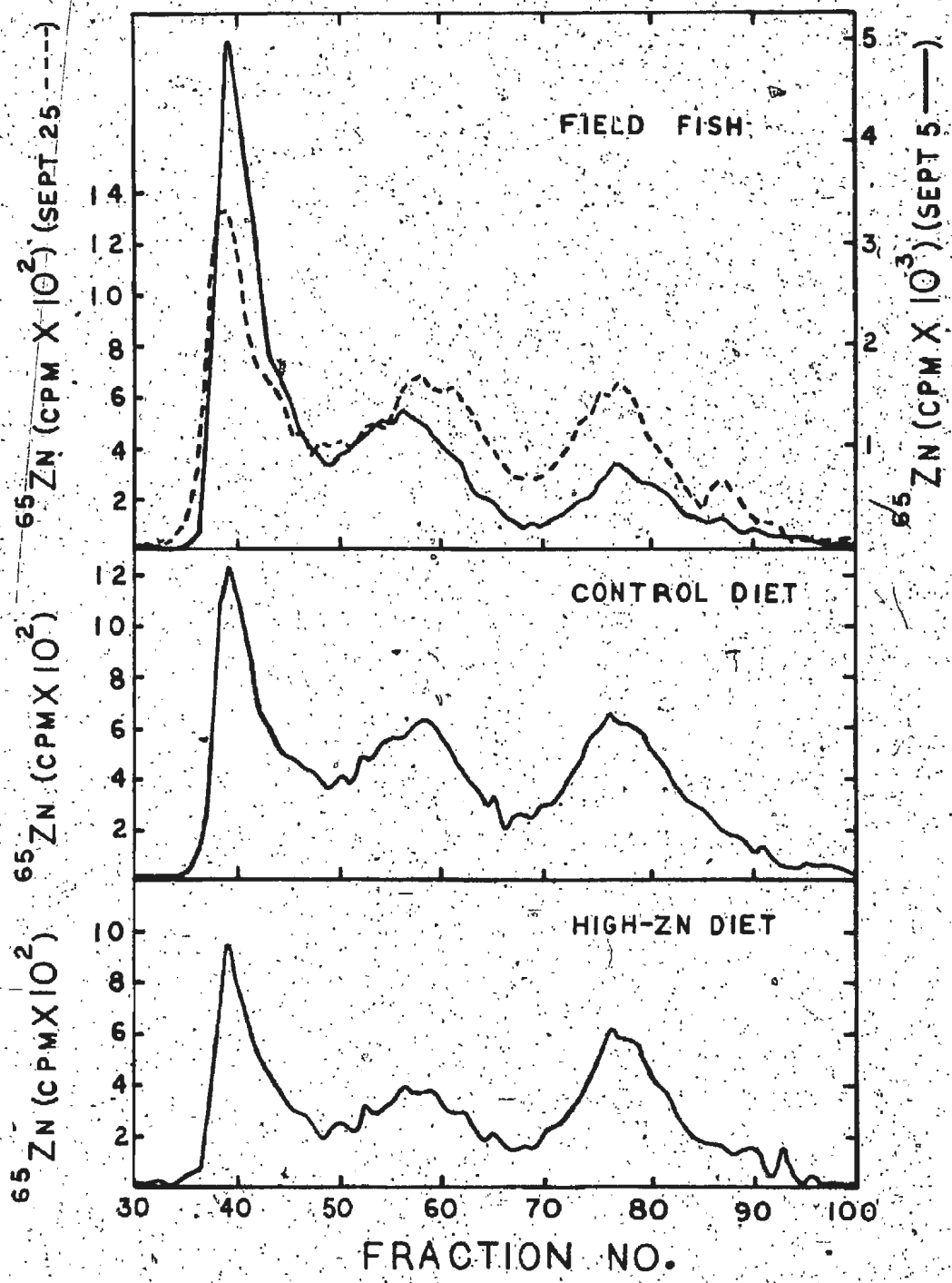

JULY
μG Zn++/ML (—●—)
0.5
0.4
0.3
0.2
0.1
0
1.5
1.0
0.5
Absorbance 280 NM (-o--o-)
EXAMINED IN:
DECEMBER
JULY
SEPTEMBER
% OF TOTAL 65Zn
5
4
3
2
1
30
40
50
60
70
80
90
100
FRACTION NO.

Fig. 33. Separation of Zn^{2+}-binding proteins in the mucosal cytosol of flounder fed a control diet (60 µg Zn^{2+}/g wet weight), a high Zn^{2+} diet (600 µg Zn^{2+}/g wet weight), or recently brought in from the field, using Sephadex G-75 (column dimensions = 2.5 X 90 cm). Fraction size collected = 4 mL.


FIELD FISH
CONTROL DIET
HIGH-ZN DIET
^{65}ZN (CPM X 10^2) (SEPT 25 ----)
^{65}ZN (CPM X 10^3) (SEPT 5 ——)
^{65}ZN (CPM X 10^2)
^{65}ZN (CPM X 10^2)
FRACTION NO.

Section B. Isolation and purification of low molecular weight (L.M.W.) Zn^{2+}-binding proteins in the mucosal cytosols of winter flounder.

The Sephadex G-75 elution pattern of the partially purified mucosal cytosol (i.e. following heat treatment and ammonium sulfate fractionation) of Zn^{2+}-injected flounder is depicted in Fig. 34 A. Most of the protein (Absorbance 230 nm) eluted in the void volume of the column. A small protein peak (Absorbance 230 nm) was found in the position corresponding to that of low molecular weight (L.M.W.) proteins; the major portion of the ^{65}Zn and stable Zn^{2+} eluted with this fraction.

When this L.M.W. fraction was applied to ion-exchange chromatography (DEAE-cellulose) most of the protein (A230 nm), ^{65}Zn and stable Zn^{2+} eluted in a single peak (Fig. 34 B). There was little absorbance at 280 nm associated with this peak.

Further chromatography of the major DEAE peak on Biogel P-30 produced the elution profile shown in Fig. 34 C. The ^{65}Zn and stable Zn^{2+} eluted in a single peak which was associated with high absorbance at 230 nm and nearly zero absorbance at 280 nm.

The main peak eluting from the Biogel P-30 column was lyophilized; 45 g of mucosal scrape resulted in 3.6 mg of "purified" L.M.W. Zn^{2+}-binding protein. The amino acid composition of the protein is shown in Table 19. Cysteine is the most predominant residue (33.1%), followed by threonine (13.4%), glycine (10.6%), serine (10.4%) and lysine (10.3%).

The Sephadex G-75 and DEAE-cellulose elution profiles for a pooled sample of mucosal scrape (50 g), obtained from normal flounder in October, are shown in Fig. 35 A and B. Most of the protein (A 230 nm) eluted in the void volume of the Sephadex G-75 column. The major portion of ^{65}Zn and Zn^{2+} eluted in the position of L.M.W. proteins, but there was not a correspondingly clear absorbance peak (A 230 nm).

Fig. 34. Separation of Zn^{2+}-binding proteins in the mucosal cytosol of Zn^{2+}-injected flounder.

A. Separation of Zn^{2+}-binding protein on Sephadex G-75 (2.5 X 90 cm) following heat treatment and ammonium sulfate fractionation of the mucosal cytosol. Fraction size collected = 4.1 mL.

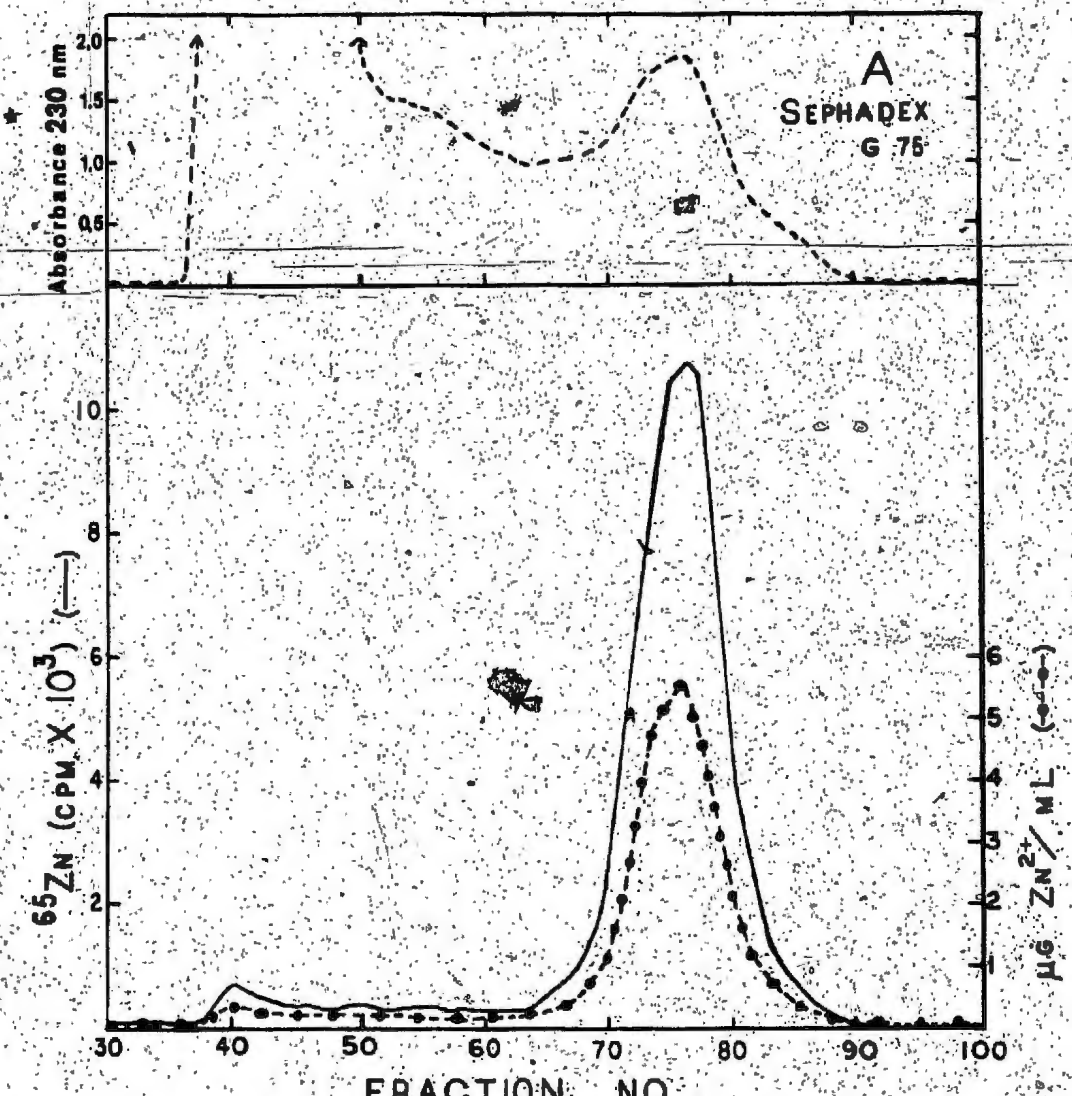
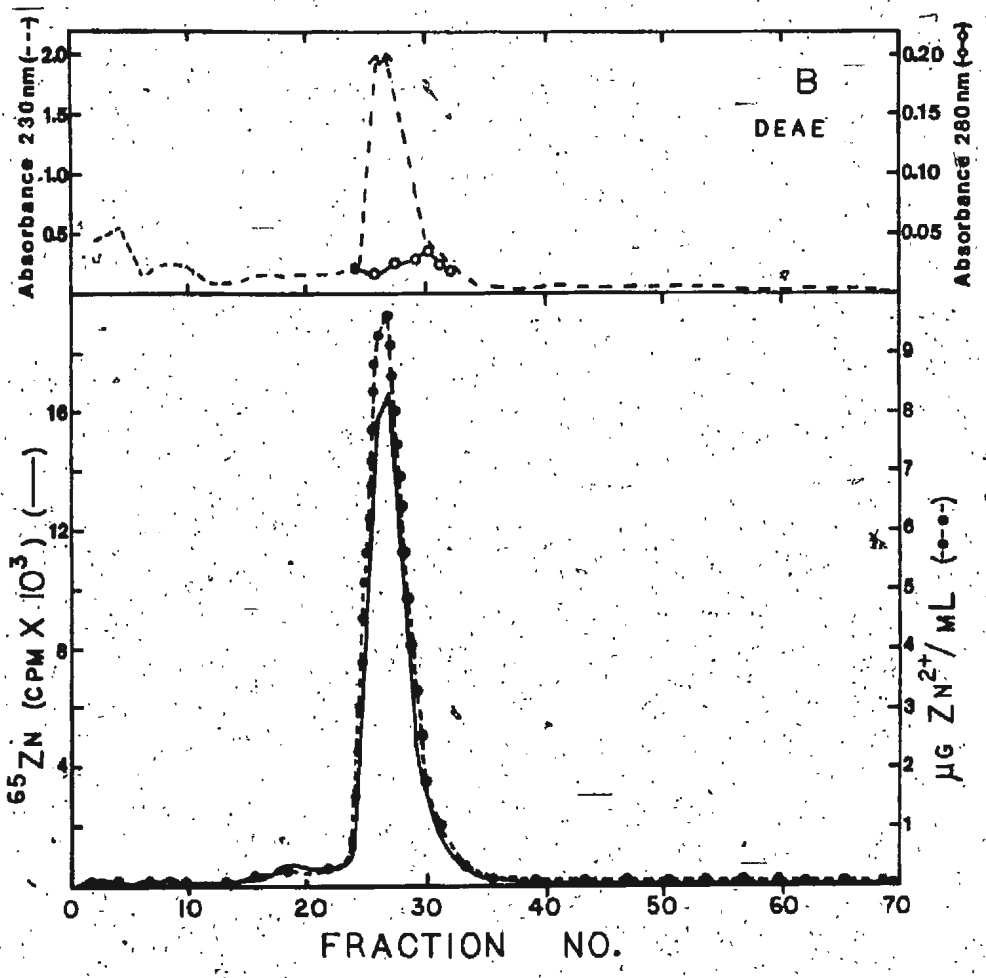

A
SEPHADEX
G 75
Absorbance 230 nm
2.0
1.5
1.0
0.5
^{65}Zn (CPM X 10^3) (——)
10
8
6
4
2
μG Zn^{2+}/ML (●—●)
6
5
4
3
2
1
30
40
50
60
70
80
90
100
FRACTION NO.

Fig. 34. Separation of Zn^{2+}-binding proteins in the mucosal cytosol of Zn^{2+}-injected flounder.

B. Further separation of the L.M.W. Zn^{2+}-binding fraction (eluting from the Sephadex G-75 column) on DEAE-cellulose (1 X 30 cm) using a 0.05 to 0.3 M Tris HCl gradient, pH 8.6. Total vol. 400 mL. Fraction size collected = 4.0 mL.

C. Separation of the major Zn^{2+}-binding fraction, eluting from the DEAE-cellulose column, on Biogel P-30 (2 X 80 cm). Fraction size collected = 4.2 mL.


B
DEAE
Absorbance 230nm(---)
Absorbance 280nm(o-o)
^{65}Zn (CPM X 10^3) (——)
μG Zn^{2+}/mL (•-•-)
FRACTION NO.

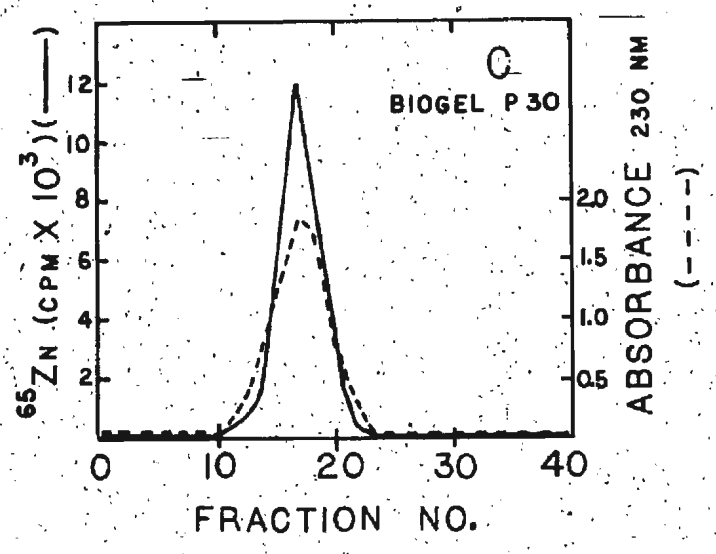

C
BIOGEL P 30
^{65}Zn (CPM X 10^3)(——)
ABSORBANCE 230 NM (- - - -)
FRACTION NO.

Fig. 35. Separation of Zn^{2+}-binding proteins in the mucosal cytosol of normal flounder.

A. Separation of Zn^{2+}-binding protein on Sephadex G-75 (2.5 X 90 cm) following heat treatment and ammonium sulfate fractionation of the mucosal cytosol. Fraction size collected = 4.8 mL.

B. Further separation of the L.M.W. Zn^{2+}-binding fraction (eluting from Sephadex G-75) on DEAE-cellulose (1 X 30 cm) using a 0.05 to 3.0 M Tris HCl gradient, pH 8.6. Total vol. 400 mL. Fraction size collected = 3.6 mL.

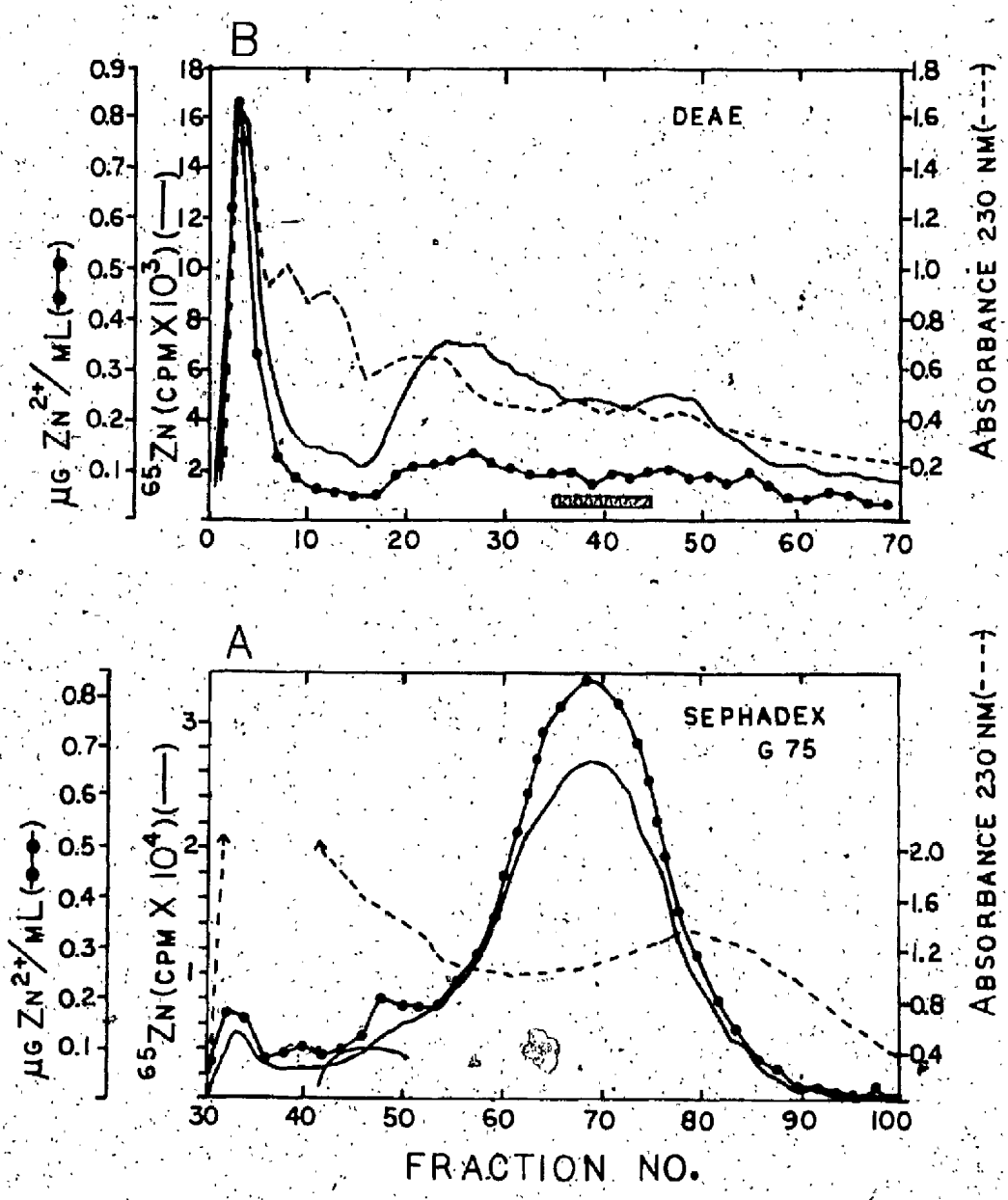

B
DEAE
A
SEPHADEX
G 75
μG Zn2+/ML(●—●)
65Zn(CPM X 10³)(——)
65Zn(CPM X 10⁴)(——)
ABSORBANCE 230 NM(- - -)
FRACTION NO.

Table 19. Amino acid composition of L.M.W. Zn^{2+}-binding protein isolated from the mucosal cytosol of Zn^{2+}-injected winter flounder by Sephadex G-75, DEAE-cellulose ion-exchange, and Biogel P-30 chromatography.

Amino acid	% total residues
Aspartic acid	9.5
Threonine	13.4
Serine	10.4
Proline	6.4
Glutamic acid	3.6
Glycine	10.6
Alanine	1.9
Cysteine*	33.1
Valine	1.7
Methionine+	1.7
Isoleucine	0.1
Leucine	0.3
Phenylalanine	0.1
Lysine	10.3
Histidine	0.1
Arginine	0.2
Tyrosine	0

* cysteine determined as cysteic acid

+ methionine determined as methionine sulfone

When subjected to ion-exchange chromatography, a major portion of the L.M.W. proteins was not retained, eluting with the bed volume of the column. A large portion of the ^{65}Zn and stable Zn^{2+} eluted with these proteins. A peak was not eluted at an ionic strength equivalent to that of the major DEAE peak observed for mucosal cytosols from Zn^{2+}-injected fish (expected position designated by [redacted], Fig. 35 B). Nor was such a peak evident in mucosal cytosol preparations of fish examined at other times of the year.

Section C. The incorporation of ^{35}S-cystine into the low molecular weight (L.M.W.)-Zn^{2+} binding proteins in the mucosal cytosol of the winter flounder.

The injection of Zn^{2+} (25% of the estimated total body Zn^{2+}) enhanced the incorporation of ^{35}S-cystine into the L.M.W. protein fraction in the intestinal cytosols applied to a Sephadex G-75 column (Fig. 36). The flounder which were injected with saline and ^{35}S-cystine did not exhibit a peak of radioactivity in this elution position. A broad peak of absorbance (230 nm) was observed in both treatments, but a peak of stable Zn^{2+} in the same elution position, was only observed in the Zn^{2+}-injected fish (Fig. 36).

It was not possible to purify the Zn^{2+}-binding protein from the ^{35}S-cystine injected flounder (i.e. not enough material) but the protein was isolated from a pooled sample obtained from fish injected with Zn^{2+} or saline according to the same protocol. The ^{65}Zn elution profiles (Sephadex G-75, DEAE-cellulose and Biogel P-30) of the pooled mucosal cytosols from the saline- and Zn^{2+}-injected flounder are shown in Fig. 37 A and B. In the Zn^{2+}-injected fish most of the ^{65}Zn added to the mucosal cytosol eluted in the position of the L.M.W. proteins (i.e. same position

Fig. 36. Fractionation of ^{35}S-cystine labelled proteins on Sephadex G-75 (2.5 X 90 cm). Mucosal cytosol (heat treated and fractionated with ammonium sulfate) obtained from Zn^{2+}-injected (-●-●-●-) and saline-injected (-o-o-o-) flounder. Fraction size collected = 4.0 mL.

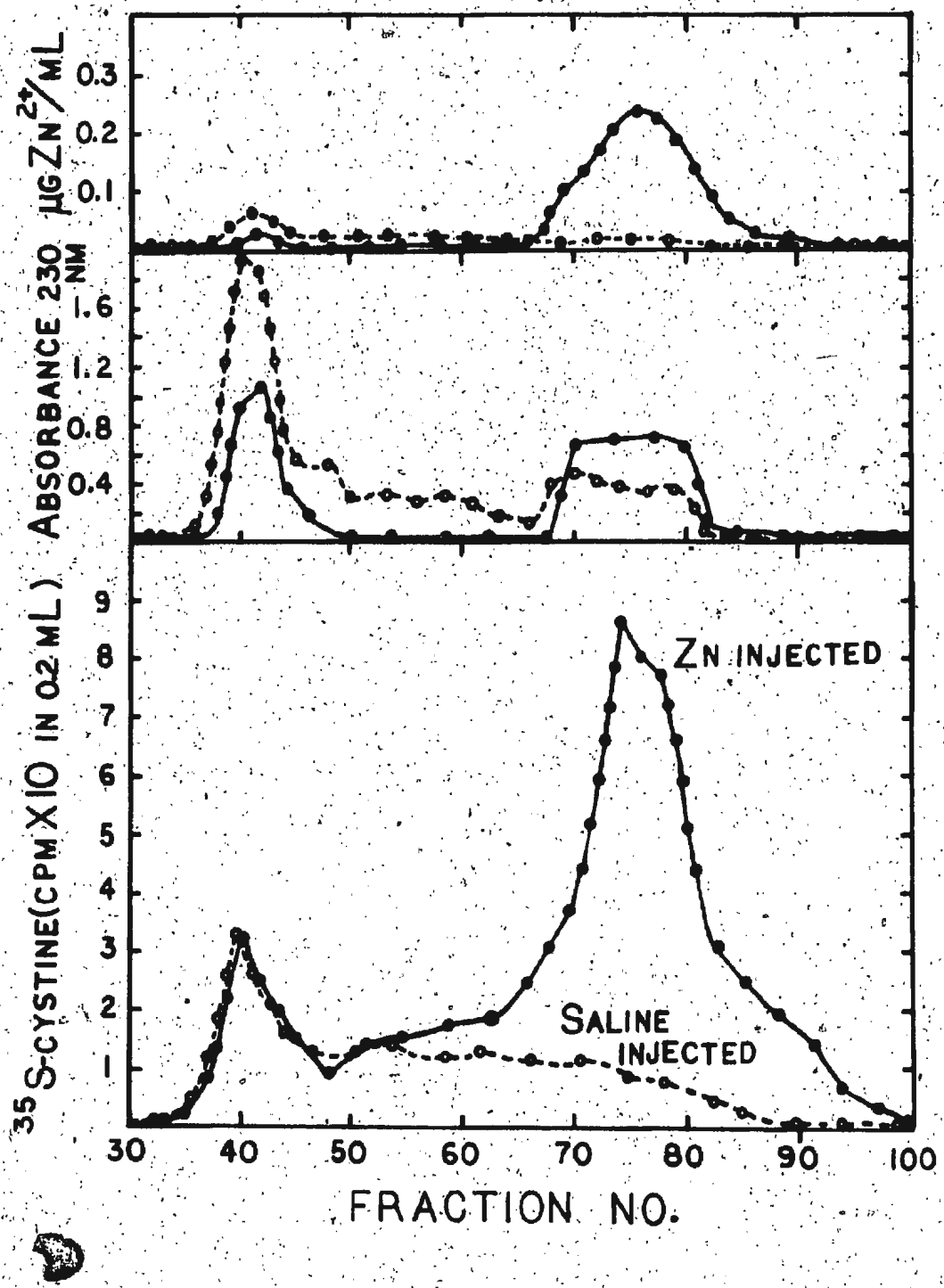
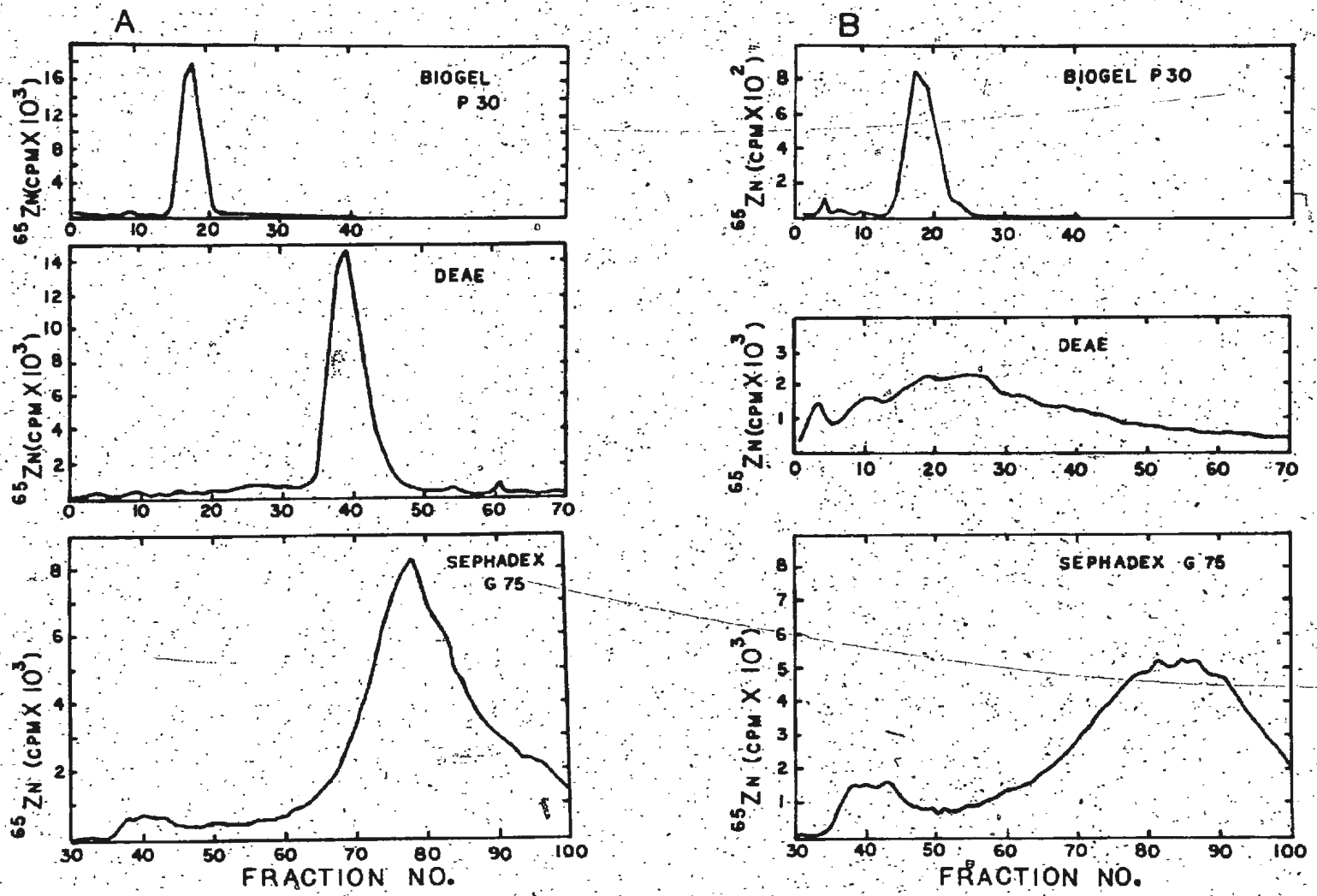

µG Zn2+/ML
0.3
0.2
0.1
ABSORBANCE 230 NM
1.6
1.2
0.8
0.4
35S-CYSTINE(CPM X 10 IN 0.2 ML)
9
8
7
6
5
4
3
2
1
ZN INJECTED
SALINE INJECTED
30
40
50
60
70
80
90
100
FRACTION NO.

Fig. 37. Separation of Zn^{2+}-binding proteins in pooled samples of mucosal scrape obtained from flounder injected according to the same protocol as that followed to examine ^{35}S-cystine incorporation (see Fig. 36).

A. Sephadex G-75 (2.5 X 90 cm), DEAE-cellulose (1 X 30 cm, 0.05 to 0.3 M Tris HCl, pH 8.6, total vol. = 400 mL), and Biogel P-30 (2 X 80 cm) elution profiles of mucosol cytosol obtained from Zn^{2+}-injected flounder.

B. .....from saline-injected flounder.


A
BIOGEL P 30
DEAE
SEPHADEX G 75
^{65}ZN (CPM X 10^3)
FRACTION NO.
B
BIOGEL P 30
DEAE
SEPHADEX G 75
^{65}ZN (CPM X 10^2)
^{65}ZN (CPM X 10^3)
FRACTION NO.

as ^{35}S-cystine). Following DEAE-cellulose and Biogel P-30 chromatography, this fraction was further examined using high pressure liquid chromatography. The Zn^{2+}-binding fraction eluted in a single peak (i.e. 99.9999% of the material eluting with absorbance at 230 nm) with an apparent molecular weight of 14-14,600 (Fig. 38).

Most of the ^{65}Zn added to the mucosal cytosol of the saline-injected flounder also eluted from the Sephadex G-75 column in the position corresponding to low molecular weight proteins (Fig. 37 B). The peak tended to be broader than that observed for Zn^{2+}-injected fish (Fig. 37 A). When this peak was applied to an ion-exchange column (DEAE-cellulose), a peak of radioactivity corresponding to the major peak in the Zn^{2+}-injected fish was not observed. However, those fractions which eluted at the "expected" position were pooled and applied to a Biogel P-30 column (Fig. 37 B).

The amino acid composition of a sample of the L.M.W. Zn^{2+}-binding protein isolated from the mucosal cytosols of both the Zn^{2+} and saline injected flounder was determined. The results are presented in Table 20. As observed for other mucosal preparations from Zn^{2+}-injected flounder, cysteine is the predominant residue in the L.M.W. protein isolated from the Zn^{2+}-injected fish. However, cysteine is only present in low amounts (2.3 vs 28%) in the protein similarly isolated from saline-injected flounder.

Fig. 38. Further separation of the L.M.W. Zn^{2+}-binding fraction from the mucosal cytosol (obtained following Sephadex G-75, DEAE-cellulose and Biogel P-30 chromatography) using high pressure liquid chromatography. TSK-125 BioRad gel filtration column eluted with 0.1 M Tris HCl, 0.1 M sodium sulfate buffer, pH 7.4. Flow rate 1.0 mL/min. Eluant monitored at Absorbance 230 nm. Arrows designate the elution times of proteins of known molecular weight: A = thyroglobulin, MW 690,000 (i.e. void volume); B = bovine serum albumin, MW 67,000; C = ovalbumin, MW 47,000; D = myoglobin, MW 17,500; E = ribonuclease A, MW 13,700.

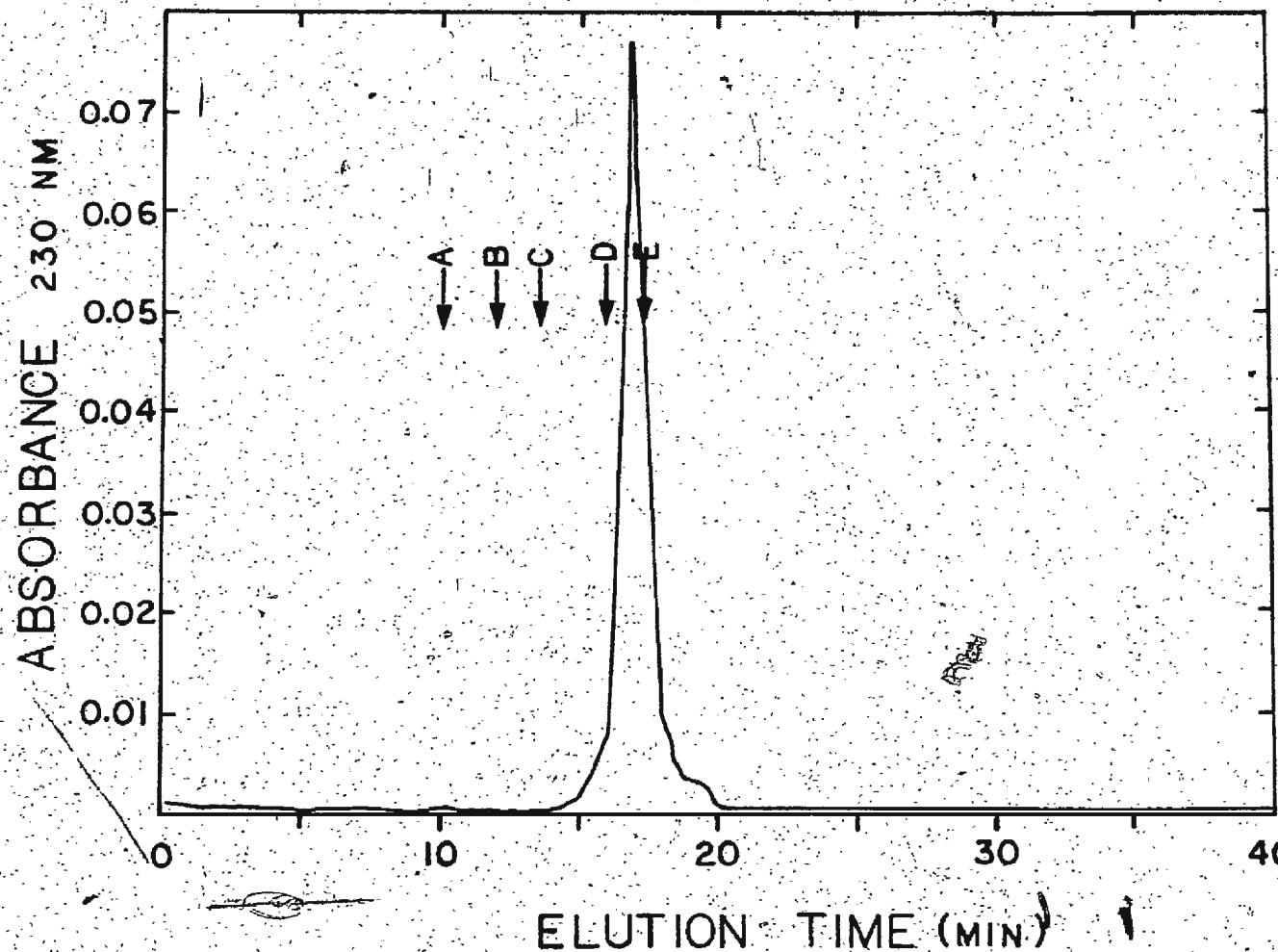

ABSORBANCE 230 NM
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
A
B
C
D
E
10
20
30
40
ELUTION TIME (MIN)

Table 20. Comparison of the amino acid composition of L.M.W. Zn^{2+}-binding protein isolated from the mucosal cytosols of flounder injected with a Zn^{2+} load (25% of estimated body load) or an equivalent volume of saline.

Amino acid	% total residues	
	Zn^{2+}-injected	Saline-injected
Aspartic acid	9.0	12.8
Threonine	11.0	5.2
Serine	8.6	8.3
Proline	5.9	6.5
Glutamic acid	5.3	14.0
Glycine	10.3	11.7
Alanine	3.3	10.6
Cysteine*	28.0	2.3
Valine	3.2	3.5
Methionine+	2.1	2.5
Isoleucine	2.8	2.6
Leucine	2.6	8.1
Tyrosine	0	0.3
Phenylalanine	0	3.0
Lysine	9.1	5.3
Histidine	0.6	1.7
Arginine	0.4	1.8

* determined as cysteic acid

+ determined as methionine sulfone

Section D. The relationship between low molecular weight (L.M.W.) Zn^{2+}-binding proteins in the mucosol cytosols of winter flounder and *in situ* uptake of Zn^{2+} from the upper intestine.

While there were no significant differences in the uptake of Zn^{2+} and ^{65}Zn from the ligated upper intestine of Zn^{2+}- or saline-injected flounder (see Chapter I, Section A, p. 49), there were differences in the chromatographic elution profiles of the Zn^{2+}-binding proteins isolated from these fish.

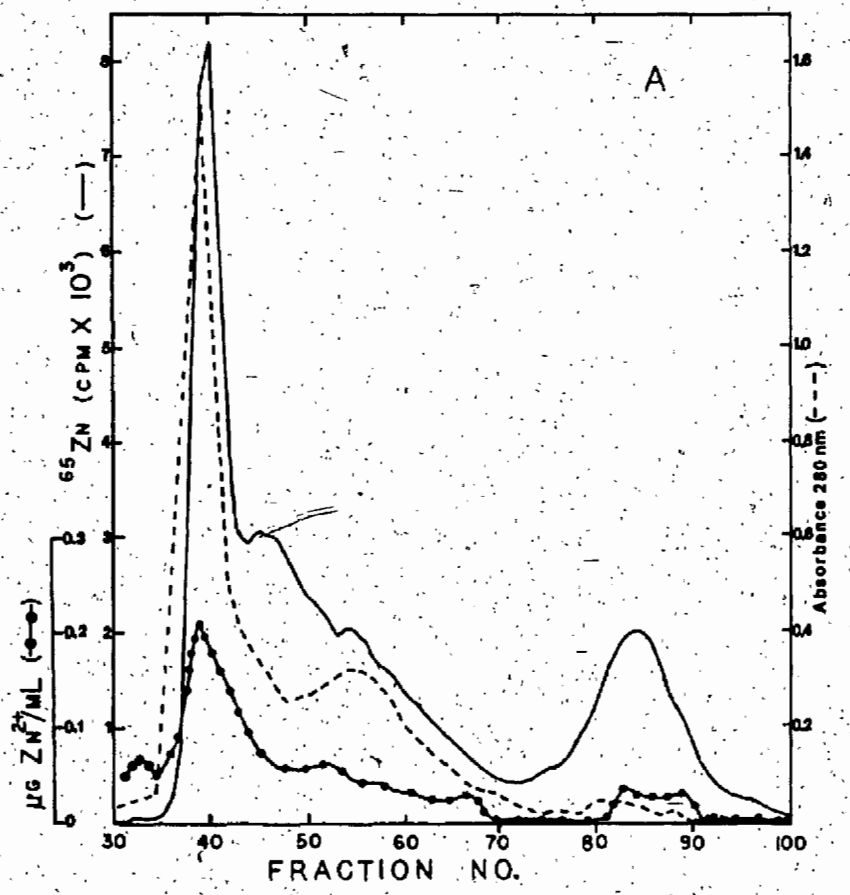
In the mucosal cytosol of Zn^{2+}-injected flounder, the major portion of ^{65}Zn and stable Zn^{2+} eluted in the position corresponding to low molecular weight proteins on Sephadex G-75. When this Zn^{2+}-binding fraction was applied to an ion-exchange column (DEAE-cellulose), the major portion of the ^{65}Zn and Zn^{2+} eluted in a position corresponding to that of proteins identified as metallothionein (by amino acid composition) in other experiments.

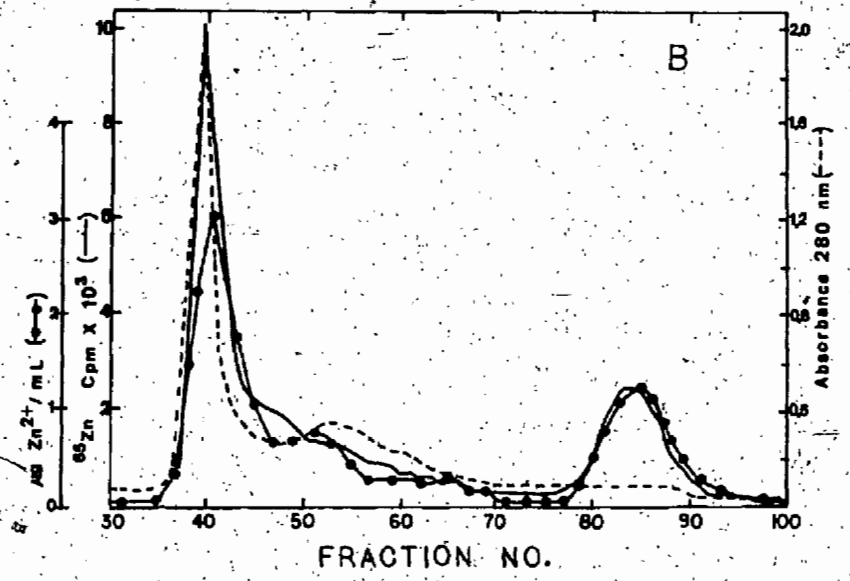
In the mucosal cytosol of saline-injected flounder, while a portion of the ^{65}Zn eluted with low molecular weight proteins, a large part was associated with proteins excluded by the Sephadex G-75 gel. When the L.M.W. fraction was applied to an ion-exchange column (DEAE-cellulose), a Zn^{2+}-binding fraction did not elute in a position equivalent to that observed for the Zn^{2+}-injected fish.

Section E. Chromatographic separation of Zn^{2+}-binding proteins in the liver cytosols of normal and Zn^{2+}-injected winter flounder.

Fig. 39 A demonstrates the elution profile obtained by chromatographing liver cytosol from normal winter flounder on Sephadex G-100. The ^{65}Zn eluted with protein fractions having molecular weights of ≥150,000, 40-45,000 and 10-15,000. Most of the protein (A 280 nm) eluted with the larger molecular weight fraction; very little absorbance at 280 nm was

Fig. 39. Separation of Zn^{2+}-binding proteins in the liver cytosol of normal (A) and Zn^{2+}-injected (B) winter flounder using Sephadex G-100 (column dimensions 2.5 X 90 cm). Fraction size collected = 4.0 mL.


A
^{65}Zn (CPM X 10^3) (—)
µG Zn^{2+}/ML (●—●)
Absorbance 280 nm (- - -)
FRACTION NO.


B
^{65}Zn Cpm X 10^3 (—)
µG Zn^{2+}/mL (●—●)
Absorbance 280 nm (- - -)
FRACTION NO.

associated with the low molecular weight (L.M.W.) fraction. The elution profile depicted is from a flounder examined in April but similar Zn^{2+}-binding protein fractions were observed in normal fish examined at other times of the year. The L.M.W. Zn^{2+}-binding fraction was present in the liver cytosol of fish examined from February to May; the L.M.W. Zn^{2+}-binding fraction was not detected in the intestinal cytosol of these fish.

Zn^{2+}-binding fractions, similar to those found in normal fish, were also observed when liver cytosols of Zn^{2+}-injected flounder were chromatographed on Sephadex G-100 (Fig. 39 B).

As determined by amino acid analysis, the L.M.W. Zn^{2+}-binding fraction eluting from a Sephadex G-100 column had a variable cysteine content (from 5 to 12% of total residues).

Section F. Isolation and purification of low molecular weight (LM.W.) Zn^{2+}- (and Cd^{2+})-binding proteins in the liver cytosol of winter flounder.

i) ...in the liver cytosol of Cd^{2+}-injected winter flounder.

A typical elution profile obtained by chromatographing liver cytosol obtained from Cd^{2+}-injected fish on Sephadex G-75 is illustrated in Fig. 40 A. ^{109}Cd eluted in two major peaks: with large molecular weight proteins excluded by the gel (i.e. 70,000) and with low molecular weight (L.M.W.) proteins (12-14,000 MW). When the liver cytosol was partially purified by heat treatment and ammonium sulfate fractionation before application to the Sephadex G-75 column, the major portion of the ^{109}Cd was associated with the L.M.W. proteins (Fig. 40 A).

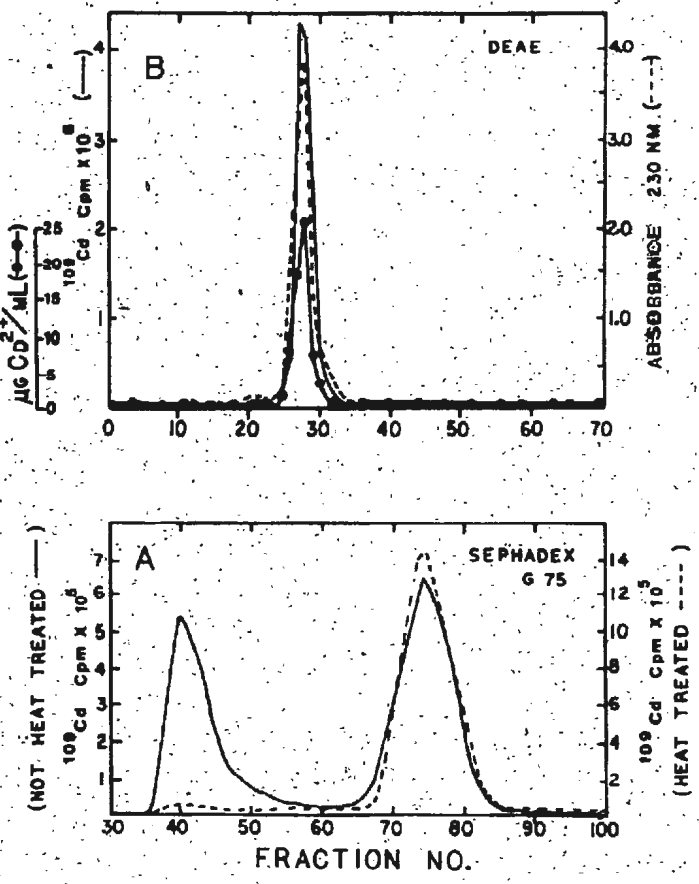
Regardless of which procedure was used to obtain the liver sample, when the L.M.W. proteins from the Sephadex G-75 column were applied to DEAE-cellulose and Biogel P-30 columns, the majority of the ^{109}Cd eluted

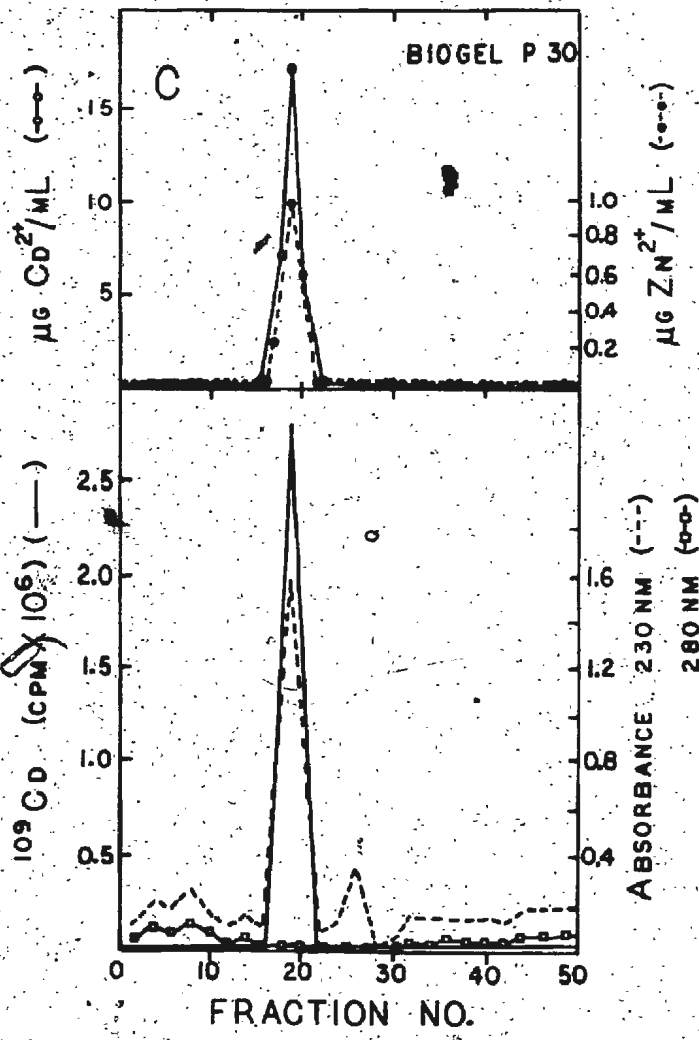
Fig. 40. Separation of metal-binding proteins in the liver cytosol of Cd^{2+}-injected flounder.

A. Separation of Cd^{2+}-binding proteins on Sephadex G-75 (2.5 X 90 cm) before (———) and after (- - —) heat treatment and ammonium sulfate fractionation of the liver cytosol. Fraction size collected = 4.0 mL.

B. Further separation of the L.M.W. Cd^{2+}-binding fraction (eluting from the Sephadex G-75 column) on DEAE-cellulose (1 X 15 cm) using a 0.05 to 0.3 M Tris HCl gradient, pH 8.6. Total vol. = 200 mL. Fraction size collected = 2.0 mL.

C. Separation of the major Cd^{2+}-binding fraction, eluting from the DEAE-cellulose column, on Biogel P-30 (2 X 80 cm). Fraction size collected = 3.8 mL.


B
DEAE
A
SEPHADEX
G 75
(NOT HEAT TREATED ——)
(HEAT TREATED ----)
FRACTION NO.
ABSORBANCE 230 NM (---)


C
BIOGEL P 30
FRACTION NO.
ABSORBANCE 230 NM (---)
280 NM (-o-o-)

in a single peak (Fig. 40 B and C). This peak was characterized by a high absorbance at 250 nm and zero absorbance at 280 nm; stable Cd^{2+} and Zn^{2+} eluted in the same position.

The amino acid composition of the L.M.W. Cd^{2+}-binding proteins isolated from the two batches of liver were similar (Table 21). Cysteine was the most prevalent amino acid (34.0 and 31.2% of the total residues).

ii)...in the liver cytosol of Zn^{2+}-injected winter flounder.

When liver cytosol (which had been heat treated and fractionated with ammonium sulfate) from Zn^{2+}-injected winter flounder was chromatographed on Sephadex G-75, the major portion of the added ^{65}Zn eluted in the position of low molecular weight (L.M.W.) proteins (12-14,000 MW) (Fig. 41 A). When this fraction was applied to ion-exchange chromatography, the major portion of ^{65}Zn and stable Zn^{2+} eluted with a protein fraction having a high absorbance at 230 nm and zero absorbance at 280 nm (Fig. 41 B). There was also a minor ^{65}Zn-binding fraction which eluted at a lower ionic strength than the major peak. Following elution on Biogel P-30 (Fig. 41 C), the amino acid composition of the major and minor DEAE-cellulose peaks was determined (Table 22). Cysteine was the most prevalent amino acid in the major peak (29.3% of total residues) but accounted for a much lower % of the total residues in the minor peak (9%).

When the major L.M.W. Zn^{2+}-binding protein (i.e. obtained following separation on DEAE-cellulose and Biogel P-30 columns) was examined using high pressure liquid chromatography, 99.9999% of the material (A 230 nm) eluted in a single peak with an apparent molecular weight of 14,500-14,600 (Fig. 42). The retention time on the HPLC column was similar to that observed for the L.M.W. Zn^{2+}-binding protein isolated from the intestinal cytosols of Zn^{2+}-injected flounder (Fig. 38).

Table 21. Amino acid composition of L.M.W. Cd^{2+}-binding protein isolated from liver cytosols of Cd^{2+}-injected winter flounder. Comparison of protein isolated from un-treated and heat-treated liver cytosol.

Amino acid	% Total Residues	
	heat-treated	not heat-treated
Aspartic acid	9.4	9.4
Threonine	12.6	13.1
Serine	9.8	10.0
Proline	6.9	6.8
Glutamic acid	4.3	3.7
Glycine	10.7	11.7
Alanine	2.5	1.8
Cysteine*	31.2	34.0
Valine	2.3	1.1
Methionine+	1.7	1.5
Isoleucine	0.4	0.1
Leucine	0.7	0.1
Tyrosine	0	0
Phenylalanine	0.2	0
Lysine	10.0	9.9
Histidine	0.2	trace
Arginine	0.3	0.1

* determined as cysteic acid

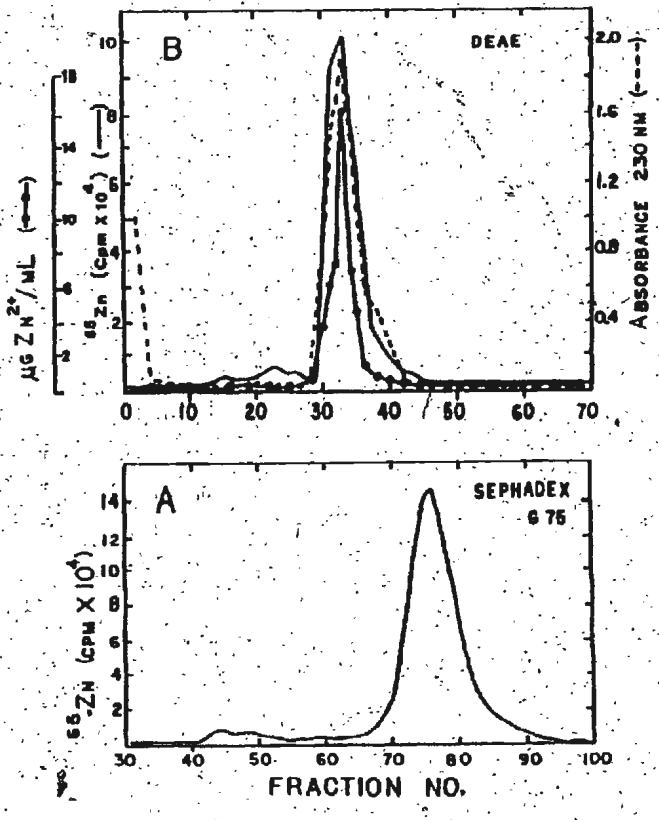
\+ determined as methionine sulfone

Fig. 41. Separation of Zn^{2+}-binding proteins in the liver cytosol of Zn^{2+}-injected flounder.

A. Separation of Zn^{2+}-binding proteins on Sephadex G-75 (2.5 X 90 cm) following heat treatment and ammonium sulfate fractionation of the liver cytosol. Fraction size collected = 4.0 mL.

B. Further separation of the L.M.W. Zn^{2+}-binding fraction (eluting from the Sephadex G-75 column) on DEAE-cellulose (1 X 30 cm) using a 0.05 to 0.3 M Tris HCl gradient, pH 8.6. Total vol. = 400 mL. Fraction size collected = 4.3 mL.

C. Separation of the major and minor Zn^{2+}-binding fractions, eluting from the DEAE-cellulose column, on Biogel P-30 (2 X 80 cm). Fraction size collected = 4.0 mL.


B
DEAE
A
SEPHADEX
G 75
FRACTION NO.

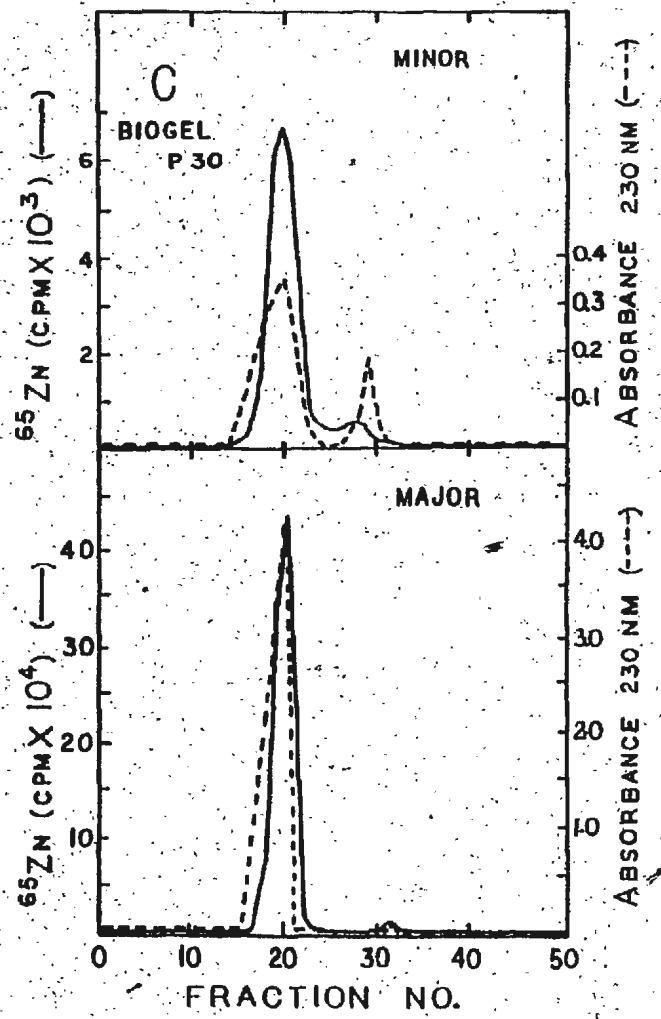

C
BIOGEL
P 30
MINOR
MAJOR
FRACTION NO.

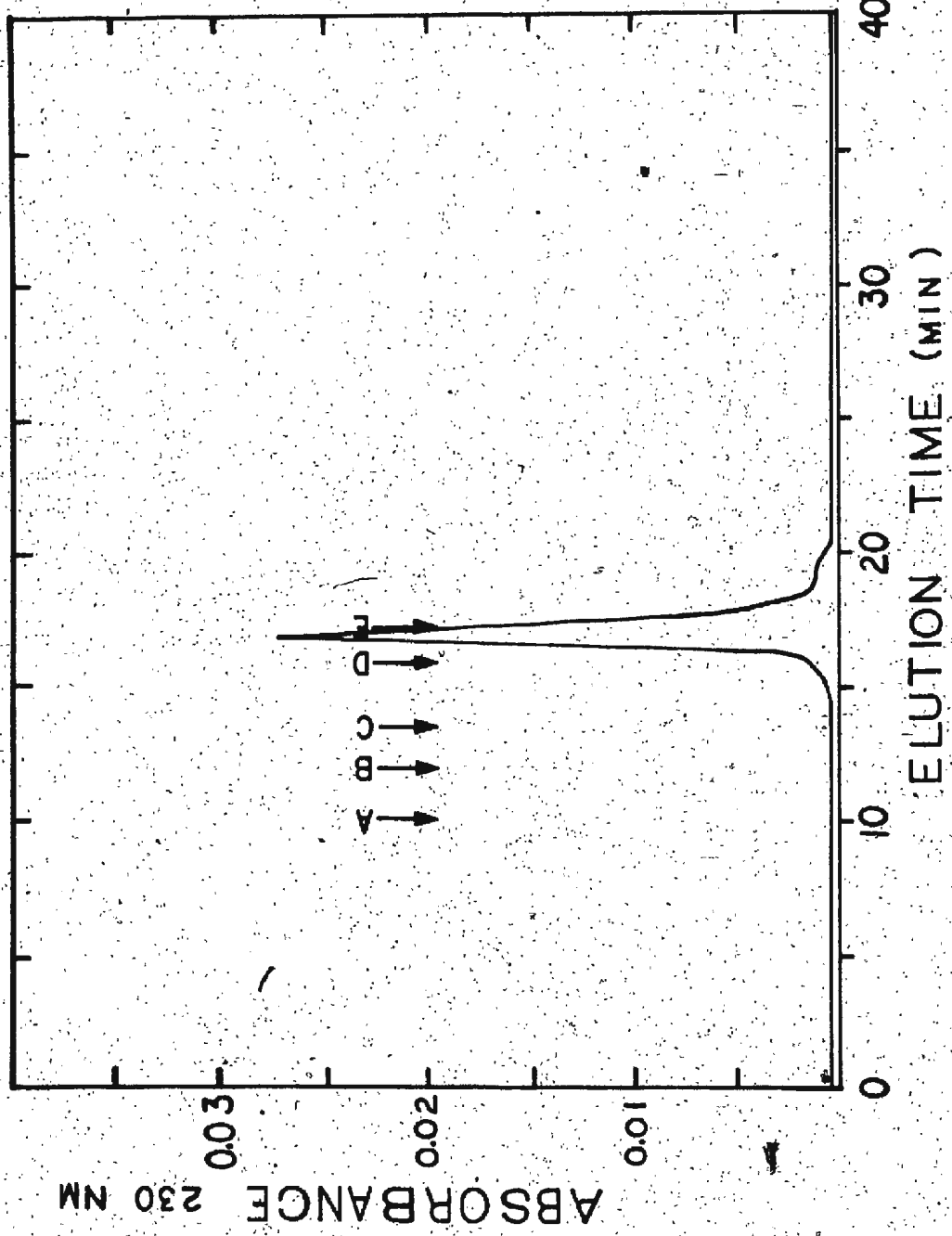
Table 22. Amino acid composition of L.M.W. Zn^{2+}-binding protein isolated from liver cytosol of Zn^{2+}-injected flounder. Comparison of the major and minor Zn^{2+}-binding fractions eluting from a DEAE-cellulose column (following application to a Biogel P-30 column).

Amino acid	% Total Residues	
	Major DEAE Fraction	Minor DEAE Fraction
Aspartic acid	10.4	9.8
Threonine	11.7	7.3
Serine	9.5	7.5
Proline	7.4	6.4
Glutamic acid	4.9	11.6
Glycine	10.1	9.4
Alanine	2.9	6.8
Cysteine*	29.3	9.2
Valine	2.3	6.3
Methionine+	1.7	2.4
Isoleucine	0.6	4.1
Leucine	1.0	5.7
Tyrosine	0	0
Phenylalanine	0.4	1.9
Lysine	9.6	8.4
Histidine	0.5	1.3
Arginine	0.8	2.9

* determined as cysteic acid

+ determined as methionine sulfone

Fig. 42. Further separation of the major L.M.W. Zn^{2+}-binding fraction from the liver cytosol (obtained following Sephadex G-75, DEAE-cellulose and Biogel P-30 chromatography) using high pressure liquid chromatography. TSK-125 BioRad gel filtration column eluted with 0.1 M Tris HCl, 0.1 M sodium sulfate buffer, pH 7.4. Flow rate = 1.0 mL per min. Eluant monitored at Absorbance 230 nm. Arrows designate the elution times of proteins of known molecular weight: A = thyroglobulin, MW 690,000 (i.e. void volume); B = bovine serum albumin, MW 67,000; C = ovalbumin, MW 47,000; D = myoglobin, MW 17,500; E = ribonuclease A, MW 13,700.


ABSORBANCE 230 NM
0.03
0.02
0.01
0
10
20
30
40
ELUTION TIME (MIN)
A
B
C
D
E

When the major L.M.W. Zn^{2+}-binding protein was examined using slab gel electrophoresis (10% acrylamide, with and without urea) only one band was apparent.

iii)...in the liver cytosol of normal winter flounder.

The typical Sephadex G-75, ion-exchange (DEAE-cellulose), and Biogel P-30 elution profiles of liver cytosol (heat treated and fractionated with ammonium sulfate) obtained from normal flounder are depicted in Fig. 43 A, B, and C. The elution characteristics are similar to those observed for liver cytosols of Zn^{2+}-injected fish (compare Fig. 42 and 43).

When the major ^{65}Zn-binding fraction from the Sephadex G-75 column was applied to a DEAE-cellulose column, two ^{65}Zn-peaks were apparent. Following further separation of the major peak on Biogel P-30, amino acid analysis revealed that while cysteine was one of the most prevalent residues (i.e. 13% of the total residues, see Table 23), it did not account for as high a % of the total as observed for the protein isolated from Cd^{2+}- or Zn^{2+}-injected flounder (compare Table 23 with 21 and 22). Cysteine accounted for a very low % of the total residues of the minor DEAE peak (2.1% of the total) (Table 23).

Section G. The incorporation of ^{35}S-cystine into the low molecular weight (L.M.W.) Zn^{2+}-binding proteins in the liver cytosol of winter flounder.

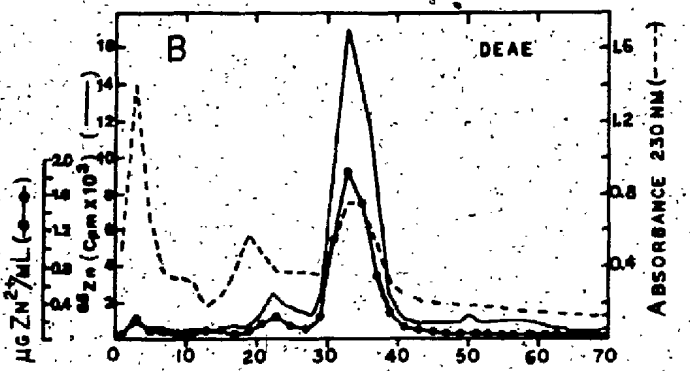
^{35}S-cystine was incorporated into the L.M.W. Zn^{2+}-binding fraction of liver cytosol obtained from Zn^{2+}-injected flounder (Fig. 44). In this instance, a peak of ^{35}S-cystine also eluted in a similar position when liver cytosol from saline-injected flounder was chromatographed. However, the level of radioactivity incorporated into the L.M.W. fraction was much lower than that observed in the Zn^{2+}-injected fish (Fig. 44).

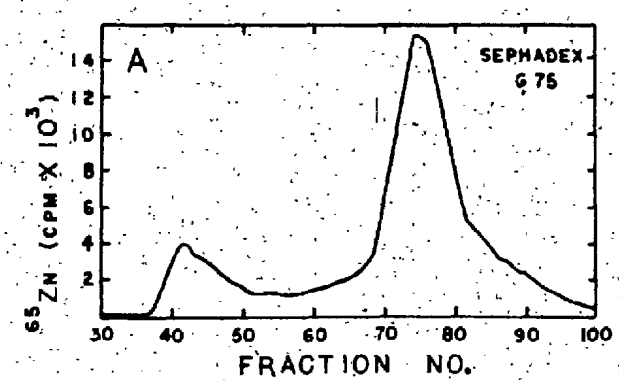
Fig. 43. Separation of Zn^{2+}-binding proteins in the liver cytosol of normal flounder.

A. Separation of Zn^{2+}-binding proteins on Sephadex G-75 (2.5 X 90 cm) following heat treatment and ammonium sulfate fractionation of the liver cytosol. Fraction size collected = 4.2 mL.

B. Further separation of the L.M.W. Zn^{2+}-binding fraction (eluting from the Sephadex G-75 column) on DEAE-cellulose (1 X 30 cm) using a 0.05 to 0.3 M Tris HCl gradient, pH 8.6 Total vol. = 400 mL. Fraction size collected = 4.0 mL.

C. Separation of the major and minor Zn^{2+}-binding fractions, eluting from the DEAE-cellulose column, on Biogel P-30 (2 X 80 cm). Fraction size collected = 4.0 mL.


B
DEAE
μG Zn²⁺/ML (●—●)
⁶⁵Zn (CPM X 10³) (——)
ABSORBANCE 230 NM (---)


A
SEPHADEX G 75
⁶⁵Zn (CPM X 10³)
FRACTION NO.

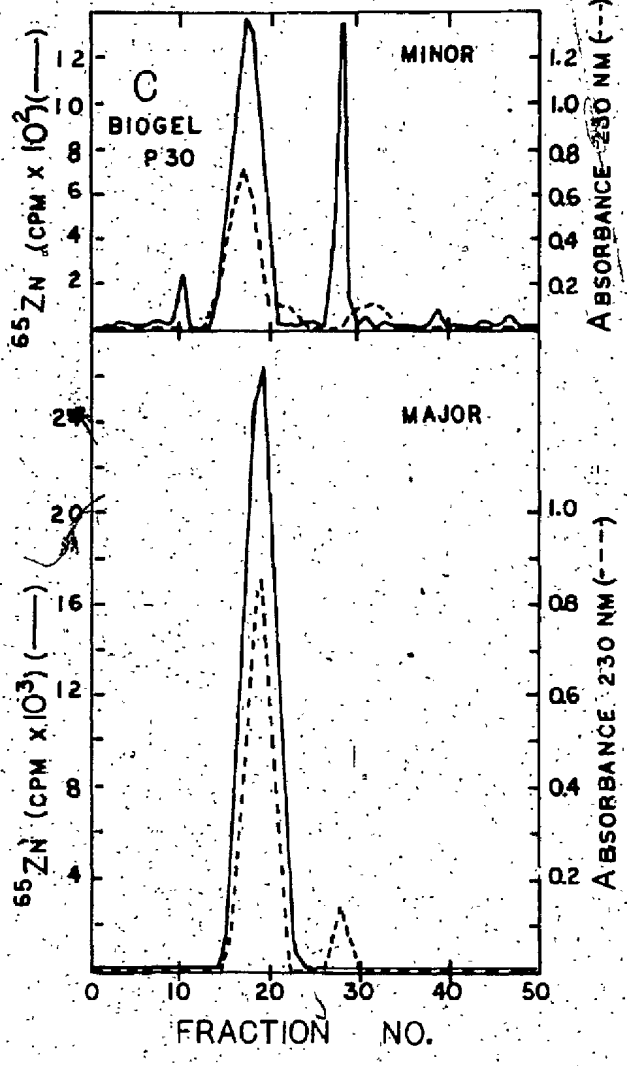
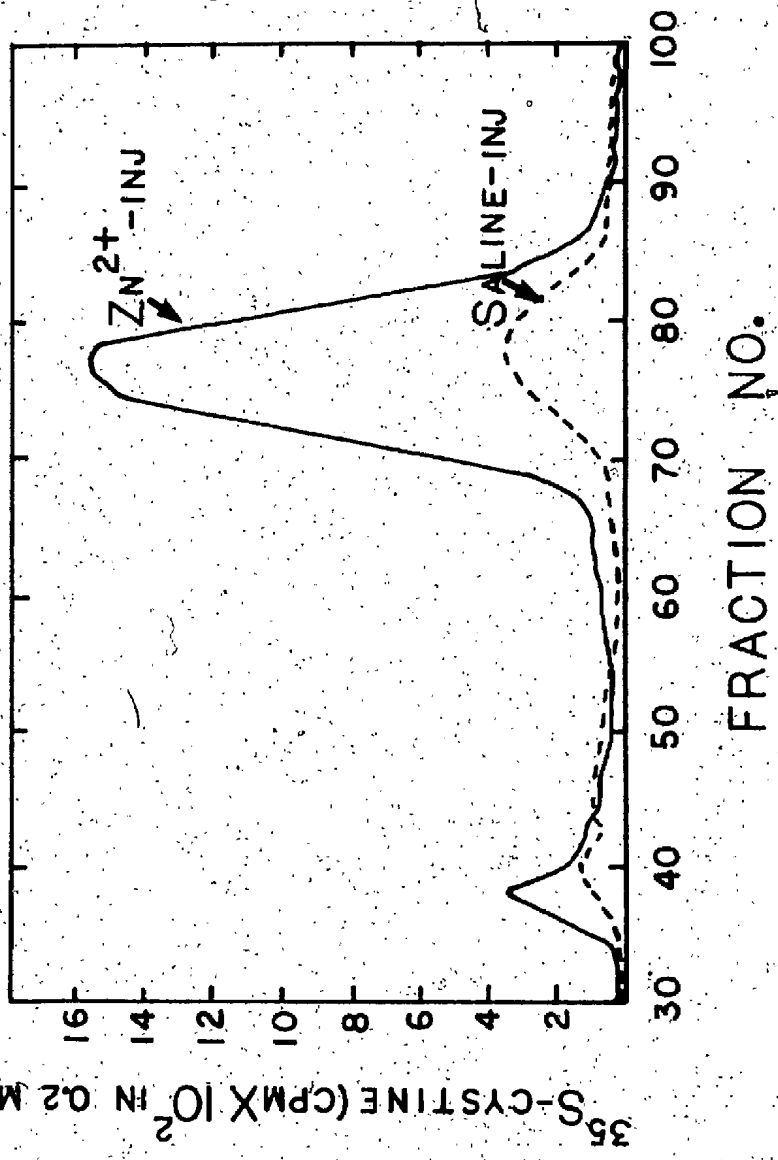

C
BIOGEL P 30
MINOR
MAJOR
⁶⁵Zn (CPM X 10²) (——)
⁶⁵Zn (CPM X 10³) (——)
ABSORBANCE 230 NM (---)
FRACTION NO.

Table 23. Amino acid composition of the L.M.W. Zn^{2+}-binding protein isolated from the liver cytosol of normal flounder. Comparison of the major and minor Zn^{2+}-binding fractions eluting from a DEAE-cellulose column (following application to a Biogel P-30 column).

Amino acid	% Total Residues	
	Major DEAE-Fraction	Minor DEAE-Fraction
Aspartic acid	10.9	11.9
Threonine	8.8	6.8
Serine	8.1	7.0
Proline	6.8	6.3
Glutamic acid	10.3	13.0
Glycine	9.3	9.4
Alanine	5.6	7.7
Cysteine*	13.0	2.1
Valine	5.4	7.4
Methionine+	2.1	2.3
Isoleucine	2.4	4.4
Leucine	4.3	6.6
Tyrosine	0.1	0.4
Phenylalanine	1.6	2.6
Lysine	8.5	8.4
Histidine	1.2	1.3
Arginine	2.9	3.5

Fig. 44. Fractionation of ^{35}S-cystine labelled proteins on Sephadex G-75 (2.5 X 90 cm). Liver cytosol (heat treated and fractionated with ammonium sulfate) obtained from Zn^{2+}-injected (———), and saline-injected (– – –) flounder. Fraction size collected = 4.0 mL.


^{35}S-CYSTINE (CPM X 10^2 IN 0.2 ML)
2
4
6
8
10
12
14
16
30
40
50
60
70
80
90
100
FRACTION NO.
Zn^{2+}-INJ
SALINE-INJ

Section H. Comparison of the chromatographic elution profiles of Zn^{2+}-binding proteins in kidney, liver, gill and intestine of normal and Zn^{2+}-injected winter flounder.

The elution profiles (from Sephadex G-75) of liver, kidney, gill and mucosal cytosols from a Zn^{2+}-injected flounder (examined 12 days following injection (i.p.) of 1 mg Zn^{2+}/100 g body weight) are shown in Fig. 45. In the mucosal and liver cytosols, ^{65}Zn eluted with a protein fraction having an estimated molecular weight of 10-12,000. A similar peak was present in the kidney cytosol but the peak was skewed indicating the presence, as well, of lower molecular weight proteins. The low molecular weight fraction was not detected in the gill cytosol. This fish was examined in March but had been maintained in heated seawater (8°C) and fed chopped capelin during what normally is a non-feeding period. The elution profiles obtained from a fish brought in from the field in April (had food in gut) are summarized in Fig. 46. A low molecular weight Zn^{2+}-binding fraction was detected in the liver but not in the gill or mucosal cytosols. A broad peak of radioactivity was present in a similar position in the kidney cytosol. However, the estimated molecular weight of the fraction containing the maximum cpm ^{65}Zn was lower in kidney than in the liver, i.e. 9,000 vs. 10-12,000.

Fig. 45. Comparison of the elution profiles (on Sephadex G-75, 2.5 X 90 cm) of Zn^{2+}-binding proteins in the kidney, liver, gill, and mucosal cytosol of Zn^{2+}-injected flounder. Fraction size collected = 4.0 mL.

Fig. 46. Comparison of the elution profiles (on Sephadex G-75, 2.5 X 90 cm) of Zn^{2+}-binding proteins in the kidney, liver, gill, and mucosal cytosol of normal winter flounder. Fraction size collected = 4.0 mL.

Fig 45

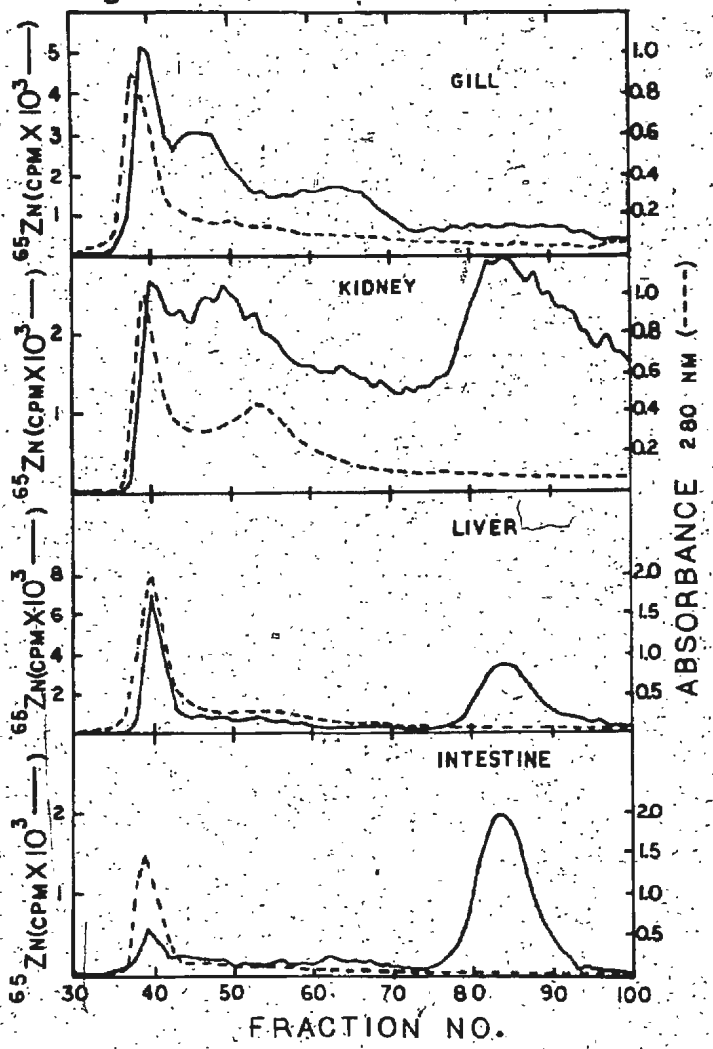
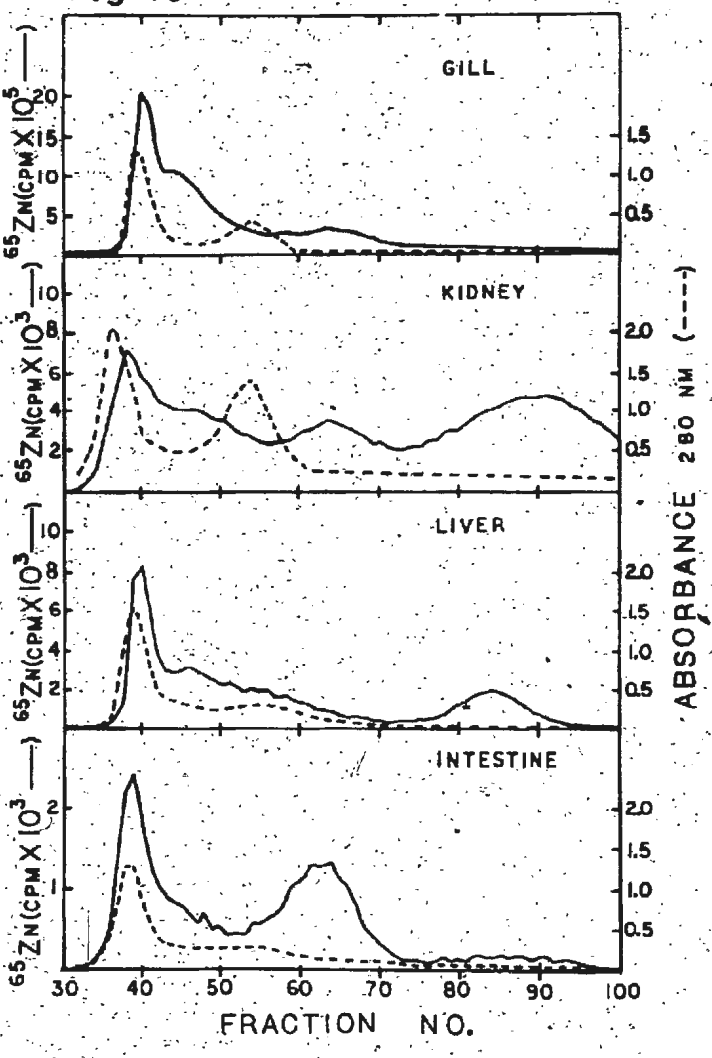

GILL
KIDNEY
LIVER
INTESTINE
^{65}ZN(CPM X 10^3 ——)
ABSORBANCE 280 NM (----)
FRACTION NO.

Fig 46


GILL
KIDNEY
LIVER
INTESTINE
^{65}ZN(CPM X 10^5 ——)
^{65}ZN(CPM X 10^3 ——)
ABSORBANCE 280 NM (----)
FRACTION NO.

DISCUSSION

Gel filtration (Sephadex G-100) of the mucosal cytosols of winter flounder indicated that ^{65}Zn (Zn^{2+}) was associated with proteins having estimated molecular weights of >150,000 (Fraction I), 80-150,000 (Fraction II), 30-40,000 (Fraction III) and 10-15,000 (Fraction IV). These ^{65}Zn-binding fractions were present in both normal flounder and in flounder which had been injected with a Zn^{2+} load. When intestinal cytosols were heat treated and fractionated with ammonium sulfate prior to application to a Sephadex G-75 column, the majority of the ^{65}Zn was associated with the low molecular weight (L.M.W.) fraction (10-15,000 M.W.). Zn^{2+}-binding proteins with elution characteristics similar to the L.M.W. fraction have been identified as metallothioneins in the tissues of a wide range of organisms (see Kägi and Nordberg 1979). Richards and Cousins (1977) isolated metallothionein from the intestinal cytosol of rats which had been injected intraperitoneally with a Zn^{2+} load. They found that the L.M.W. Zn^{2+}-binding fraction which eluted from a Sephadex G-75 column resolved into one minor and two major Zn^{2+}-binding fractions when applied to a DEAE-ion exchange column. The two major components had cysteine contents (30.5 and 28.4%) comparable to the metallothioneins isolated from liver and kidney and were designated metallothionein A and B. In contrast, only one major L.M.W. Zn^{2+}-binding fraction was isolated from the intestinal cytosol of Zn^{2+}-injected winter flounder using DEAE-ion exchange chromatography. The amino acid composition of this Zn^{2+}-binding fraction, following further purification on Biogel P-30, was quite similar to that of the metallothioneins isolated from the intestinal cytosols of the Zn^{2+}-injected rats (see Table 24). The high metal content, heat stability, lack of absorbance at 280 nm, high absorbance at 230 nm,

Table 24. Comparison of the amino acid composition of intestinal metallothionein isolated from winter flounder and from rat (Richards and Cousins 1977).

Amino acid	% Total Residues		
	Winter flounder	Rat	
		A	B
Aspartic acid	9.5	6.9	8.1
Threonine	13.4	5.4	4.0
Serine	10.4	12.3	10.6
Proline	6.4	5.0	6.8
Glutamic acid	3.6	4.8	7.5
Glycine	10.6	10.4	8.1
Alanine	1.9	5.5	9.5
Cysteine	33.1	30.5	28.4
Valine	1.7	5.4	3.6
Methionine	1.7	2.3	0.9
Isoleucine	0.1	2.7	2.4
Leucine	0.3	1.5	1.2
Phenylalanine	0.1	< 0.5	< 0.5
Lysine	10.3	6.3	8.1
Histidine	0.1	0	0
Arginine	0.2	< 0.5	< 0.5
Tyrosine	0	< 0.5	< 0.5

low molecular weight (10-15,000 based on gel filtration) and high cysteine content tend to confirm that the L.M.W. Zn^{2+}-binding fraction present in the intestinal cytosols of Zn^{2+}-injected winter flounder is metallothionein (see Kägi and Nordberg 1979).

Attempts to determine whether the L.M.W. Zn^{2+}-binding fraction isolated from the intestinal cytosols of normal winter flounder fits the criteria of metallothionein were less successful. The L.M.W. Zn^{2+}-binding fraction eluted from a Sephadex G-75 column had a relatively high cysteine content (28% of the total residues) but the protein could not be satisfactorily resolved on a DEAE-ion exchange column. When proteins (not a distinct peak) which eluted at an ionic strength similar to that observed for metallothionein in Zn^{2+}-injected flounder were subsequently chromatographed on a Biogel P-30 column, a Zn^{2+}-binding fraction eluted in the same position as metallothionein. However, the cysteine content of this protein was low (2.3% of the total residues). The discrepancies between the Zn^{2+}-binding proteins isolated from the Zn^{2+}-injected and normal winter flounder are difficult to explain. It may be related to the small amount of metallothionein present in normal fish. The amount of Zn^{2+} associated with the L.M.W. fractions (see Fig. 34 and 35) suggests that metallothionein is present in much greater concentrations in the intestinal cytosols of Zn^{2+}-injected, compared with control flounder. This is substantiated by the differences between the ^{35}S-cystine incorporation into metallothionein in saline- and Zn^{2+}-injected flounder. The incorporation of labelled cystine has been used as a measure of metallothionein synthesis in rats (Richards and Cousins 1975b). Using this criterion, the synthesis of metallothionein was induced in the intestines of flounder injected with Zn^{2+} but not in saline-injected fish. This agrees with observations made by Richards and Cousins (1975b) in rats. They concluded

that metallothionein was only present in very small amounts in the intestines of normal rats and, presumably due to the small amounts, did not report any attempt to purify it from the normal animals. However, a Cu^{2+}- and Zn^{2+}-binding protein, which had a high cysteine content and met other criteria for classification as a metallothionein, has been isolated and purified from the small intestine of 5-day old rats whose only source of metal was maternal milk (Johnson and Evans 1980).

Most speculation on the physiological function of metallothionein in the intestinal cells of mammals has been based on differences in the Sephadex G-75 chromatographic elution profiles of ^{65}Zn-binding proteins from normal animals and animals in which the Zn^{2+} status has been altered by feeding excess Zn^{2+} in the diets or by injection of Zn^{2+} loads. For example, Richards and Cousins (1975a,1976) observed that ^{65}Zn injected into the intestinal lumen of control rats was primarily associated with a large molecular weight protein fraction (MW >75,000) and a low molecular weight zinc-binding complex (MW <2000), later determined to be a degradation product (Cousins et al. 1978). In rats in which the Zn^{2+} status was elevated, the ^{65}Zn content of the cytosol was increased and this increase was associated with the metallothionein fraction. The amount of ^{65}Zn transferred into the body was inversely related to the amount of ^{65}Zn bound to the metallothionein fraction. Cousins (1979) hypothesized that metallothionein serves as an inducible ligand in the mammalian intestine which competes for available Zn^{2+} with the normal ligand involved in Zn^{2+} absorption, resulting in a reduction of the amount of Zn^{2+} transferred into the body when the Zn^{2+} status of the animal is elevated. However, metallothionein does not appear to serve this function in the intestines of the flounder. Based on the chromatographic elution profiles, metallothionein was present in the mucosal cytosols of Zn^{2+}-injected

flounder but Zn^{2+} absorption from the *in situ* ligated intestine was not significantly different in fish injected with a Zn^{2+} load or an equivalent volume of saline (see Chapter I).

In contrast to the theory advanced by Cousins (1979), Starcher et al. (1980) proposed that Zn^{2+} absorption was directly proportional to the intestinal metallothionein level. They also observed a decrease in the absorption of an oral dose of ^{65}Zn concommitant with an increase in the intestinal metallothionein content in mice injected with a Zn^{2+} load. However, they estimated that the injection of Zn^{2+} had diluted the ^{65}Zn pool in the intestinal tissue such that the amount of stable Zn^{2+} transferred into the body was actually greater than indicated by the % of ^{65}Zn. In support of their theory when the dosage of Zn^{2+} used for induction of metallothionein was low, they reported a 200-300% increase in ^{65}Zn absorption above the control level. However, it seems unlikely that metallothionein is playing a direct role in Zn^{2+} absorption in flounder. When examined on a seasonal basis the presence of the L.M.W. Zn^{2+}-binding fraction (eluting in the same position as metallothionein) in the mucosal cytosols of flounder was not in phase with the *in situ* absorption of Zn^{2+} or with the feeding period of the flounder (see Fig. 47). The fraction was still detected in flounder after the Zn^{2+} absorption had started to decline and the fish had stopped feeding.

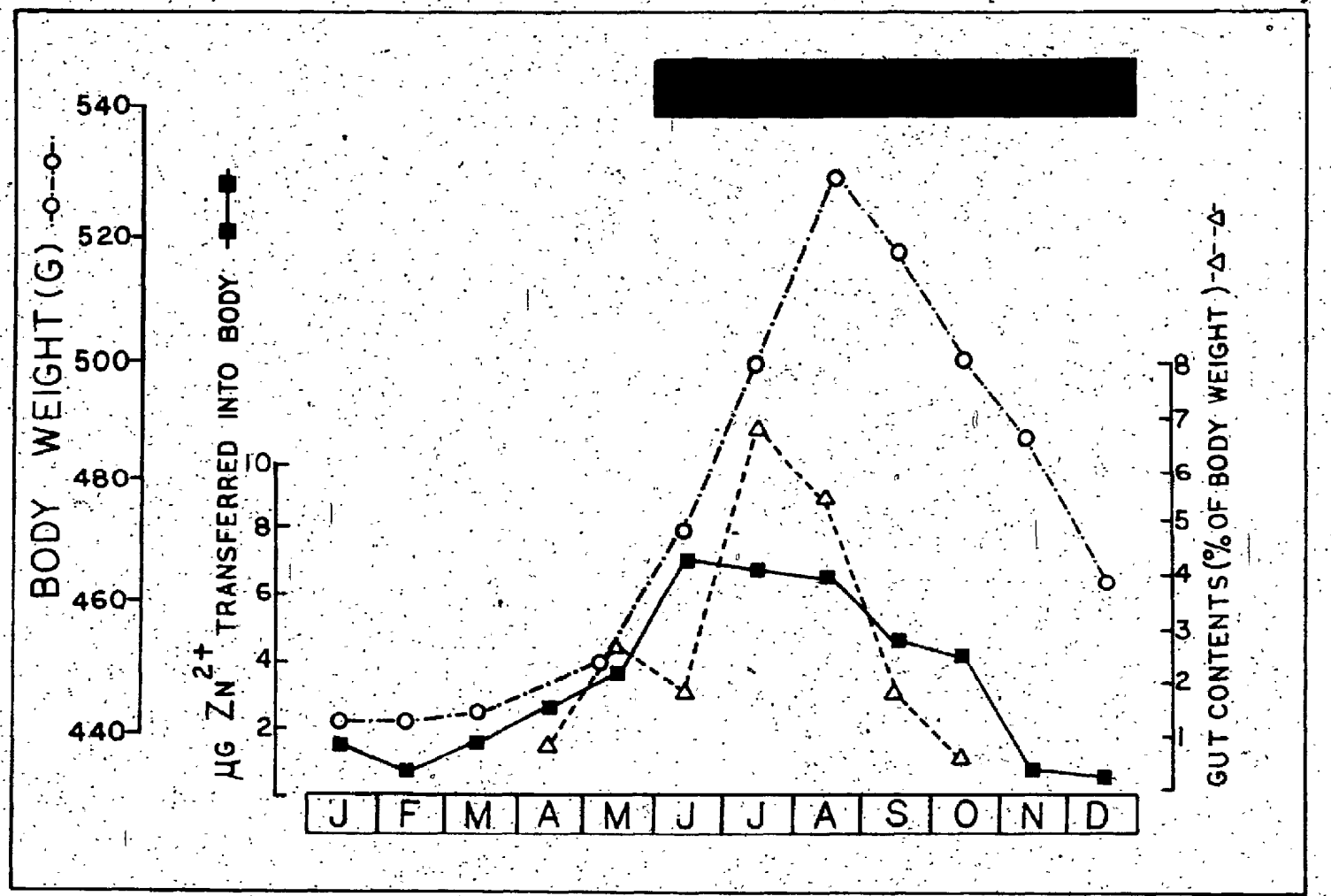
It has also been suggested that metallothionein could be playing a role in Zn^{2+} excretion. Smith et al. (1978 a) observed that ^{65}Zn presented to the perfused intestines of rats via the vascular perfusate rather than the luminal perfusate also accumulated in the mucosal cells. When the mucosal cytosols were chromatographed more ^{65}Zn was associated with metallothionein in Zn^{2+}-injected rats than in normal animals. Whether the ^{65}Zn was actually bound to the metallothionein in the intact cell is a

difficult question to resolve. The ^{65}Zn could have been redistributed during the homogenization and centrifugation steps required to obtain the mucosal cytosol. One of the routes of Zn^{2+} elimination from the winter flounder does appear to be the gastrointestinal tract (see Chapter II) but the involvement of metallothionein was not assessed.

It is possible that metallothionein in the intestines of the flounder is not involved in either absorption or excretion *per se* but is synthesized in response to a change in distribution of Zn^{2+} in the body and serves a temporary storage function. Several factors other than Zn^{2+} injections of high Zn^{2+} diets have been found to result in an elevation of the hepatic metallothionein level in mammals, i.e. restriction of food intake (Bremner and Davies 1975) and other stresses such as cold environment and strenuous excercise (Oh et al. 1978). Oh et al. (1978) proposed that one possible explanation for the observed increase in metallothionein levels could be that catabolism of proteins (including ones with Zn^{2+} bound to them) occurs during these stress conditions, resulting in the release of Zn^{2+}, which, in turn, stimulates metallothionein synthesis. The winter flounder undergoes seasonal changes in body weight (see Fig. 47); during the fish's non-feeding period muscle tissue is metabolized and used as an energy reserve. Zn^{2+} released from muscle proteins during its metabolism is redistributed within the body (see Chapter II) and possibly results in the synthesis of a low level of metallothionein in the intestinal tissue.

When the liver cytosols of the flounder were chromatographed (Sephadex G-100), ^{65}Zn (and stable Zn^{2+}) was associated with proteins having estimated molecular weights of >150,000 (Fraction I), 40-50,000 (Fraction II) and 10-15,000 (Fraction III). These fractions were present in both Zn^{2+}-injected and normal flounder. ^{109}Cd added to the liver cytosols was associated primarily with proteins eluting in the void volume of the

Fig. 47. Seasonal changes in gut content weight (see Fig 10, p. 44), body weight (gonadectomized body weight of a 35 cm female flounder, Fletcher and King, personal communication), amount of Zn^{2+} transferred into the body from the upper intestine (see Fig. 10, p. 44) and the appearance of the L.M.W. Zn^{2+} binding fraction (designated by ■) in the mucosal cytosol.


BODY WEIGHT (G)
540
520
500
480
460
440
μG Zn2+ TRANSFERRED INTO BODY
10
8
6
4
2
GUT CONTENTS (% OF BODY WEIGHT)
8
7
6
5
4
3
2
1
J
F
M
A
M
J
J
A
S
O
N
D

column (i.e. >75,000 on a Sephadex G-75) and with a low molecular weight fraction (12-14,000). The ^{65}Zn-binding profile was similar to that reported for rat liver cytosols (Bremner and Davis 1975). In rats, the Zn^{2+}-binding fraction with a molecular weight similar to Fraction II, was found to have both superoxide dismutase and carbonic anhydrase activity. Fraction III from the flounder had elution characteristics similar to rat liver metallothionein (i.e. bound ^{65}Zn and ^{109}Cd, low absorbance at 280 nm, low molecular weight). Following ion exchange chromatography (DEAE-cellulose) and gel filtration on a Biogel P-30 column, the L.M.W. metal-binding protein isolated from the liver cytosols of Zn^{2+}- and Cd^{2+}-injected winter flounder had an amino acid composition similar to that of metallothionein isolated from the intestinal cytosol. The composition was also similar to that of metallothionein isolated from the livers of Cd^{2+}-exposed eels (*Anguilla anguilla*) (Noël Lambot et al. 1978), Cd^{2+}-injected plaice (*Pleuronectes platessa*) (Overnell and Coombs 1979), Cu^{2+}-exposed coho salmon (*Oncorhynchus kisutch*) (McCarter et al. 1982), Cd^{2+}-injected carp (*Cyprinus carpio*) (Kito et al. 1982 c) and from mammalian liver (Kägi and Nordberg 1979) (see Table 25 for comparison). The molecular weight, as estimated on a calibrated HPLC column, was 14,500-14,600, the same order as that estimated for the flounder intestinal metallothionein (i.e. 14-14,600). This is somewhat larger than gel filtration estimates of the molecular weight of mammalian metallothionein (i.e. 10-13,000) but similar to the gel filtration estimate of plaice liver metallothionein (13-15,000) (Overnell and Coombs 1979). Estimates of the molecular weight of metallothionein, based on gel filtration, are considerably larger than those determined from the amino acid composition and amino acid sequence data (i.e. MW 6-7000); the discrepancy has been attributed to the non-globular shape of the protein (Kägi and Nordberg 1979).

Table 25. Comparison of the amino acid composition of metallothionein isolated from the winter flounder with that isolated from several other species of fish and from mammals.

Amino acid	Residues per molecule													
	Flounder liver[a] (Cd^{2+}-inj.)	Flounder liver[a] (Zn^{2+}-inj.)	Flounder intestine[a] (Zn^{2+}-inj.)	Eel liver[b] (Cd^{2+}-inj.)		Plaice liver[c] (Cd^{2+}-inj.)	Coho salmon liver[d] (Cu^{2+}-exposed)	Carp liver[e] (Cd^{2+}-inj.)		Horse liver[f]		Mouse liver[g]	
				MT_1	MT_2		MT_1	MT_1	MT_2	MT_{1A}	MT_{1B}	MT_1	MT_2
Asx	6	6	6	8.3	9.4	6	5	5	6	3	3	4	4
Thr	8	7	8	8.8	8.4	6	3	4	5	3	1	5	1
Ser	6	6	6	8.9	8.1	6	5	7	6	8	8	9	10
Pro	4	4	4	3.9	5.3	4	3	3	3	3	2	2	2
Glx	3	3	2	6.1	7.7	3	2	2	2	2	3	1	3
Gly	7	6	6	9.6	9.3	7	5	6	7	7	5	5	4
Ala	1	2	1	6.3	6.3	2	-	3	3	5	7	5	6
1/2 Cys	20	17	19	24.4	20.0	19	21	20	20	20	20	20	20
Val	1	1	1	4.2	4.8	1	-	1	1	1	3	2	1
Met	1	1	1	1.6	1.6	2	1	1	1	1	1	1	1
Ile	-	-	-	1.3	1.9	-	-	-	1	-	-	-	1
Leu	-	1	-	2.2	3.2	1	-	-	-	-	-	-	-
Tyr	-	-	-	-	-	-	1	-	-	-	-	-	-
Phe	-	-	-	1.1	1.3	-	-	-	-	-	-	-	-
Lys	6	6	6	10.5	9.6	6	4	7	6	6	7	7	8
His	-	-	-	0.4	0.8	-	-	-	-	-	-	-	-
Arg	-	-	-	0.8	0.7	-	-	-	-	2	1	-	-

[a] Calculated to the nearest integer on the basis of 1 residue methionine / molecule.
[b] Preliminary amino acid composition; number of residues per 10,000 mol. wt. (Noel-Lambot et al. 1978).
[c] Calculated as nearest whole unit number of residues based on 19 half-cystine residues per molecule (Overnell and Coombs 1979).
[d] Expressed to the nearest integer (McCarter et al. 1982).
[e] Expressed to the nearest integer (Kito et al. 1982 a).
[f] Number of amino acid residues per molecule as determined by sequence analysis (Kojima et al. 1979).
[g] Number of amino acid residues per molecule as determined by sequence analysis (Huang et al. 1979).

Consistent with the findings of Overnell and Coombs (1979) on livers of Cd^{2+}-injected plaice, only one form of metallothionein was isolated (following DEAE-ion exchange chromatography) in the liver cytosol of Cd^{2+}-injected flounder. Two Zn^{2+}-binding fractions, a major and a minor one, were isolated from livers of Zn^{2+}-injected flounder but only the major fraction had an amino acid composition consistent with that of metallothionein. This contrasts with mammalian tissues where, based on charge properties, at least two forms of metallothionein, having quite similar amino acid compositions, have been isolated (Kägi and Nordberg 1979). In addition at least two forms of metallothionein appear to be present in the livers of Cd^{2+}-exposed eels (Noël Lambot et al. 1978), Zn^{2+}-injected rainbow trout (Pierson 1980), Cu^{2+}-exposed coho salmon (McCarter et al. 1982) and Cd^{2+}-injected carp (Kito et al. 1982 a). Different amounts of the metallothionein variants have been detected in different species of mammals and in different tissues (Whanger et al. 1981 b). It is possible that a second variant of metallothionein is present in the liver and intestinal cytosols of the winter flounder but occurs in such low amounts that it was not resolved by the methodology employed.

A Zn^{2+}-binding fraction, with elution characteristics similar to the metallothionein isolated from the livers of Zn^{2+}-injected flounder, was detected in the liver of normal fish. However, the cysteine content of the fraction was much lower than that of the metallothionein isolated from the Zn^{2+}-injected flounder (i.e. 13 versus 30% of the total residues). As discussed for the intestinal tissue, the difficulty encountered in isolating metallothionein from the livers of non-Zn^{2+}- (Cd^{2+})-injected

flounder may be related to the small amount of metallothionein present in the normal animals. Based on the incorporation of radiolabelled cystine, the rate of hepatic metallothionein synthesis is normally very low in the flounder. In agreement with this, Overnell and Coombs (1979) observed a peak of ^{35}S-cystine incorporation, coinciding with metallothionein, in the livers of Cd^{2+}-injected plaice but did not detect any incorporation into this fraction in control fish. Similarily, appreciable rates of hepatic metallothionein synthesis (based on the incorporation of radiolabelled cystine) were only apparent in adult rats when the Zn^{2+} status was elevated by Zn^{2+}-injections (Richards and Cousins 1975 b) or altered by various stresses (Oh et al. 1978).

A heavy metal detoxification function has often been suggested as the primary role of hepatic metallothionein (Webb 1979). However, the presence of Zn^{2+} in the metallothionein induced by Cd^{2+}-injections has also led to the proposal that the ability of thioneins to "detoxify" heavy metals may only be a fortuitous consequence of the physiochemical similarity of these ions and zinc (Winge et al. 1978). In keeping with this, metallothionein isolated from livers of Cd^{2+}-injected winter flounder in the present study, also contained appreciable amounts of Zn^{2+}.

Unlike the intestinal tissue of the flounder, Zn^{2+} in the liver was associated with a L.M.W. protein fraction, eluting in the same position as metallothionein, in fish examined throughout the year. If metallothionein has a function in the normal metabolism of Zn^{2+} in the flounder liver, it might be expected that the protein would be present all year round since the turnover of Zn^{2+} in the liver (based on the changes in the concentration of ^{65}Zn in the liver following a single i.m. injection, see Chapter II) was similar in the feeding and non-feeding periods. While it is generally

accepted that hepatic metallothionein is involved in the homeostasis of Zn^{2+} in mammals, no single physiological function has yet been assigned to the protein (Brady 1982; Webb and Cain 1982). Richards and Cousins (1975 a,b) proposed that metallothionein could function in the uptake of Zn^{2+} into the liver cells. They observed that when synthesis of metallothionein in the livers of Zn^{2+}-injected rats was blocked by administration of actinomycin D, the serum levels of Zn^{2+} remained high and Zn^{2+} was not taken up into the liver until the effect of the protein synthesis inhibitor had worn off. In addition, in certain species of mammals it has been suggested that metallothionein could be serving as a Zn^{2+} storage protein (Whanger et al. 1981 a).

Preliminary investigations, based only on the elution of $^{65}Zn(Zn)$-binding proteins from Sephadex columns, indicated that metallothionein is also present in the kidney cytosols of normal and Zn^{2+}-injected winter flounder. However, a metallothionein-like fraction was not apparent in the cytosols of gill tissue from these same flounder. Based on the information reported for other fish, it appears that the level of metallothionein in different tissues varies with the species of fish and the metal to which it is exposed. Pierson (1980) reported that injections of Zn^{2+} stimulated metallothionein synthesis in the liver but not in the gill or kidney of rainbow trout (*Salmo gairdneri*). Injections of Zn^{2+} resulted in the appearance of a metallothionein-like fraction in both the kidney and liver of goldfish (*Carassius auratus*). However, much less ^{65}Zn was associated with this fraction in the kidney than in the liver (i.e. 1.9% of total ^{65}Zn in the kidney versus 39.5% in the liver) (Marafante 1976). Bouquegneau et al. (1975) reported that exposure of eels (*Anguilla anguilla*) to inorganic mercury resulted in the appearance of a protein

fraction with elution characteristics similar to metallothionein in the liver, gills and kidney. Mercury was also associated with a metallothionein-like protein in the gills and kidney of rainbow trout exposed to methyl mercury, but the amounts were much lower than detected in the liver (Olson et al. 1978). Noël Lambot et al. (1978) found that Cd^{2+} accumulated with a metallothionein fraction in both gill and liver of eels after chronic exposure to Cd^{2+}. The fraction was also present in the liver of non-exposed eels but could not be detected in the gills. The level of metallothionein in mammalian tissues also varies with the metal administered. In rats fed high-Zn^{2+} or high-Cd^{2+} diets, Cd^{2+} accumulated to a greater extent in kidney than in liver metallothionein; the opposite was observed for Zn^{2+} (Oh et al. 1978). Similarily, Kägi et al. (1974) found that metallothioneins isolated from non-exposed equine and human liver contained predominantly Zn^{2+} whereas the metallothionein isolated from kidney contained more Cd^{2+}. Durnam and Palmiter (1981) observed that the rate of transcription of metallothionein and the metallothionein mRNA levels were increased in both kidney and liver of mice injected with Cd^{2+}, Zn^{2+}, Cu^{2+} or Hg^{2+}. However, Cd^{2+} and Zn^{2+} resulted in the greatest amount of induction in the liver; Cd^{2+} and Hg^{2+} were the best inducers of metallothionein in the kidney. Cd^{2+} induced metallothionein synthesis in 7 of the 8 tissues which were examined (i.e. in liver, kidney, intestine, heart, muscle, brain and spleen). However, the tissues did not respond identically to the Cd^{2+} which they absorbed. It took ten times more Cd^{2+} in the intestine than in the muscle to induce equivalent amounts of metallothionein mRNA. Therefore, the apparent differences in the metallothionein levels in the tissues of the winter flounder and other species of fish examined is possibly related to the amount of metal to which the fish

was exposed. If a more sensitive assay procedure was used, it may be found, as observed in mammals, that metallothionein is present or can be induced in most tissues of the body.

In conclusion, in agreement with observations made in mammals, both the intestine and the liver of the flounder respond to injections of a load of stable Zn^{2+} by synthesizing metallothionein. The question which arises is what role, if any, does metallothionein play in the normal metabolism of Zn^{2+} in the flounder. That it could play a role in the homeostasis of Zn^{2+} in fish, as hypothesized for mammals, is suggested by the presence (albeit in low amounts) of a L.M.W. Zn^{2+}-binding protein fraction, with elution characteristics similar to metallothionein, in the tissues of non-Zn^{2+}-(Cd^{2+})-injected flounder. However, the difficulties encountered in characterizing the metallothionein-like fraction from the liver and intestinal tissue of "normal" flounder point to the need for a specific assay (such as the radioimmune assay developed for mammals by Vander Mallie and Garvie 1978, 1979) to quantitate the levels of metallothionein in the tissues. Development of such an assay would seem necessary before any possible relationship between the level of metallothionein in the tissues and the normal metabolism of Zn^{2+} can be delineated.

SUMMARY AND CONCLUSIONS

Chapter I

1. Results from *in situ* studies on Zn^{2+} uptake indicate that the entire digestive tract of the winter flounder is capable of absorbing Zn^{2+}, with the uppermost portion of the intestine having the highest and the stomach the lowest capacity.

2. Feeding studies with the non-absorbed marker ^{141}Ce, suggest little Zn^{2+} absorption in the stomach, a net secretion of Zn^{2+} into the uppermost portion of the intestine and net absorption of Zn^{2+} along the rest of the tract. However, the apparent secretion of Zn^{2+} into the upper intestine may be an artifact of the method.

3. Based on the *in situ* findings, Zn^{2+} absorption appears to involve at least two steps, the first a rapid accumulation of Zn^{2+} by the intestinal tissue and the second, a slower transfer of Zn^{2+} into the body.

4. When different loads of Zn^{2+} were instilled in the lumen, the proportion of Zn^{2+} accumulated in the intestinal tissue was greater from lower than from higher doses, implying that the first step in Zn^{2+} absorption involves binding to specific sites on or within the intestinal cells.

5. The amount of Zn^{2+} transferred into the body appeared to be directly proportional to the amount of Zn^{2+} accumulated in the intestinal tissue, suggesting that the transfer step from the mucosal tissue into the blood could be a passive process.

6. When an equilibrium dialysis technique was used to examine the affinity of the mucosal cytosol proteins for Zn^{2+}, more than one binding system was apparent. The highest affinity binding system

($K=2.42 \times 10^7$) appeared to be undersaturated with Zn^{2+}. The affinity of this system for Zn^{2+} was lower than that reported for the high affinity Zn^{2+}-binding system in the plasma, which (if the system is involved in Zn^{2+} absorption) would facilitate the transfer of Zn^{2+} from the mucosal cells to the blood.

7. Several intraluminal factors appear to influence Zn^{2+} absorption:

i) The level of Zn^{2+} in the lumen appears to be an important determinant in the extent of Zn^{2+} absorption. Although the proportion of Zn^{2+} absorbed *in situ* declined as the intraluminal Zn^{2+} load increased, the amount of Zn^{2+} absorbed actually increased with increasing Zn^{2+} loads.

ii) Several metals (i.e. Cu^{2+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Ni^{2+}, Fe^{3+}, Mn^{2+} and Hg^{2+}) exert an inhibitory effect on Zn^{2+} uptake when examined *in situ*. In equilibrium dialysis experiments only Cu^{2+} interferred with binding of Zn^{2+} to mucosal cytosol proteins. In view of this, the inhibitory effect of the other metals observed *in situ* could be mediated at the surface of the cells, whereas Cu^{2+} could also interfere with the transfer step.

iii) Feeding studies using the non-absorbed marker, ^{141}Ce, suggest that the Zn^{2+} content and/or the digestibility of the diet could influence the extent of absorption.

iv) *In situ* studies indicate that dietary constituents which chelate Zn^{2+} (i.e. histidine) may, at certain ratios, reduce Zn^{2+} accumulation and transfer.

8. A seasonal study (using the *in situ* technique) revealed that the capacity of the digestive tract to absorb Zn^{2+} was greatest during the summer feeding period. There was a seasonal fluctuation in the stable

Zn^{2+} concentration of the intestinal tissue but there was no apparent relationship between the endogenous stable Zn^{2+} content of the tissue and the amount of Zn^{2+} accumulated from the lumen. Nor was any relationship apparent when the concentration of Zn^{2+} in the intestinal tissue was elevated by injections of stable Zn^{2+}.

9. Artificially elevating the Zn^{2+} status of the flounder by parenteral injections of Zn^{2+} had no significant effect on *in situ* uptake of ^{65}Zn or stable Zn^{2+}.

10. Feeding the flounder a high-Zn^{2+} diet for 5 weeks did not result in any significant elevation of the Zn^{2+} concentrations in the tissues examined. Nor was the capacity of the digestive tracts of these fish to absorb Zn^{2+} detectably altered.

11. Prior injection of flounder with a Zn^{2+} load did result in a reduction of net absorption of ^{65}Zn from radiolabelled capelin. Based on the ratio of stable Zn^{2+} to ^{141}Ce in the rectum contents, there was a net secretion of Zn^{2+} in the Zn^{2+}-injected flounder.

12. Based on the findings of the above studies it is suggested that in a marine fish such as the winter flounder, which has only a portion of the year to obtain its annual Zn^{2+} requirement, uptake of Zn^{2+} may not be limited by the Zn^{2+} status of the animal. In the event of exposure to elevated dietary levels of Zn^{2+}, elimination mechanisms may play a greater role in Zn^{2+} homeostasis than limitation of gastrointestinal uptake.

Chapter II

1. Different tissues in the winter flounder exhibit different rates of accumulation and release of ^{65}Zn following a single, intramuscular

injection. For a substantial time period following the injection, the concentrations of ^{65}Zn were an order of magnitude higher in tissues such as the kidney, liver, gill and gastrointestinal tract than in muscle and bone. In addition, during the period when the concentration of ^{65}Zn in these tissues was declining rapidly, the concentration of ^{65}Zn in the muscle and bone remained constant or increased.

2. The concentration of stable Zn^{2+} in most somatic tissues of the flounder remain relatively constant throughout the year. However, based on the distribution of ^{65}Zn there does appear to be a seasonal change in the turnover of Zn^{2+} in several of the tissues.

3. During the non-feeding period several tissues decline in weight and Zn^{2+} lost from these tissues appears to be redistributed within the body. In particular, the female flounder continues to incorporate Zn^{2+} into the gonads during the non-feeding period.

4. The whole-body retention of ^{65}Zn in live flounder (monitored in the area of the peritoneal cavity), injected in late August or December and monitored through to June, could be resolved into two exponential rate functions. The first component of the retention plot, accounting for 28% of the initial activity, had a very short half-time ($TB_{1/2}$ = 2 days). The second component of the retention plot had by far the longer half-time ($TB_{1/2}$ = 1510 days) and accounted for the major portion of the activity (72%). The latter component is probably more representative of the exchange of Zn^{2+} in the flounder with Zn^{2+} in its environment during the winter, non-feeding period.

5. In agreement with the changes in ^{65}Zn retention observed in several of the tissues, the loss of ^{65}Zn from the whole flounder also appears to change seasonally. When flounder were monitored from June to

August, the rate of ^{65}Zn loss increased over that seen in the winter.

6. The theoretical half-time for exchange of the total body burden of Zn^{2+} in a 35 cm male flounder, consuming a diet of capelin (retention 37% based on the non-absorbed marker technique), was estimated to be 259 days. This is comparable to the half-time observed using whole body retention of ^{65}Zn (i.e. approx 200 days).

7. In view of the slow rate of ^{65}Zn loss observed in flounder during the non-feeding period, compared to the increased rate of ^{65}Zn loss during the feeding period, one could speculate that the rate of Zn^{2+} intake influences the rate of Zn^{2+} elimination. However, examination of ^{65}Zn loss in flounder injected with stable Zn^{2+} plus ^{65}Zn tends not to support this hypothesis. The whole body ^{65}Zn retention patterns were similar in flounder injected with saline or a load of stable Zn^{2+}, i.e. the rate of ^{65}Zn loss does not appear to be affected by an excess of stable Zn^{2+}.

8. Flounder examined in ^{65}Zn retention and tissue distribution studies were maintained under ambient conditions; as such they were subject to changes in water temperature as well as feeding. Therefore, an alternative explanation for the observed seasonal changes in ^{65}Zn retention is that it is related to differences in the metabolism of the fish associated with these changes.

9. Following injection of ^{65}Zn into the winter flounder, the concentration of ^{65}Zn was relatively high in all three of the tissues thought to be possible routes of excretion, i.e. kidney, gill and gastro-intestinal tract. In addition, a similar ^{65}Zn retention pattern was observed in these tissues over the duration of the long-term study. However, without direct evidence it is not possible to say whether ^{65}Zn decline in these tissues represented ^{65}Zn loss from the body at these

sites. ^{65}Zn loss may also occur via the body surface. The concentration of ^{65}Zn in the skin was high; relatively more ^{65}Zn than stable Zn^{2+} was found in the skin up to 528 days post-injection.

10. Experiments conducted to determine the possible site(s) of Zn^{2+} excretion into the digestive tract following i.v. injections of ^{65}Zn indicated that while the greatest amount of radioactivity was detected in the lumen contents of the upper intestine, ^{65}Zn was "secreted" into the lumen contents all along the digestive tract.

11. The level of ^{65}Zn in the lumen contents was similar in flounder injected with ^{65}Zn plus a Zn^{2+} load or ^{65}Zn plus an equivalent volume of saline. However, due to differences in the specific activity, the ^{65}Zn in the lumen contents of the Zn^{2+}-injected flounder presumably represents a greater amount of stable Zn^{2+} than in the saline-injected fish. In other words, while the rate of ^{65}Zn loss appears not to be altered by excess Zn^{2+} in the body, the absolute quantities of Zn^{2+} eliminated are probably greater.

Chapter III

1. Gel filtration (Sephadex G-100) of mucosal cytosols of the winter flounder indicated that ^{65}Zn (Zn^{2+}) was associated with proteins having estimated molecular weights of >150,000 (Fraction I), 80-150,000 (Fraction II), 30-40,000 (Fraction III) and 10-15,000 (Fraction IV). These Zn^{2+}-binding fractions were present in both normal and Zn^{2+}-injected flounder.

2. The low molecular weight (L.M.W.) Zn^{2+}-binding fraction (further separated using DEAE-ion exchange and Biogel P-30 chromatography) isolated from the mucosal cytosol of Zn^{2+}-injected flounder had

properties characterizing it as metallothionein (i.e. high metal content, heat stability, lack of absorbance at 280 nm, high absorbance at 230 nm and high cysteine content).

3. Attempts to determine whether the L.M.W. Zn^{2+}-binding fraction present in the mucosal cytosol of normal flounder fit the criteria of metallothionein were less successful.

4. Metallothionein present in the mucosal cytosol of the Zn^{2+}-injected flounder did not appear to be serving the same physiological functions as commonly hypothesized for intestinal metallothionein in mammals.

i) In mammals it has been proposed that metallothionein serves as an inducible ligand in the intestine which competes for available Zn^{2+} with the normal ligand involved in Zn^{2+} absorption, resulting in a reduction in the amount of Zn^{2+} transferred into the body when the Zn^{2+} status of the animal is elevated. Based on the chromatographic elution profiles, metallothionein was present in the mucosal cytosols of Zn^{2+}-injected flounder but Zn^{2+} absorption from the *in situ* ligated intestine was not significantly different in fish injected with a Zn^{2+} load or an equivalent volume of saline.

ii) An alternative theory holds that Zn^{2+} absorption is directly proportional to the metallothionein level. However, it seems unlikely that metallothionein is playing a direct role in Zn^{2+} absorption in the flounder. When examined on a seasonal basis, the presence of the L.M.W. Zn^{2+}-binding protein (eluting in the same position as metallothionein), in the mucosal cytosol of the flounder was not in phase with the *in situ* absorption of Zn^{2+} or with the feeding period of the flounder.

5. It is possible that metallothionein in the intestinal tissue of the winter flounder is synthesized in response to a change in the distribution of Zn^{2+} in the body and serves a temporary storage function.

6. Metallothionein was also isolated from the liver cytosols of Zn^{2+}- (and Cd^{2+})-injected flounder.

7. A Zn^{2+}-binding fraction, with elution characteristics similar to metallothionein (i.e. similar profiles on Sephadex G-75, DEAE-ion exchange and Biogel P-30 columns), was also present in the liver cytosol of normal flounder. However, the cysteine content of this fraction was lower than that of metallothionein isolated from Zn^{2+}-injected flounder (i.e. 13 versus 30% of the total residues).

8. Unlike the intestinal tissue, Zn^{2+} in the liver was associated with a L.M.W. fraction, eluting in the same position as metallothionein, in fish examined throughout the year.

9. Preliminary investigation, based only on elution of ^{65}Zn (Zn^{2+}) binding proteins from Sephadex columns, indicated that metallothionein may also be present in the kidney cytosol of normal and Zn^{2+}-injected flounder. Its presence was not apparent in gill cytosol.

10. In conclusion, in agreement with observations in mammals, both the intestine and liver of the flounder respond to injections of a load of Zn^{2+} by synthesizing metallothionein. However, it remains to be resolved what role, if any, metallothionein plays in the normal metabolism of Zn^{2+} in the flounder. The difficulties encountered in characterizing the metallothionein-like fraction from the tissues of normal flounder point to the need for a specific assay to quantitate the level of metallothionein in the tissues. Such an assay would seem

necessary before any possible relationship between the normal metabolism of Zn^{2+} and the involvement of metallothionein can be delineated.

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APPENDIX A. EQUATIONS USED TO ESTIMATE WEIGHTS OF TISSUES OF THE WINTER FLOUNDER.

The following equation was used to estimate the total dry weight of a given tissue:

$$y = e^{x \cdot b + a}$$ (Fletcher and King, personal communication)

where, x = LN Body length (cm)

y = dry weight of tissue (g)

a and b (tabulated on the following page) were obtained from individual scattergrams for each tissue where n = number of fish examined; winter refers to fish dissected in April, i.e. at the end of the winter before summer feeding resumes; summer refers to fish dissected in October, i.e. at the end of the summer feeding period.

D. – refers to tissue sampled from the "dark" side (top) of the flounder.

L. – refers to tissue sampled from the "light" side (bottom) of the flounder.

The dry weights of the skin, muscle (white) and gills can be converted to wet weights using the average % water in the tissues, i.e. 70, 81 and 77%, respectively.

Tissue	Males (winter)			Males (summer)			Females (winter)			Females (summer)		
	a	b	n	a	b	n	a	b	n	a	b	n
Int. tract	-12.9	3.72	14	-12.9	3.77	13	-12.7	3.69	12	-16.1	4.71	12
Heart	-13.4	3.11	12	-15.3	3.66	10	-14.8	3.43	9	-20.9	5.20	10
Kidney	-19.1	5.04	14	-15.4	4.07	13	-12.4	3.08	12	-15.8	4.14	12
Gills	- 8.24	2.40	19	-11.0	3.18	18	- 9.45	2.74	12	-14.1	4.04	12
Eyes	- 8.31	1.82	14	- 6.07	1.18	13	- 8.36	1.82	12	- 9.33	2.08	12
Scales (D.)	- 9.69	3.02	24	-11.2	3.40	23	- 9.46	2.90	17	- 9.49	2.89	17
Scales (L.)	-11.8	3.54	19	-12.5	3.70	18	-11.72	3.43	17	-10.1	2.95	17
Skin scrapings (D.)	-10.7	3.17	14	- 8.80	2.64	13	- 4.50	1.42	12	-10.2	3.08	12
Skin (D.)	-10.3	3.24	14	-10.8	3.35	13	- 9.48	2.98	12	- 6.49	2.16	12
White muscle (D.)	- 6.39	2.61	14	- 7.15	2.90	13	- 2.25	1.41	12	- 5.39	2.42	12
Belly muscle (D.)	- 9.24	2.75	13	- 6.86	2.12	13	- 5.26	1.63	12	-11.8	3.58	12
Fin muscle (D.)	- 9.81	3.18	14	- 8.38	2.90	13	- 9.59	3.09	12	- 4.29	1.82	12
Skin scrapings (L.)	- 7.73	2.34	14	- 8.10	2.43	13	- 8.14	2.44	12	- 9.35	2.85	12
Skin (L.)	-13.7	4.18	14	-11.9	3.68	13	-10.9	3.39	12	- 7.88	2.54	11
White muscle (L.)	- 7.00	2.74	14	- 6.31	2.62	13	- 1.37	1.12	12	- 5.14	2.31	12
Belly muscle (L.)	-11.6	3.34	14	- 8.19	2.44	13	- 7.35	2.16	12	-14.6	4.31	12
Fin muscle (L.)	- 8.94	2.97	14	- 8.99	3.10	13	- 2.09	0.99	12	- 5.95	2.30	12
Total fins	- 7.19	2.57	14	- 8.44	2.90	13	- 8.63	2.91	12	- 8.50	2.92	12
Skeleton and head	- 7.62	3.09	14	- 7.17	2.96	13	- 6.79	2.85	12	- 6.62	2.84	12
Spleen	-18.5	4.62	14	-25.2	6.52	13	-15.7	3.72	12	-16.2	3.95	12
Interhaemal spine	-13.6	3.79	11	-13.0	3.61	11	-11.4	3.12	10	- 9.86	2.73	11

APPENDIX B. ESTIMATED ZINC CONTENT OF TISSUES OF 35 CM MALE AND FEMALE WINTER FLOUNDER.

The dry weights of tissues of a 35 cm flounder were estimated using the equations in Appendix A. The stable Zn^{2+} concentrations of the tissues (determined by atomic absorption spectrophometry) represent the $\bar{X}$ values of tissues sampled from 2-19 fish ranging in body length from 30 to 40 cm (Fletcher and King, personal communication).

Estimated tissue Zn^{2+} concentrations of a 35 cm female winter flounder.

Tissue	Start of summer			End of summer	
	Dry wt. (g)	µg Zn^{2+}/g dry wt.	Total µg Zn^{2+}	Dry wt. (g)	Total µg Zn^{2+}
Int. tract	1.5290	92.6	142	1.8733	173
Heart	0.0933	114	10.6	0.0927	10.6
Kidney	0.2390	117	28.0	0.3584	41.9
Gill filaments	0.9205	92.1	84.8	0.9247	85.2
Gill arches	0.4261	74.9	31.9	0.4278	32.0
Eyes	0.1522	390	59.4	0.1441	56.2
Scales (D.)	2.2664	144	326	2.1694	312
Scales (L.)	1.5508	103	160	1.4921	154
Skin scraping (D.)	1.7416	66.4	116	2.1596	143
Skin (D.)	3.1625	43.6	138	2.3030	100
White muscle (D.)	16.1466	35.3	570	24.7160	872
Belly muscle (D.)	1.7418	83.1	145	2.4662	205
Fin muscle (D.)	4.0711	69.1	281	8.8310	610
Skin scraping (L.)	1.6916	35.5	60.0	2.1639	76.8
Skin (L.)	3.1155	18.3	57.0	3.2024	58.6
White muscle (L.)	13.5999	26.01	354	21.3473	555
Belly muscle (L.)	1.4159	48.7	69.0	2.0520	99.9
Fin muscle (L.)	4.1582	52.6	219	9.1617	482
Fins	5.5816	88.8	496	6.6028	587
Skeleton and head	28.39	69.0	1960	32.2472	2230
Spleen	0.1116	92.8	10.4	0.1137	10.6
Interhaemal spine	0.8328	66.3	55.2	0.8527	56.5
	Wet wt. (g)	µg Zn^{2+}/g wet wt.	Total µg Zn^{2+}	Wet wt. (g)	µg Zn^{2+}/g wet wt.	Total µg Zn^{2+}
Liver	10.38	25	260	9.62	44	423
Gonad	13.60(July)	65	884	39.62	47	1860

Estimated tissue Zn^{2+} concentrations of a 35 cm male winter flounder.

	Start of summer			End of summer		
Tissue	Dry wt. (g)	µg Zn^{2+}/g dry wt.	Total µg Zn^{2+}	Dry wt. (g)	µg Zn^{2+}/g dry wt. where differs	Total µg Zn^{2+}
Int. tract	1.3860	104	144	1.6056	-	167
Heart	0.0955	181	17.3	0.0995	125	12.4
Kidney	0.2974	161	47.9	0.4124	-	66.4
Gill filaments	0.93	122	113	0.879	-	107
Gill arches	0.43	118	50.7	0.4091	-	48.3
Eyes	0.1602	1047	168	0.1529	-	160
Scales (D.)	2.8319	341	966	2.4662	-	841
Scales (L.)	2.1907	148	324	1.9464	-	288
Skin scraping (D.)	1.7872	183	327	1.8257	-	334
Skin (D.)	3.2577	139	453	3.1264	-	435
White muscle (D.)	18.0618	55.8	1008	23.3579	-	1300
Belly muscle (D.)	1.7373	209	363	1.9844	-	415
Fin muscle (D.)	4.5230	168	760	6.8723	-	1160
Skin scraping (L.)	1.8128	39.0	70.7	1.7396	-	67.8
Skin (L.)	3.1492	25.3	79.7	3.1497	-	79.7
White muscle (L.)	15.5201	27.1	421	20.1939	-	547
Belly muscle (L.)	1.2621	67.3	84.9	1.6036	49.3	79.1
Fin muscle (L.)	4.9657	68.3	339	7.6407	-	522
Fins	7.0327	170	1200	6.9782	-	1190
Skeleton and head	28.3937	106	3010	28.0917	-	2980
Spleen	0.1239	82.9	10.3	0.1376	104	14.3
Interhaemal spine	0.9083	84.6	76.8	0.8355	-	70.7
	Wet wt. (g)	µg Zn^{2+}/g wet wt.	Total µg Zn^{2+}	Wet wt. (g)	µg Zn^{2+}/g wet wt.	Total µg Zn^{2+}
Liver	4.56	27	123	5.29	30	159
Gonad	7.95(July)	40	318	58.19(Oct.)	20	1160

END

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FIN