A STUDY OF THE PANCREATIC ISLETS AND ZINC TOXICITY IN THE RAINBOW TROUT, SALMO GAIRDNERI RICHARDSON

by

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A study of the pancreatic islets and zinc toxicity in the Rainbow Trout, Salmo gairdneri Richardson

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ABSTRACT

A study was carried out on the pancreatic islets in rainbow trout, Salmo gairdneri Richardson, and also the effects of zinc pollution upon insulin secretion in this species.

In the first experiment, a histological study of the pancreatic islets was done in which polypeptide hormone-producing cells were localized, using immunocytochemical staining techniques. Four different cell-types were identified in this manner. These were the insulin, somatostatin, pancreatic polypeptide and glucagon/gastric inhibitory polypeptide (GIP) cells. The glucagon/GIP cell was designated thus as antisera to both hormones cross-reacted with a common population of cells. A fifth cell-type, commonly referred to as a clear cell, was also identified although its secretory product is as yet undetermined.

The second experiment involved the localization of zinc within the pancreatic islets of Salmo gairdneri. Individual fish were injected with ^{65}Zn and the distribution of the isotope within the islets was determined by autoradiography. It was found that the insulin cells accumulated approximately twice as much zinc per unit area as the rest of the islet tissue. It is presumed that this zinc is involved with the crystallization and storage of insulin within the insulin cells.

The third experiment involved a study on the effects of zinc upon carbohydrate metabolism and insulin secretion in Salmo gairdneri. An LC_{50} study was first done to determine the toxicity of zinc to this species in Vancouver water. A 48-hour LC_{50} of 0.99 ppm of zinc was established. The upper and lower confidence limits of this LC_{50} were 1.73 and 0.56 ppm respectively. A 31-day exposure of Salmo gairdneri to water containing 0.081, 0.096, 0.352 and less than 0.04 (control) ppm of zinc was then conducted. At all the experimental concentrations of zinc, significant hyperglycemia resulted after one day of exposure. In fish exposed to 0.352 ppm of zinc this hyperglycemia persisted until day 5 and was accompanied by severe mortalities. Liver glycogen and plasma insulin levels were monitored in the control and the 0.352 ppm zinc groups. In the experimental group, there was a significant depletion of liver glycogen on days 3 and 5. The levels then became significantly higher than controls on day 9. Plasma insulin levels were significantly lower in the experimental group on days 2, 3 and 7 and only returned to control levels on day 9. The changes in carbohydrate metabolism and insulin levels are discussed in relation to increased plasma corticosteroids and catecholamines as a result of zinc-induced stress.

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GENERAL INTRODUCTION

Zinc is a naturally occurring trace metal in Canadian freshwater and is also important in biological systems. It is often found in elevated concentrations in waters adjacent to stripmining and pulpmilling operations, resulting in severe fish mortalities (Sprague et al., 1965). Consequently, much research has been devoted to the toxic action of zinc upon morphological and physiological parameters, utilizing rainbow trout, Salmo gairdneri Richardson, as the test species because of its sensitivity to zinc.

A primary physiological response of Salmo gairdneri to zinc toxicity is stress-induced hyperglycemia (Watson 1975), which may involve changes in plasma insulin, an important hormone in teleost carbohydrate metabolism (Ince and Thorpe 1977b). These alterations in the levels of plasma insulin may be due to the combined actions of other hormones, or to the direct action of zinc upon the insulin cells, as it is important in insulin storage in many vertebrates (Petkov 1970).

While assayable insulin has been found in the plasma of Salmo gairdneri (Thorpe and Ince 1976), the localization of the insulin cells and their requirements for zinc have not yet been determined. Therefore, experiments were conducted to determine a) the localization of insulin and other cell types within the pancreatic islets of Salmo gairdneri,

b) the presence of zinc within the insulin cells, and c) the effects of zinc-induced hyperglycemia stress upon insulin secretion and glycogen metabolism in this species.

CHAPTER I

The immunocytochemical localization of hormone-producing cells within the pancreatic islets of *Salmo gairdneri* Richardson

Introduction

Interest in the pancreatic islets of teleosts has grown considerably in the last decade and there is now a large body of literature on this subject (Brinn, 1973, 1975, Epple and Brinn, 1975, Epple and Lewis 1973, Johnson et al., 1976, Klein and Lange 1977, Klein and Van Noorden 1978, Kobayashi et al., 1976, Lange et al., 1975 and Thomas 1970, 1975). In much of the early work, cell types were identified according to their affinity for tinctorial stains. In recent years however, many authors have relied upon immunocytochemical techniques as the means of cell identification (Johnson et al., 1976, Klein and Van Noorden 1978, Lange et al., 1975, Van Noorden and Patent 1978). As a result, the identity of at least four cell types has been confirmed. These are the insulin (β) cell, the glucagon (α) cell, the somatostatin (D) cell and the pancreatic polypeptide (F) cell. In comparison, the human pancreatic islets are comprised of these same four cell types.

Furthermore, a fifth cell type, designated as a clear cell on account of its appearance under the light microscope, has been identified in teleosts by at least two laboratories (Klein and Lange 1977, Kobayashi et al., 1976). Secretory granules have been observed in these clear cells, although their content is not known.

The pancreatic islets of the Rainbow Trout, Salmo gairdneri (Richardson), have received only cursory attention in the literature to date (Brinn 1973, Epple and Brinn 1975) and their histology has not been studied.

The purpose of this study was to map the pancreatic islets of Salmo gairdneri, using the best histological techniques available. These included both tinctorial stains which were noted for their affinity for individual cell types, and the peroxidase anti-peroxidase (PAP) staining technique developed by Sternberger (1974).

Materials and Methods

Juvenile Rainbow and Steelhead Trout, Salmo gairdneri (Richardson), obtained from the Abbotsford Hatchery, Abbotsford, B. C., were maintained in 760-liter fibreglass tanks supplied with dechlorinated tap water (6 - 12° C). The lighting was adjusted to a natural photoperiod (Vancouver) and the fish were fed ad libitum daily with Oregon Moist Pellets. Tissue was collected by first stunning the fish (20 - 50 g) with a blow to the head and then immediately removing the islet organ and intestine.

Histology. Tissues were fixed in either Bouin's fluid, Bouin-Hollande solution or 3% Glutaraldehyde buffered with 0.1M cacodylate to pH 7.4. Bouin's and Bouin-Hollande-fixed tissues were dehydrated in graded ethanols, cleared

in toluene and embedded in paraplast (56° C). Tissues fixed in glutaraldehyde were dehydrated in graded ethanols, cleared in absolute ethanol and embedded in Spurr's low-viscosity embedding media. The clearing stage which normally employs propylene oxide was omitted, since this solvent has been found to reduce subsequent immunoreactivity of fixed endocrine tissue to specific antibodies (Van Noorden and Pearse 1974). Paraplast-embedded tissue was sectioned on a rotary microtome (4 μ) whereas Spurr's embedded tissue was sectioned on an LKB Ultramicrotome (0.5 μ). All sections were mounted on gelatinized glass slides. Prior to staining, paraplast embedded sections were dewaxed in toluene and rehydrated. Sections embedded in Spurr's media were also rehydrated after removing the resin with sodium methoxide according to Mayor et al., 1961).

Islet tissue was stained for insulin cells with aldehyde fuchsin following permanganate oxidation (Scott 1952), for glucagon cells by Grimelius' (1968) silver impregnation technique and for somatostatin cells by the silver impregnation procedure described by Hellman and Hellerstrom (1960). Following the staining procedures, all slides were dehydrated, cleared in toluene and mounted under glass coverslips with permount (Fisher Sc. Co.).

Immunocytochemistry

Islet organs from both rainbow and steelhead trout were stained according to the PAP immunocytochemical technique of Sternberger (1974) after the application of one of the following primary antisera: guinea pig anti-porcine insulin serum (GP-APIS, Dr. R. A. Pederson, U. B. C.), rabbit anti-porcine glucagon serum (R-APGS, Dr. B. A. McKeown, S. F. U.), rabbit anti-somatostatin serum (R-ASS, Dr. C. McIntosh, U. B. C.), rabbit anti-porcine gastric inhibitory polypeptide serum (R-APGIPS, Dr. J. C. Brown, U. B. C.) and rabbit anti-bovine pancreatic polypeptide serum (R-ABPPS, Dr. R. E. Chance, Eli Lilly Labs Ltd., Indiana, U. S. A.). The dilutions of primary antisera used (diluted with 0.15 M NaCl which contained 0.05 M Tris-HCl buffer and 1% normal goat serum, pH 7.6) were GP-APIS 1/200, R-APGS 1/700, R-APGIPS 1/200, R-ASS 1/200 and R-ABPPS 1/200. Antisera could generally be used at a much higher dilution on Bouin's-fixed material than on glutaraldehyde-fixed material.

All primary antisera were applied overnight at 4° C. Goat anti-rabbit gamma globulin serum was then applied (1/10 dil. for 10 min. at 22° C) followed by rabbit peroxidase anti-peroxidase serum (Miles Yeda, Ltd.) at a 1/50 dilution for 10 min. at 22° C.

After each antiserum application, the slides were washed for at least 10 minutes in Tris-buffered saline (0.15 M NaCl containing 0.05 M Tris-HCl buffer, pH 7.6). The sites of peroxidase activity were then visualized by transferring the slides to 0.01% H_2O_2 containing 0.025% (W/V) 3,3-diaminobenzidene (Graham and Karnovsky, 1966). The slides were then post-fixed in 0.1% OsO_4, washed thoroughly in distilled water, dehydrated and mounted with Permount (Fisher Sc. Co).

Several control procedures were carried out to confirm the specificity of antibody binding. These included the application of a non-immune serum as primary antiserum, the omission of the primary antiserum step altogether and the saturation of the primary antiserum with its complement antigen prior to application (saturation was always carried out overnight at 4° C). Because glucagon and GIP antisera have been shown to bind to and stain a common cell type in the rat (Smith et al., 1977), additional control procedures were carried out for the GIP antiserum, which has been well characterized and found to be non-reactive with glucagon under radioimmunoassay conditions (Brown, pers. comm.). Individual aliquots of GIP antisera were saturated with either glucagon (Sigma) or GIP (Dr. J. C. Brown). To retard any molecular degradation of the glucagon and GIP the diluent buffer contained 1200 K. I. U./ml. of Trasylol (Boehringer Ingelheim Ltd.) and 0.05 M benzamidine (Sigma).

The saturation was carried out at 4° C for either four hours or overnight. The saturated antisera were then applied to tissue sections for one hour after which time the PAP procedure was followed. The application of the primary antisera for only one hour was found to be a good alternative to the overnight application. Non-saturated GIP antiserum was also maintained at 4° C alongside the saturating antisera. This served as a control against any potential failure of the PAP technique.

All stained material was microphotographed (Carl Zeiss, Photomicroscope) using Kodak Panatomic X film.

Results

Some of the pancreatic islets of Salmo gairdneri were found to be concentrated into an islet organ that is located alongside the wall of the gall bladder. Other islets were also found in the curvature of the spleen, dispersed in exocrine pancreatic tissue. The islet organ consisted of several islets fused together and surrounded by a capsule of exocrine pancreatic tissue. The most conservative arrangement consisted of a single, large islet surrounded by exocrine tissue. In this case there was no invasion and subsequent subdivision of the islet by exocrine tissue. In more extreme cases, the exocrine tissue

had invaded the islet to such an extent that the latter had been subdivided into its individual component islets. The single, large islet arrangement was seen to predominate in the smaller fish (< 30 grams) while the subdivided islet arrangement was more prevalent in fish greater than 30 grams.

Histology. Aldehyde fuchsin was found consistently to be the most reliable of the staining methods used. A large number of insulin cells were revealed by this staining method. These cells were arranged in simple aggregates or in cords that varied in length. The insulin cells occurred in most parts of the islet but were never found on the extreme periphery. Morphologically, the insulin cells are elliptical in appearance and the nucleus is situated at the pole distal to the capillaries. In glutaraldehyde-fixed, resin-embedded tissue, the cells appeared quite granular. The use of fresh paraldehyde in the preparation of the stain was found to be essential for dense staining, thus confirming Gairdner's (1969) recommendations.

The silver impregnation procedure of Grimelius (1968) was unreliable, but often revealed a population of cells. Although these cells were not confirmed as being glucagon cells (by staining the adjacent section using the PAP technique) their pattern of distribution within the islet

was similar to that of the glucagon cells. Material fixed with glutaraldehyde and embedded in Spurr's media revealed that these cells took up the stain in two different ways. One group of cells stained a deep reddish-brown while the second group exhibited granular black staining. The reddish brown cells also appeared to be the smaller of the two types.

The silver impregnation procedure of Hellman and Hellerstrom (1960) stained some cells. However, their numbers were fewer than the population of cells revealed by the PAP technique using anti-somatostatin serum.

In all specimens that were examined, large numbers of oval, clear cells were observed. These cells generally demonstrated no affinity for any stains. However they were stained non-specifically by Grimelius's (1968) technique, showing marked granulation. Individual large granules appeared to be scattered sparsely throughout the cytoplasm which may explain the general lack of affinity of these cells for other stains. These cells appeared larger and more prominent in steelhead than in rainbow trout.

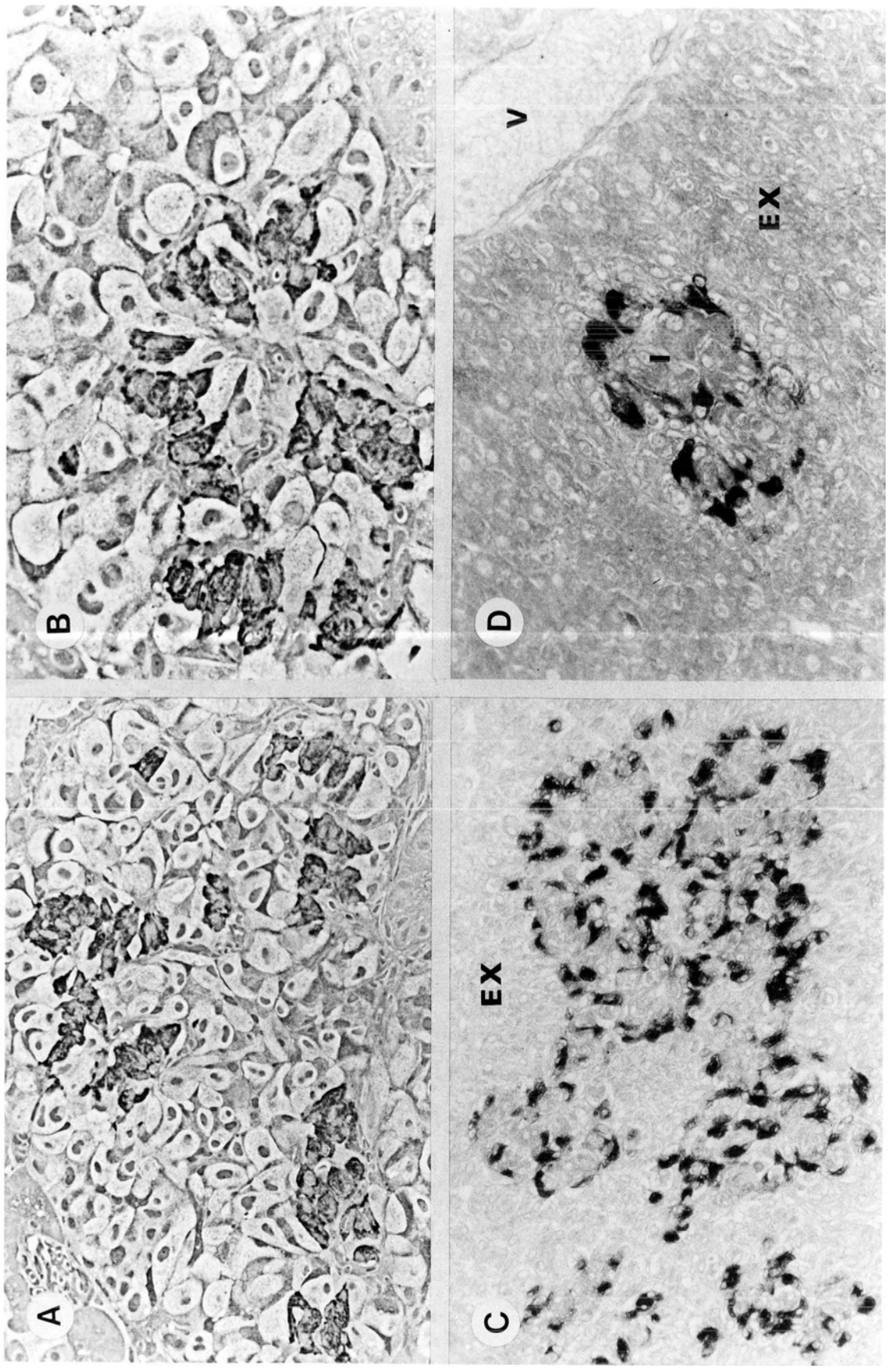
Immunocytochemistry. The peroxidase-anti-peroxidase technique (PAP) was found to be both specific and reliable in its staining of individual cell types. Non-specific background staining, which inevitably resulted when testing

Figure 1. A) A portion of the islet tissue stained immunocytochemically for insulin cells. Note that the cells (dark staining) appear quite granular and occur in clusters or cords. Glutaraldehyde fixed, resin embedded. X350

B) A higher magnification of the islet tissue also stained for insulin cells. Tissue preparation same as in "A". X560

C) Islet tissue stained immunocytochemically for somatostatin cells. These cells form "halos" inside of which insulin cells may be found. All of the islet tissue is surrounded by exocrine (EX) tissue. Bouin's fixed, paraffin embedded. X260

D) One isolated islet surrounded by exocrine (EX) tissue and stained for somatostain cells. The "halo" arrangement of the cells is evident here. Insulin (I) cells are located within the "halo". A vein (V) is situated in the upper right corner. Tissue preparation same as in "C". X450.


A
B
C
EX
D
V
I
EX

a new primary antiserum, could be reduced, but not usually eliminated, by diluting the antiserum. Pre-treating all sections with undiluted normal goat serum, for 10 minutes at room temperature, also reduced background staining as well as shortening the washing times of the slides. This procedure apparently blocks all non-specific binding sites on the tissue sections to which the goat anti-rabbit gamma globulin serum (secondary antiserum) tends to bind (Smith, pers. comm.).

Anti-insulin serum reacted specifically with the aldehyde fuchsin positive cells. There was a high intensity of staining in Bouin's fixed tissue and a complete absence of background staining at even low dilutions. Lower dilutions of antiserum were required in glutaraldehyde-fixed tissue and the insulin cells appeared quite granular in such material.(Figure 1A). Capillaries were more prevalent amongst the insulin cells than any other cell type.

Staining with anti-somatostatin serum revealed a population of cells that was always closely associated with the insulin cells (Figs. 1C,D). Each aggregate of insulin cells was found to be surrounded by an intermittent 'halo' of somatostatin cells. These cells were ellipsoidal in shape, the nucleus situated at one pole while the other

Figure 2. A comparison of glucagon cells in the islet tissue of Steelhead and Rainbow Trout.

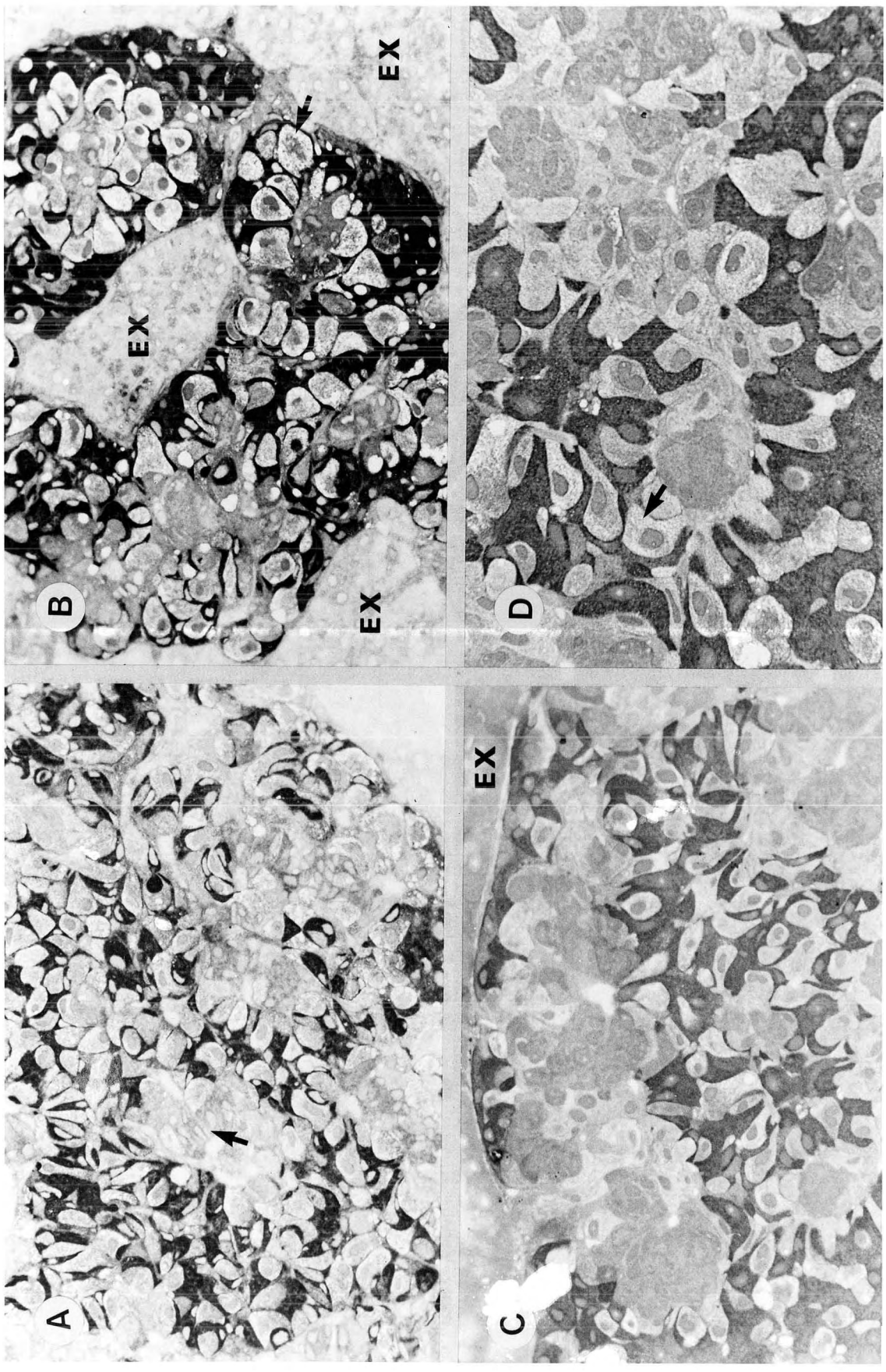
A) A portion of Steelhead islet tissue stained immunohistochemically for glucagon cells. Although the cells appear to be randomly scattered, they do form several circles in which insulin and somatostatin cells may be found (arrow). X370

B) A portion of Steelhead (> 30 g) islet tissue into which there has been an invasion of exocrine tissue (EX). Note the large clear cells containing a granular cytoplasm (arrow). X370

C) A portion of the islet tissue from Rainbow Trout also stained for glucagon cells. These cells are similar in shape and distribution to the Steelhead. (EX) Exocrine tissue. X370

D) Rainbow islet tissue stained for glucagon cells and exhibiting large, distinctly granular clear cells (arrow). At the base of the arrow are a cluster of insulin cells surrounded by glucagon and clear cells. X550

All tissue was glutaraldehyde fixed, resin embedded.


A
B
EX
EX
EX
C
EX
D

pole tapered to a point.

Staining with anti-glucagon serum revealed a population of cells that was confined to the periphery of the islet. However periodically these cells were observed to have invaded the centre of the islet (Figure 2B). The glucagon cells were always found encircling groups of insulin and somatostatin cells. As was observed with the Grimelius (1968) silver impregnation technique, use of the PAP technique with anti-glucagon serum resulted in some cells staining more densely than others. There were very few capillaries found amongst the glucagon cells.

Anti-GIP serum was found to cause staining in the glucagon cells as well. Examination of serial sections stained with antisera to glucagon and GIP, confirmed that both of these antisera reacted with a common population of cells (Figs. 3C,D).

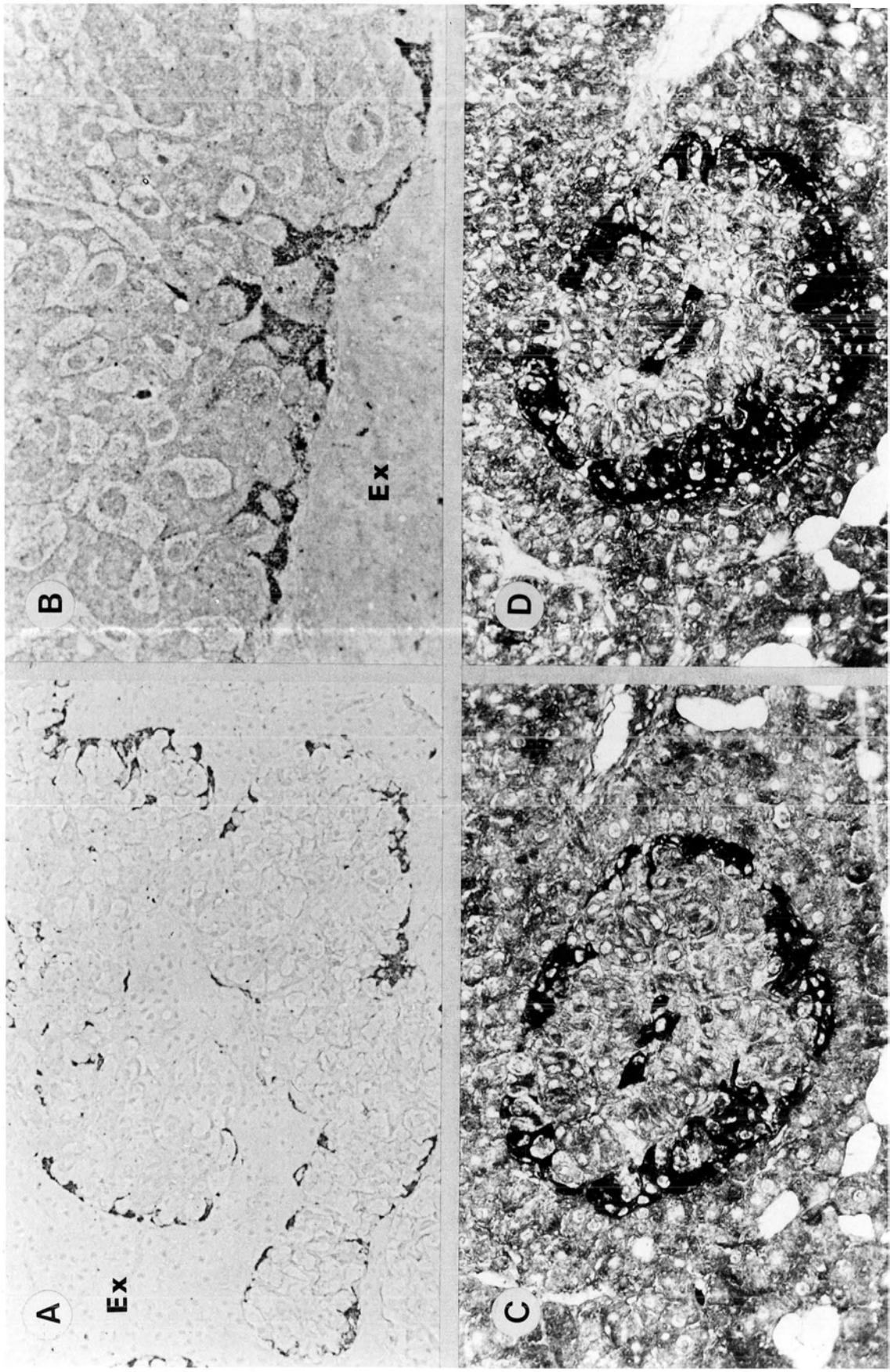
Staining with anti-pancreatic polypeptide serum revealed a very small population of cells (Figs. 3A,B). These pancreatic polypeptide cells were found on the extreme periphery of the islet, between the glucagon cells and the connective tissue capsule. Here the cells formed a single layer with periodic interruptions. Occasionally, these cells were found invading the centre of the islet, but not to the extent observed for the glucagon cells. The pancreatic polypeptide cells were irregular in shape and had many

Figure 3. A) Islet tissue stained immunocytochemically for pancreatic polypeptide cells. The cells (dark staining) are confined to the islet periphery. (EX) exocrine tissue. Glutaraldehyde fixed, resin embedded. X175

B) Islet tissue stained for pancreatic polypeptide cells. Note the granular nature of the dark staining cells. Tissue preparation same as in "A". X410

C) Islet tissue stained immunocytochemically for glucagon cells showing a single islet. Bouin's fixed, paraffin embedded. X365

D) Section immediately adjacent to "C" and stained immunocytochemically for GIP cells. Tissue preparation same as in "C". X365


A
Ex
B
Ex
C
D

cytoplasmic processes.

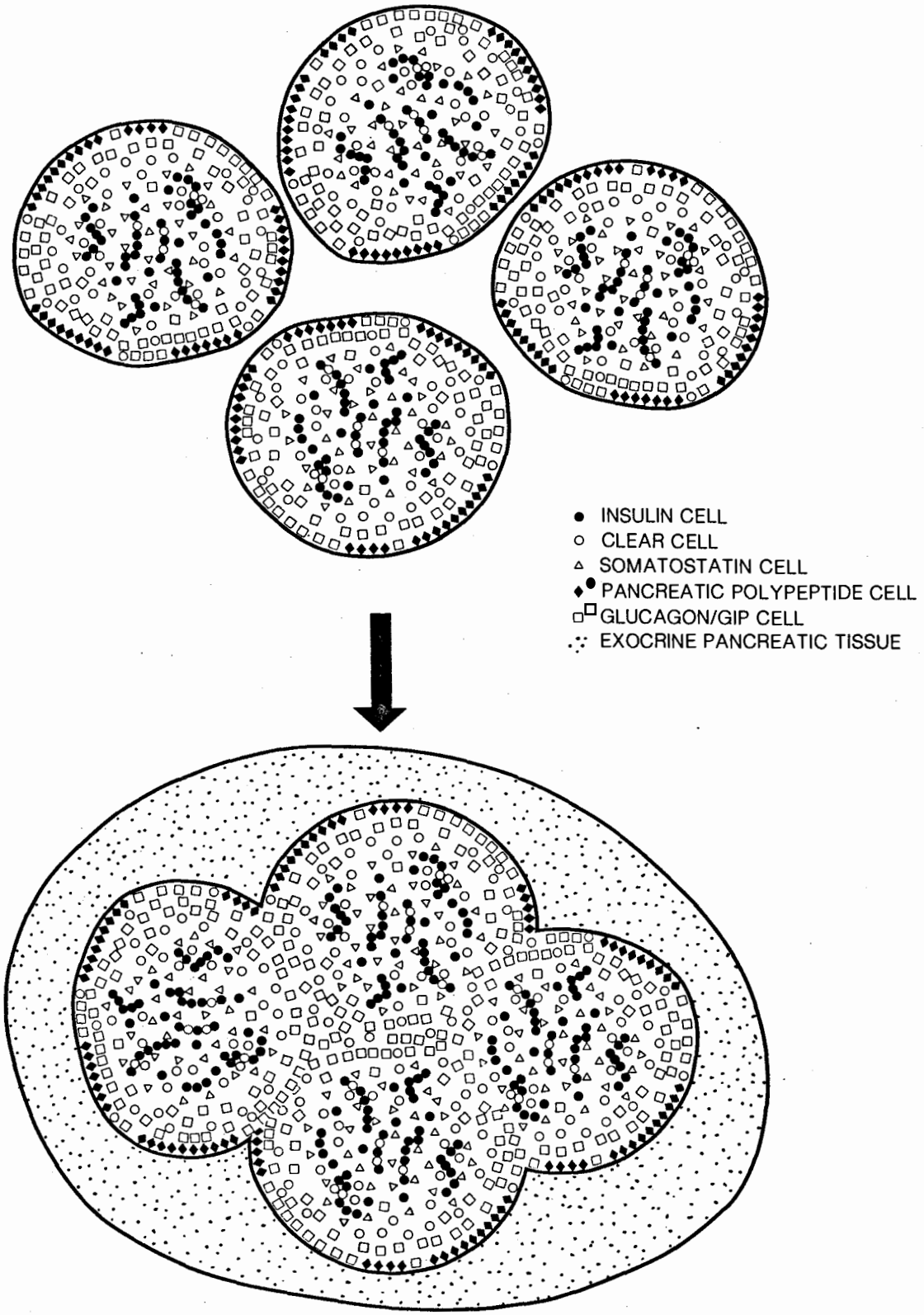
Although none of the antisera used reacted specifically with the clear cells, some non-specific staining resulted after staining with anti-glucagon and anti-GIP serum. Both of these antisera revealed a granular material within the clear cells that was not evident in unstained sections (Figs. 2B,D). The granular material was similar to that revealed non-specifically by the Grimelius (1968) silver impregnation technique. This non-specific staining could be reduced, but not eliminated, by diluting the antiserum further and it was only observed in glutaraldehyde fixed, resin embedded material.

When standard control procedures were carried out, negative staining resulted in all cases. When the GIP antiserum was saturated with glucagon, negative staining also resulted after either four hours of saturation or overnight saturation. The control antiserum that was treated similarly, but which did not receive any antigen, demonstrated no deterioration.

Discussion

The islet organ of Salmo gairdneri consists of at least four different endocrine cells, each of which occupies a unique position within the islet. These are

Figure 4. The fusion of individual pancreatic islets to form the islet organ in Salmo gairdneri. The cell types in each islet are distributed in an identical fashion that is maintained upon their being integrated into the islet organ. The distributive pattern of each cell type was determined as a result of extensive observations on several islet organs. The frequencies of each cell type were assessed subjectively and do not reflect actual cell counts. The islet organ shown is representative of fish under 30 grams in which there has been no invasion by exocrine tissue.


INSULIN CELL
CLEAR CELL
SOMATOSTATIN CELL
PANCREATIC POLYPEPTIDE CELL
GLUCAGON/GIP CELL
EXOCRINE PANCREATIC TISSUE

the insulin cells, the glucagon /GIP cell, the stomatostatin cell and the pancreatic polypeptide cell. Although each cell does in fact occupy a specific location in the islet organ, at first glance this does not appear to be the case. For example, the glucagon/GIP cells which are generally peripherally located, frequently invade the centre of the islet organ. Further microscopical observations revealed that the islet organ actually consists of several individual pancreatic islets that have fused together and then been encapsulated by exocrine tissue.

Figure 4 illustrates the manner in which these individual islets fuse to form an islet organ. The cell types in each islet are distributed in an identical fashion that is maintained upon the islets being integrated into the islet organ. It is a result of islet fusion, that a portion of the glucagon/GIP cells from each islet become incorporated into the centre of the islet organ.

It is not known at what point in the early life history the islet organ is formed or when the integration of the islets is at a maximum. However, in smaller fish (< 30 g) the islet organ is very compact, while in larger fish (> 30 g) there may be considerable invasion and subdivision of the islet organ by exocrine tissue.

The anatomical substructure reported here for Salmo

gairdneri has not been reported in other teleosts to date. Islet fusion has only recently been reported in the human pancreatic islets (Unger et al., 1978).

Although each of the cell types described in Salmo gairdneri has been identified immunohistochemically in other teleosts (Johnson et al., 1976, Klein and Van Noorden 1978, Lange et al., 1975 and Van Noorden and Patent 1978), this is the first reported identification of four cell types and their secretory products in one species, using this technique. The distributive pattern of these cell types may differ radically between species. In the channel catfish, Ictalurus punctata, for example (Johnson et al., 1976) there is no obvious pattern of cell type distribution. However, the distribution of islet cells (insulin, glucagon and somatostain cells) is similar for Salmo gairdneri, Cottus corpius (Falkner 1961), Gadus callarias (Thomas 1970) and Limanda limanda (Thomas 1975). The significance of islet cell distribution patterns and anatomical associations between cell types is speculative at best. In Salmo gairdneri for example, the somatostatin cells are closely associated anatomically with the insulin cells and the region around these cells is extensively vascularized. In mammals, heterocellular regions, in which insulin, glucagon and somatostatin cells are in close proximity to one another (Orci and Unger, 1975) and

which are highly vascularized and innervated (Fujita et al., 1976), have been identified. It has been suggested that coordinated secretion of pancreatic hormones may be facilitated by these heterocellular regions (Unger et al., 1978). The vascular networks around the insulin and somatostatin cells in Salmo gairdneri may in fact be the sites of islet organ heterocellular regions. Vascularization of the islet tissue in Salmo gairdneri occurs almost exclusively in the regions of these two cell types, which however are in close proximity to the clear cells and are surrounded by glucagon/ /GIP cells. As in teleosts, the cellular distribution patterns in mammalian islets also differ between species, but a heterocellular region has been nevertheless identified in each species (Orci and Unger 1975). This region might permit intercellular communication and coordinated secretion regardless of cellular distribution patterns. In fact, the different islet cell distribution patterns may only reflect species diversity and not necessarily any fundamental change in islet physiology.

On a comparative basis, Salmo gairdneri has as many pancreatic hormones as man (Pelletier 1977). However, their modes of action and secretion still remain the subject of considerable controversy. Insulin has been purified from several teleosts (Grant and Reid 1968, Neuman

and Humbel 1969, Neuman et al., 1969) and the subsequent development of radioimmunoassays employing teleost insulin components (Noe et al., 1977, Patent and Foa 1971, Thorpe and Ince 1976) have greatly assisted the investigations on teleost islet physiology. Cod insulin lowers plasma glucose and amino acid nitrogen levels in Esox lucius (Thorpe and Ince 1974) as well as cholesterol levels in Anguilla anguilla (Ince and Thorpe 1974). The studies by Thorpe and Ince (1974) also point out the importance of using at least teleost insulin, if not species specific insulin, when investigating its physiology in fishes. They found that bovine insulin exerted only hypoaminoacidemic effects in Anguilla anguilla, at the same dose level (2 I. U./kg) at which cod insulin lowered plasma glucose, amino acid nitrogen and cholesterol levels (Ince and Thorpe 1974).

Studies on glucagon physiology in fishes have employed mammalian glucagon exclusively, due to the unavailability as yet of the teleost hormone. In all species under study, glucagon invoked significant hyperglycemic responses at doses ranging from 50 µg/kg to 2 mg/kg (Chan and Woo 1978, Ince and Thorpe 1977, Inui and Yokote 1977, Larsson and Lewander 1972, Wagner and McKeown, unpublished observations). Any effects of mammalian glucagon upon amino acid metabolism are questionable, however. Of all the forementioned

studies only Anguilla japonica responded with lowered amino acid nitrogen levels, and only after repeated injections (500 µg/kg/4 hours) over twelve hours (Inui and Yokote 1977). Glucagon also elevates free fatty acid levels in Esox lucius (Ince and Thorpe 1975), but not in Anguilla anguilla (Larsson and Lewander 1972).

Somatostatin effects upon the endocrine pancreas in teleosts have not been given serious attention to date. One study has demonstrated that somatostatin inhibits insulin release in the hagfish, Epatretatus stouti (Stewart et al. 1978). The hormone demonstrates a similar activity in mammals (Vale et al., 1975).

The physiology of pancreatic polypeptide has not been investigated in teleosts as yet. In fact its true function in mammals is not known (Floyd et al. 1978).

Due to the fact that glucagon was successful in blocking anti-GIP serum staining, the significance of both anti-GIP and anti-glucagon serum cross-reacting with a common cell type cannot be properly evaluated. Glucagon has been found to not cross-react with this same anti-GIP serum under radioimmunoassay conditions (Brown, pers. comm.). Similarly, immunohistochemical studies carried out on rat islets (Fujimoto et al. 1978, Smith et al. 1977) demonstrated no cross-reactivity for this antiserum with glucagon added for control procedures. In the light of

this evidence, the results reported here are contradictory.

In the first twenty-nine amino acids of the GIP molecule, there are several sequences which are also common to glucagon (Brown et al. 1975). It may therefore be possible that, despite any precautions taken, some alteration of the glucagon molecule occurred to permit its cross-reactivity with the anti-GIP serum. This change may have occurred as a result of enzymes still active in the exocrine portion of the tissue sections used. Another possibility is that the glucagon may have blocked the anti-GIP serum in some manner other than by binding to the antibody. Clearly, further studies need to be carried out to verify the designation of this cell type in the endocrine pancreas of Salmo gairdneri, as a glucagon/GIP cell.

The presence of a distinct population of clear cells in Salmo gairdneri merits further investigation of this cell type. Clear cells were first described in the guinea pig by Bensley (1911). They have no counterpart in other mammals, but are found frequently in teleost pancreatic islets (Brinn 1973). They have not been recognized as a distinct endocrine cell yet, on account of conflicting reports. Brinn (1973) maintains that their numbers vary with the fixative employed and that they may in fact be glucagon cells. He further states that the clear cells cannot be "considered as comprising a significant propor-

tion, if any, of the Brockmann body (islet organ) endocrine cell population". Although the clear cells did not stain specifically in Salmo gairdneri by any method employed, they did stain non-specifically following Grimelius' (1968) silver impregnation and the PAP technique for glucagon and GIP. A granular cytoplasm was revealed in the clear cells following these two staining techniques. That this granular material represents a secretory granule population is speculative at best. However, in Fugu rubripes (Kobayashi et al. 1976) and Xiphophorus helleri (Klein and Lange 1977), clear cells which were also unstainable, had a distinct secretory granule population when viewed under the electron microscope. In Salmo gairdneri, clear cells were not easily discerned in Bouin's fixed tissue, whereas they were immediately identifiable in glutaraldehyde fixed, resin embedded tissue. The controversy over their existence may be due in part to the different methods of tissue preparation employed.

The difficulties encountered here in the use of tinctorial stains, may be due in part to the lack of specificity of these stains, for the peptide hormones in each cell type. Epple and Lewis (1973) maintain that aldehyde fuchsin stains only the granular membranes. The same is

true for Grimelius' (1968) silver impregnation technique (Vassallo et al. 1971). Hellman and Hellerstrom's (1960) stain for somatostatin cells is not even understood (Klein and Lange 1977). Furthermore, these staining techniques have been optimized in other laboratories, using other species (generally mammals).

Although many staining techniques may be followed verbatim, many more require experimenting to optimize their use. It is for these reasons that the PAP technique appears to be a more preferable alternative. Although teleost cell types localized with antibodies to mammalian hormones must be qualified in terms of identity, the reaction is understood, acceptable control procedures can be carried out and the final product has excellent contrast for photographic reproduction.

CHAPTER II

The localization of zinc within the pancreatic islets of *Salmo gairdneri* using ^{65}Zn autoradiography

Introduction

The role of zinc in the pancreatic islets has been debated by many authors in the past (Maske 1957, Voigt 1958, Phil 1967, Petkovand Galabowa, 1968), Petkov, 1970) and it is now known to be bound to stored insulin within the insulin cells (Greider et al., 1969, Howell et al., 1969). However, the importance of zinc in insulin storage is poorly understood. In cultured mouse islets for example, the removal of zinc from the incubation media does not interfere appreciably with either insulin biosynthesis or storage (Howell et al., 1978). Furthermore, zinc is more prevalent in the glucagon cells in the rat and it is altogether absent from the islets of both sheep and cattle (Petkov 1970). Therefore, it appears that zinc is not a requirement of all vertebrate insulin cells and that in those species lacking zinc, it has most likely been supplanted by another heavy metal (Petkov 1970).

In teleosts, zinc has been found in the insulin cells of Gadus callarias (Grant et al., 1971) and in the islets of Cottus scorpius (Falkmer et al., 1970), but its role in the pancreatic islets has not been investigated. In Salmo gairdneri, the islet organ also contains zinc (Szeto, pers. comm.) however, its origin (exocrine or endocrine) has not yet been determined.

The purpose of this study was to determine if zinc is found in the insulin cells of Salmo gairdneri, using a modi-

fied version of Timm's (1958) silver-sulphide technique in combination with ^{65}Zn autoradiography.

Materials and Methods

Rainbow trout, Salmo gairdneri Richardson, were obtained and maintained as described in Chapter 1. At the start of the experiment, four groups (N = 2 or 3) of fish (20 - 40 grams) were transferred to individual, five gallon glass aquaria containing zinc-free water. This water was prepared by adding NaCl, KCl, $CaCl_2$ and $MgSO_4$ to glass distilled water in quanities equivalent to those found in Burnaby tap water. This water was determined to be zinc free (< 40 ppb) by atomic absorption spectrophotometry (Pye Unicam, Model SP 191). The fish were not fed during the experiment and the tanks were flushed daily with fresh, zinc-free water. Kept in this water, the normal losses of zinc via the urine and feces would take place without being replaced. The fish were maintained in these tanks for ten days during which time each fish received an intraperitoneal glucose injection equivalent to their total plasma glucose every second day. Total plasma glucose calculations were made based upon a hematocrit of 42% (Watson 1975) and a total blood volume equalling 3% of body weight (Randle 1960). The specific gravity of plasma was considered to be 1.0 for these calculations.

As insulin in teleosts is secreted in response to raised plasma glucose levels (Ince 1979, Ince and Thorpe 1977b), these injections served to increase the turnover rate of pancreatic insulin and any zinc present within the insulin cells. Similarly, an increased turnover rate would also be expected for any zinc present in the glucagon cells. During conditions of starvation, the secretory activities of cells which synthesize gluconeogenic hormones, such as glucagon, are probably increased to maintain normal plasma glucose levels. Therefore, by increasing the turnover rate of pancreatic zinc and by limiting the sources of replacement, a percentage of an injection of ^{65}Zn should enter the pancreas to fulfill real physiological needs.

After ten days each fish in groups 2, 3 and 4 received an intraperitoneal injection of ^{65}Zn as zinc chloride (1 μCi/μg, sp. act.) equivalent to 0.9 μCi/gram body weight. The ^{65}Zn came solubilized in 0.1N HCl which was neutralized with 1N NaOH before use. The ^{65}Zn was allowed to be assimilated in the body tissues for 48 hours. Fish in group 1 received a saline injection.

Tissue Preparation. The islet organs from fish in Groups 1, 2 and 4 were processed according to a modified version (Popham and Webster 1976) of Timm's (1958) silver-sulphide method for localization of heavy metals. The fish were

stunned by a blow to the head and the islet organ, which is attached to the wall of the gall bladder, was removed. Each islet organ was placed in a separate test tube containing 5 mls. of ice-cold 3% glutaraldehyde in 0.1 M cacodylate, pH 7.4, for ten minutes (in all succeeding steps the islet organs were treated in test tubes containing 5 mls. of each solution). They were then transferred to 1% (V/V) ammonium sulphide for 13 minutes followed by fresh glutaraldehyde for six hours. The islet organs were then treated with fresh ammonium sulphide for 16 hours. The tissues were then rinsed in distilled water (three changes for a total of 60 minutes) to remove all of the residual sulphide. Islet organs from Groups 1 and 2 were then dehydrated in graded ethanol, cleared in propylene oxide and embedded in Spurr's epoxy resin. Islet organs from Group 4 were developed with a modified (Phil and Falkmer, 1967) Timm's (1958) developer according to Popham and Webster (1976). The developer was prepared by adding 34 mg. of quinol to 20 mls. of 30% (W/V) gum arabic containing 10% (W/V) sucrose. The pH of the solution was adjusted to 4.0 with 10% citric acid. Immediately before use, 0.2 mls. of 10% silver nitrate was added in complete darkness. Each islet organ was diced into 0.5 mm cubes and rinsed further with distilled water. The cubes were then transferred to the developer for 90 minutes with constant agitation. Following development, the tissues were

rinsed well in distilled water, dehydrated, cleared and embedded in Spurr's resin.

Islet organs from Group 3 were fixed in three changes of ice-cold 3% glutaraldehyde for a total of six hours and then washed, dehydrated, cleared and embedded in Spurr's resin.

The initial activity of each islet organ and the activity remaining in each of the solutions used were determined in a gamma well counter (Nuclear Chicago) by counting the test tube and its contents. For each step in a given procedure, the percentage of the initial ^{65}Zn activity leached into the solution used and the leaching rate were calculated. Table I compares the leaching of ^{65}Zn from islet organs treated with glutaraldehyde and ammonium sulphide versus glutaraldehyde alone. Table II shows the effects of development upon ammonium sulphide-precipitated ^{65}Zn.

Autoradiography. The islet organs from Group 2, in which the ^{65}Zn had been precipitated but which had not been developed, were used for autoradiography. Initially, 1.0 μ-sections were cut using wet glass knives and then were flattened onto slides on a drop of water with gentle heat. However, on processing these slides for autoradiography, all of the radioactivity was found to have leached out of the sections and onto the slides. Therefore, 1.0 μ-sections were cut on a Porter-Blume LKB Ultramicrotome using dry glass knives and the sections were flattened onto slides with a drop of acetone.

No leaching was observed using this technique. The slides were then carbon-coated to minimize chemography (Sechrist and Upson, 1974) and dipped vertically in Kodak NTB-2 Nuclear Track Emulsion at 45° C, and then were hung vertically overnight to dry in a light-proof box containing drierite. The next day, the slides were taped to the bottom of black plastic slide boxes (2 per box) which were then sealed with black tape and stored at 4° C. The humidity was kept to a minimum by a vial of drierite, which was taped inside the top of each box.

The autoradiograms were developed between 24 and 106 days after dipping. The slides were developed in Kodak D-19 developer for four minutes at 18° C, stopped in 30% sodium thiosulphate for fifteen minutes, rinsed in running water for thirty minutes and then air dried. The slides were dipped in complete darkness, while the boxing, development and stopping procedures were carried out under Wratten No. 2 illumination.

Control procedures included preparing autoradiograms with 'cold' sections from the islet organs in Group 1 and deliberately fogging some of the positive autoradiograms to test for latent image fading (Rogers 1973).

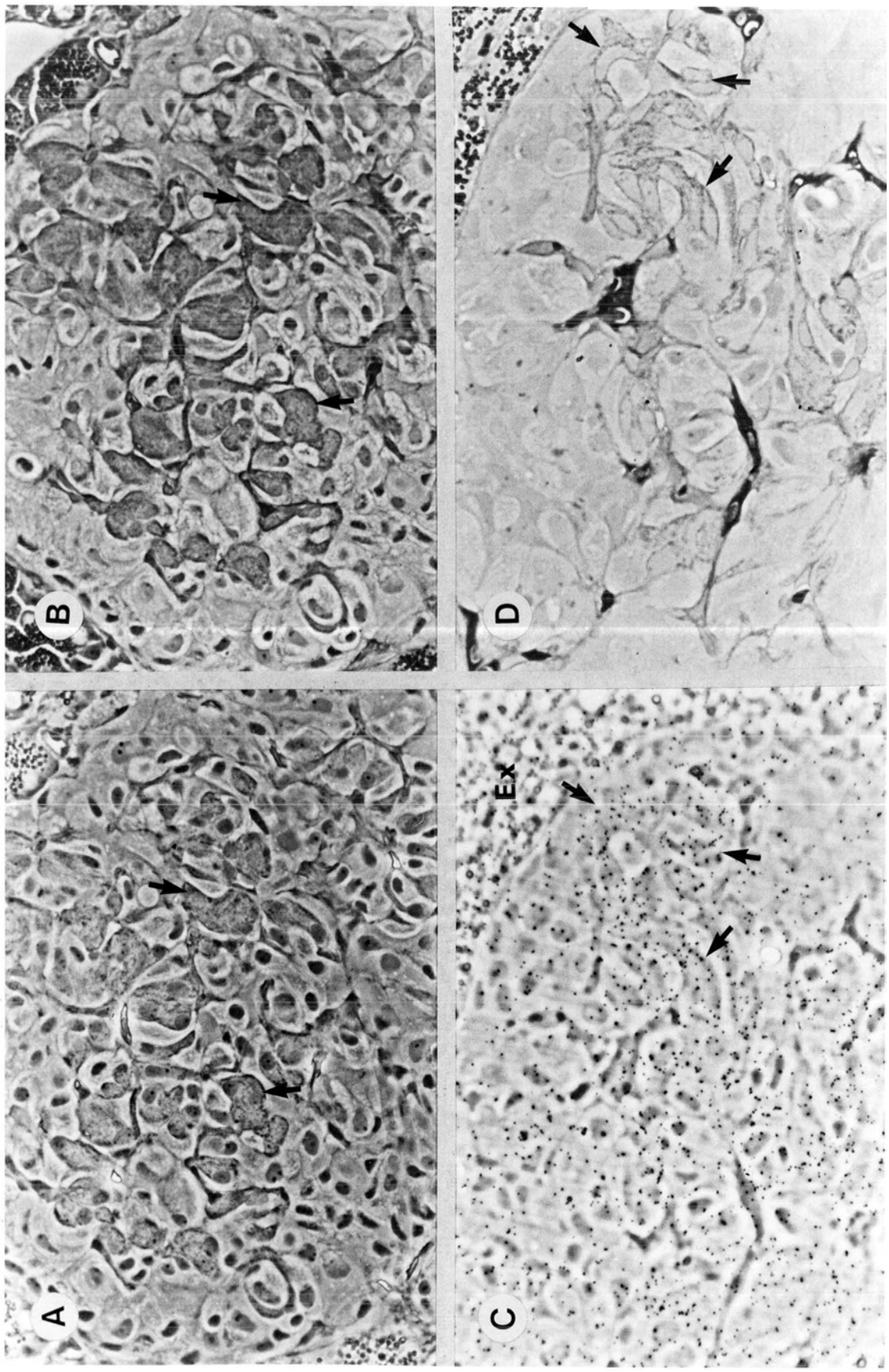
Grain Counting. Microscopic observations on the islet of this species revealed that the insulin cells appear much darker than the other cell types when viewed under phase contrast micro-

Figure 5. A) An unstained section of islet tissue from Salmo gairdneri viewed under phase contrast microscopy. The insulin cells (arrows) are easily identifiable in this preparation. Glutaraldehyde fixed, resin embedded. X365

B) The same section as in "A" stained with aldehyde fuchsin. Note that the same cells are stained (arrows). X365

C) A developed autoradiogram showing the distribution of grains over the islet tissue. The grains are concentrated over specific loci (arrows). X445

D) The same section as in "C" in which the emulsion has been removed and the section stained with aldehyde fuchsin. Note that the location of the insulin cells (arrows) corresponds to areas underlying the grain concentrations in "C". Glutaraldehyde fixed, resin embedded. X445


B
D
A
Ex
C

scopy (Fig. 1) and thus could be identified in an unstained section. These were confirmed to be insulin cells by their selective staining with aldehyde fuchsin, which is specific for insulin cells in Salmo gairdneri (Chapter 1). This proved to be useful as, upon viewing the autoradiograms, it was evident that the grains were more prevalent over the darker, insulin cells. Therefore, as the grains over the other cells appeared to be distributed at random, it was decided to compare the grain density over the insulin cells with that over the 'non-insulin' cells. This was accomplished as follows.

Areas of varying grain density on the autoradiograms were photographed (Carl Zeiss, Photomicroscope) with the grains in focus, at powers of 100X and 160X. The nuclear track emulsion was then removed with 0.5% potassium hydroxide (Rogers 1973) and the resin was dissolved from the sections with sodium methoxide (Mayor et al., 1961). The slides were then stained with aldehyde fuchsin (Scott 1952) and mounted with Permount (Fisher Sc. Co.). These stained slides were then re-photographed in the same locations and at the same magnifications as before. A micrometer scale mounted on a slide (Carl Zeiss) was also photographed at these magnifications. All of the negatives were then printed the same size (5 x 7 inches). The photos of the micrometer scale were used to construct a grid of squares on clear plastic.

The area enclosed by each grid square equaled 400 μm^2 for photographs taken at 100X and 156 μm^2 for those taken at 160X.

To count grains over aldehyde fuchsin positive, insulin cells, the grid was first taped in place over a photograph of the stained section. The insulin cells were then outlined on the plastic with a marking pen. Several reference points which were common in both photographs (generally capillaries) were traced over as well, but in another colour. The grid was then removed, re-positioned over the photograph of the unstained section (with the assistance of the reference points) and secured in place. The grid squares within the insulin cell regions and over the non-insulin cell regions were then counted for total grains. To eliminate any possible subjectiveness in the selection of grid squares, the whole photograph was counted in each case.

Because all of the autoradiograms from Group 2 exhibited identical patterns of grain distribution, only a few sections were counted. However, the islet organ consists of numerous smaller islets fused together and, therefore, each section that was counted actually represented a large number of islets. For the first islet organ, a total of 251 grid squares (77,800 μm^2) overlying the islet tissue were counted. For the second islet organ, 248 grid squares (38,700 μm^2) overlying the islet tissue were counted. All of the data were

adjusted so that grain density is expressed as the number of grains/400 μm^2. Further information on the grain counting may be found in Table III.

Results

Of the total activity injected into each fish 3.9±1.59% (N = 8) was taken up by the islet organ. This represents a substantial proportion of the total as the islet organ weighs only a few milligrams in these fish.

The treatment of tissues in Group 2 with ammonium sulphide (without development) resulted in a mean total loss of 49.98% of the initial ^{65}Zn activity compared with 68.05% for the tissues fixed in glutaraldehyde alone (See Table I). Most of this activity was lost during the first five steps of tissue preparation (fixation to water wash). In step 1, the glutaraldehyde fixation resulted in double the leaching rate of ^{65}Zn from Group 3 tissues compared with Groups 2 and 4. In step 2, the leaching rate for Groups 2 and 4 in ammonium sulphide was greater than that of the Group 3 tissues in glutaraldehyde. Hereafter, the leaching rates declined in all groups but persisted much longer in ammonium sulphide treated tissues. Of the ^{65}Zn lost in total by the sulphide treated tissues, 43.8% was lost during steps 1 - 5. For Group 3, 64%, of the 68% lost in total was lost during the same sequence.

TABLE I

A Comparative Study on the Effects of Glutaraldehyde and Ammonium Sulphide versus Glutaraldehyde alone on the Leaching of ^{65}Zn from the Islet Organ of Salmo gairdneri.

	1% Ammonium Sulphide (Groups 2,4)*					Glutaraldehyde (Group 3)				
Step	Fluid	Min. in Fluid	% Leached	±S.D.	Leaching Rate %/Min.	Fluid	Min. in Fluid	% Leached	±S.D.	Leaching Rate %/Min.
1	Glut.	10	11.84	4.48	1.184	Glut.	10	23.62	8.55	2.36
2	$(NH_4)_2S$	13	7.74	2.82	0.595	Glut.	10	3.34	4.10	0.334
3	Glut.	360	9.01	1.96	0.025	Glut.	350	36.80	4.02	0.105
4	$(NH_4)_2S$	960	15.08	1.53		D.W.	966	0.36	0.13	0.0004
5	D.W.	32	0.131	0.136		D.W.	52	0.289	0.07	0.05
			43.80	(Sub)				64.12	(Sub)	
6	D.W.	15	0.02	0.05	0.0017	D.W.	15	N.D.	-	-
7	D.W.	15	0.098	0.13	0.006	D.W.	15	N.D.	-	-
8	E-75%	1020	0.335	0.07	0.0003	E-75%	1020	N.D.	-	-
9	E-95%	75	N.D.	-	-	E-95%	75	N.D.	-	-
10	E-100%	150	N.D.	-	-	E-100%	150	N.D.	-	-
11	E-100%	60	N.D.	-	-	E-100%	60	N.D.	-	-
12	P.O.	60	0.06	0.08	0.001	P-O.	60	N.D.	-	-
13	P.O.	60	N.D.	-	-	P.O.	1905	N.D.	-	-
14	P.O.-R.	1905	4.62	0.05	0.002	P.O.-R.	1440	3.02	2.19	0.0015
15	P.O.-R.	1440	0.83	0.18	0.0005	P.O.-R.	1080	0.54	0.04	0.0004
16	R.	1080	0.155	0.08	0.0001	R.		0.09	0.099	0.00008
			6.18	(Sub)				3.93	(Sub)	
			49.98	(Total)				68.05	(Total)	

*Steps 1-5 include groups 2 and 4. Hereafter Group 2 was embedded for autoradiography while Group 4 was developed. See Table II for the effects of development upon the leaching of ^{65}Zn from the islet organs in Group 4.

Abbreviations: D.W. (Distilled Water), E. (Ethanol), Glut. (Glutaraldehyde), N.D. (Non-Detectable); 35 counts per minute), $(NH_4)_2S$ (Ammonium Sulphide), P.O. (Propylene Oxide), R. (Spurr's Resin), S.D. (Standard Deviation), Sub. (Sub-total).

Comparatively little activity was lost during the dehydration and embedding (steps 8 - 16) in either group 2 (6.18%) or group 3 (3.93%).

The subsequent development of Group 4 (Table II) resulted in the further loss of 53.14% of the initial ^{65}Zn activity. Leaching occurred primarily during the development and subsequent washing of the tissues. When these losses are combined with those in steps 1 - 5, a total loss of 96.64% of the initial ^{65}Zn in the tissues is obtained. This clearly demonstrates that the zinc is displaced from the tissue during development.

Grain counting revealed a pronounced concentration of grains over the insulin cells (Table III). In the first islet organ the insulin cells had a grain density of 34.05 compared with 14.01 for the non-insulin cells. This represents a 2.4 fold difference in grain density. In the second islet organ, the insulin cells had a grain density of 32.74 compared with 16.56 for the non-insulin cells. This represents a 1.97 fold difference in density.

Of the cells comprising the non-insulin cell fraction (glucagon/GIP, somatostatin, pancreatic polypeptide and clear cells) only the clear cells tended to accumulate zinc. However, this was observed in only some cells.

TABLE II

A Study on the Effects of Development on Ammonium Sulphide-Precipitated ^{65}Zn in the Islet Organ of Salmo gairdneri Richardson

Step	Fluid	Development (Group 4) Min. in Fluid	% Leached S.D.		Leaching Rate %/Min.
6*	D.W.	185	N.D.	-	-
7	D.W.**	105	1.73	0.49	0.016
8	D.W.	60	8.07	4.58	0.134
9	Dev.	90	20.06	2.60	0.222
10	D.W.	30	7.98	1.10	0.266
11	D.W.	10	6.43	2.46	0.643
12	D.W.	10	5.36	1.77	0.536
13	E-50%	30	1.14	0.59	0.038
14	E-70%	825	0.95	0.33	0.001
15	E-95%	235	0.30	0.06	0.001
16	E-100%	65	0.12	0.20	0.002
17	E-100%	45	0.81	0.54	0.018
18	P.O.	30	0.03	0.05	0.001
19	P.O.	75	N.D.	-	-
20	P.O.-R.	315	0.04	0.06	0.0001
21	P.O.-R.	1440	0.10	0.11	0.00006
22	R.	1080	0.02	0.03	0.00002
			53.14	(Sub)	
			43.80	(Sub; Steps 1-5, Table I)	
			96.94	(Total)	

* See Table I for steps 1-5 in which the tissues are treated with ammonium sulphide and glutaraldehyde.

** Tissues were diced into 0.5 mm cubes.

Abbreviations: D.W. (Distilled Water), Dev. (Developer), E. (Ethanol), N.D. (Non-Detectable; 35 counts per minute), P.O. (Propylene Oxide), R. (Spurr's Resin), S.D. (Standard Deviation), Sub. (Sub-Total).

TABLE III

A Comparison of Grain Counts Over Insulin and Non-Insulin Cells

Islet Organ		No. of Grid Squares Counted	Total Area Counted -μm^2	Total Grains Counted	Density (Grains/400μm^2)
1)	Insulin Cells	117	36.230	2878	34.05±2.9
	Non-Insulin Cells*	134	42,630	1414	14.01±3.0
	Background	184	73,600	367	3.08±1.1
2)	Insulin Cells	124	19,375	1572	32.75±3.0
	Non-Insulin Cells	124	19,375	802	16.56±1.9
	Background	144	22,500	365	2.54±2.0

* glucagon/GIP, somatostatin, pancreatic polypeptide and clear cells

Discussion

Zinc has been localized histochemically with dithizone (Mager et al., 1953, McNary 1954, Rixon and Whitfield 1958), Timm's (1958) silver sulphide method, and modifications thereof (Haug 1967, Okamoto and Kawanashi 1966, Phil 1967, Phil and Falkmer 1967) and by autoradiography with ^{65}Zn (Bawden and Hammarstrom 1977, McIssac 1955). Dithizone is reputed to be specific for zinc (Mager et al., 1953), however Petkov (1970) maintains that zinc present in tightly bound enzyme-metal complexes, escapes detection by this method. The silver sulphide method precipitates all heavy metals and therefore cannot be considered as being specific for zinc. However, autoradiography can provide an accurate distribution pattern of heavy metals in tissues, if diffusion of the isotope during tissue preparation can be minimized. Both freeze substitution (Gielink et al., 1966) and freeze drying followed by vacuum embedding (Wilske and Ross, 1965) have been used successfully in the autoradiography of diffusible substances. In this study, the silver sulphide technique was combined with autoradiography in an attempt to localize zinc in the pancreatic islets of Salmo gairdneri.

With the silver sulphide technique, the sulphide penetrates the tissue where it binds to heavy metals, rendering them insoluble. If the tissue is then treated with

a developer containing silver nitrate, the sulphide-metal complexes catalyze the reduction of ionic silver to molecular silver, which then coats each sulphide-metal complex (Brunk et al., 1968). The resulting silver grains are visible in the light microscope and the method has also been adapted for use in the electron microscope (Haug 1967, Okamoto and Kawanashi 1966, Phil 1967, Popham and Webster 1976). The tissue may be fixed briefly before sulphide treatment (Haug 1967, Popham and Webster 1976) or sulphide treatment may be combined with fixation (Brunk et al., 1968, Phil 1967, Phil and Falkmer 1966) with similar results.

In this study the tissue was treated with ammonium sulphide following a brief fixation. Several of the islet organs were also developed to determine the fate of the sulphide-metal complex. The results showed that ammonium-sulphide treatment reduces to some extent (18%) the leaching of ^{65}Zn from pancreatic islet tissue. However, this reduction in leaching obtained with sulphide treatment cannot all be attributed to sulphide precipitation, because in step 1, the leaching rate of Group 3 (untreated) tissues in glutaraldehyde was double that of Groups 2 and 4 which were also in glutaraldehyde. The Group 3 tissues lost a further 11% of their initial activity due to this excessive leaching. Therefore, the 18% reduction in leaching obtained with sulphide treatment is actually a 7% reduction and in spite of

this treatment, 49.98% of the initial activity was still lost.

One can only speculate about the sources of the leachate in this experiment. It may represent that fraction of the zinc which is loosely bound or even unbound within the cytoplasm and therefore most susceptible to leaching. In addition, the zinc may have leached from the cells and plasma of the islet organ microcirculation or from the exocrine pancreatic ducts. This is an important question that requires further studies to be resolved.

The subsequent development of sulphide treated tissues resulted in the loss of almost all (96.94%) of the initial activity. It appears then that the zinc is actually displaced, perhaps by the silver, during development, which contradicts Brunk and co-workers (1968), who stated that the sulphide-metal complex is coated with silver in situ.

Popham and Webster (1976) found that while embedding undeveloped sulphide treated tissues in epoxy resin a large proportion of the sulphide-metal complexes were lost. They attributed this to the oxidation of the complex by the resin. Phil (1967) also encountered losses during embedding. Results reported here substantiate in part these earlier findings. There were losses during the embedding process, but these occurred in both the untreated (3.93%) and ammonium sulphide treated (6.18%) tissues. However, when sulphide treated tissue was sectioned, extensive leaching was observed

from sections that were cut on wet glass knives. This problem was eliminated by using dry knives and by flattening the sections with acetone. Therefore, the sulphide-metal complex does appear to be de-stabilized by the embedding process. However, the onset of leaching (during embedding or sectioning) may be determined by the resin and organic solvent employed. Consequently, it may be advisable to develop the tissue before embedding it, when using the silver sulphide technique.

The results demonstrate conclusively the presence of zinc within the insulin cells of Salmo gairdneri. Twice the number of grains were found over these cells as compared with the non-insulin cells. This may reflect a preferential uptake of zinc by the insulin cells or the selective leaching of zinc from the non-insulin cells during tissue processing. However, the latter would imply that zinc is less tightly-bound in these cell types, which remains to be proven.

In fractionated islet tissue from the teleost, Gadus callarias, 32% of the detectable zinc was found in association with the secretory granules while the remainder was found primarily in the cytosol and nuclear fractions (Grant et al., 1971). Therefore, it may be assumed that the zinc in the insulin cells of Salmo gairdneri is involved with insulin storage, as in other vertebrates (Greider et al., 1969, Howell et al., 1969). In those clear cells which accumulated

zinc, the grains were often observed directly over the nucleus. The non-granular zinc may be associated with enzyme activity (Petkov and Galabowa, 1968).

CHAPTER III

The effects of zinc-induced hyperglycemia on carbohydrate metabolism and insulin secretion in *Salmo gairdneri* Richardson

Introduction

The effects of environmental pollutants on teleost physiology have been evaluated through changes in hematocrit, growth rate, carbohydrate reserves, serum electrolytes and plasma glucose and corticosteroids, to name a few. Many of these changes have been attributed to stress owing to the exposure of the fish to noxious or harmful substances in the water (Crandall and Goodnight 1962, 1963, Silbergeld 1974, Watson and McKeown 1976a, b). Sublethal concentrations of zinc, a common pollutant, have caused hyperglycemia in rainbow trout, Salmo gairdneri Richardson (Watson and McKeown, 1976a). While this has been attributed to stress and increased corticosteroid production, it may also be due to a direct effect of zinc upon carbohydrate metabolism. The insulin cells of Salmo gairdneri contain zinc, which is involved with insulin storage in many vertebrates (Greider et al., 1969, Howell et al., 1969, Petkov 1970). Therefore, exposure to zinc may also have a direct effect upon insulin storage and secretion. This study was designed to determine the effects of zinc-induced hyperglycemia upon insulin secretion and carbohydrate metabolism in Salmo gairdneri.

In a previous study, Watson (1975) exposed rainbow trout to sublethal zinc concentrations for 80 days. Plasma glucose was first monitored after one week of exposure and significant elevations were observed among zinc-treated fish. In this study, rainbow trout were exposed to three concentrations of

zinc, including a control (< 40 ppb Zn^{++}) for up to 31 days. Plasma glucose was monitored every second day for the first 11 days, and then every five days thereafter. With a glucose profile for each group, information such as the onset of hyperglycemic stress (which may occur before seven days) could be readily obtained. Those fish demonstrating the most severe hyperglycemia were then analyzed along with the controls for changes in plasma insulin and liver glycogen.

While many acute toxicity studies with zinc have been done on rainbow trout, it was necessary to determine the sensitivity of this stock to zinc in Burnaby water before doing the long-term exposure. Therefore an acute toxicity study was done to determine the 48-hour LC_{50}. This task was facilitated considerably with the knowledge of previously reported LC_{50} data for rainbow trout, exposured to zinc under similar conditions of water temperature and hardness. Once the LC_{50} was determined, sub-lethal and lethal concentrations were derived for the long-term exposure.

Materials and Methods

Rainbow trout, Salmo gairdneri Richardson, were obtained and maintained as described in Chapter 1.

Acute Toxicity of Zinc. A 48-hour LC_{50} was carried out in 760 l, flow-through fibre-glass tanks, to determine the acute toxicity of zinc in Burnaby water. A diluting apparatus,

described by Watson (1975), was constructed for each of three tanks, to ensure the delivery of a fixed concentration of zinc through the water supply. The water to each tank was supplied by a single fibre-glass headtank (120 l) which was continually repleted with dechlorinated Burnaby tap water. This headtank served to maintain a constant water pressure and flow rate (2.5 l/min.) for all diluting apparatus. The dilution factor of each apparatus was calculated (Watson 1975) using a stock solution of zinc (100 ppm). These dilution factors were then used to determine the concentration of zinc sulphate needed in each diluting apparatus to achieve the desired tank concentration. Three test concentrations were used to determine the LC_{50} value and a fourth tank (water also supplied by the headtank) served as a control.

Due to the length of the replacement time of the tank water (9.5 hours) it was not possible to acclimate the fish to the test tanks and then start the dilutors. Therefore, the fish were maintained in similar tanks and in the same room for their acclimation period (2 months). To commence the toxicity study, the dilutors were started and the water in all tanks was allowed to reach the test concentrations, determined by atomic adsorption spectrophotometry (Pye Unicam Model SP 191). Ten fish were then randomly selected and placed in each tank. The fish were not fed during the experiment. The tanks were examined at hourly intervals for the

first eight hours and then every four hours thereafter. Fish were considered to be dead when there was no opercular movement and no response to handling. These fish were removed from the tanks and weighed. An analysis of variance was performed on the weights of the fish from each test tank. Temperature, pH, hardness, oxygen and zinc concentration were determined once each during the first and second day of the experiment. Oxygen was measured by the Winkler method, hardness by the EDTA titrimetric method (Taras et al., 1971), and zinc by atomic adsorption spectrophotometry. The physical and chemical properties of the water may be found in Table IV. The percent mortalities after 48 hours were plotted against zinc concentrations on log-probit paper. A regression line was then drawn through these points and tested for goodness of fit. The LC_{50} was then derived from this line and the 95% confidence limits were calculated, according to Litchfield and Wilcoxon (1949).

Long Term Exposure. For the protection of fish and other forms of aquatic life, the concentration of zinc in the water should not exceed 1/100 of the LC_{50} value at any time. This ensures a safe concentration of zinc, including an allowance for sublethal effects. However, to establish strictly sublethal concentrations, the zinc content of the water should not exceed 1/10 of the LC_{50} value. This ensures against mortality, but not sub-lethal effects (Ontario Ministry of

TABLE IV

Physical and Chemical Properties* of the Water Used to Determine the Acute Toxicity of Zinc (48-h LC_{50}) to Salmo gairdneri

Tank	Zinc Concentration mg/L ±S.D.	Oxygen Concentration Range mg/L	Temperature** Range °C	pH Range	Hardness (EDTA)* Range mg/L
1	0.40±0.010	6.0-7.5	12.0-12.5	6.5-6.8	6.0-6.5
2	0.95±0.001	6.2-7.6	12.0-12.5	6.7-6.8	6.0-6.5
3	1.36±0.043	6.3-8.0	12.0-12.5	6.3-6.5	6.0-6.5
4	Control < 0.04	6.0-8.0	12.0-12.5	6.2-6.6	6.0-6.5

* Determined once each during first and second day of experiment

** Determinations made from one tank

the Environment 1974). For the long term exposure (31 days), zinc concentrations that were above and below 1/10 of the 48-hour LC_{50} of 0.99 ppm were desired. Four tanks were used in the experiment, three containing test concentrations of zinc (0.081, 0.096 and 0.352 ppm) and the fourth serving as the control (< 0.04 ppm of zinc).

Eighty fish with a mean length of 20.5±2.7 cm (standard deviation) were placed in each tank and then allowed to acclimate for two months. The tanks were the same as those used in the LC_{50} experiment and were supplied with dechlorinated tap water. Water anlyses for temperature, pH, hardness, ozygen and zinc were done on each day that fish were sampled (days 1, 2, 3, 5, 7, 9, 11, 16, 21, 26 and 31). The physical and chemical characteristics of the water are shown in Table V. The fish were fed daily ad libitum with Oregon Moist Pellets. Following the acclimation period, each dilutor was set up to deliver $ZnSO_4$ through the water supply to achieve the desired tank concentrations. Because of the large number of fish to be sampled from each tank (8) on any given day, Tanks 1 (0.081 ppm) and 3 (0.352 ppm) were started one day in advance of Tanks 2 (0.096 ppm) and 4 (control). It was considered that any physiological differences that might occur over 24 hours as a result of monthly or circannual rhythms would be negligible. On the sampling days eight fish were removed from each tank except on day 2, when five fish were

TABLE V

Physical and Chemical Properties of the Water Used in the Long Term Exposure of Rainbow Trout to Different Concentrations of Zinc*

Tank	Zinc Concentration mg/L ±S.D.	Oxygen Concentration Range mg/L	Temperature** Range °C	pH Range	Hardness (EDTA)* Range mg/L
1	0.081±0.0019	6.9-7.8	7.6-8.3	6.5-6.7	6.0-6.5
2	0.096±0.043	5.3-7.2	7.6-8.3	6.3-6.5	6.0-6.5
3	0.352±0.015	6.9-9.2	7.6-8.3	6.7-6.8	6.0-6.5
4	Control < 0.04	6.0-8.0	7.6-8.3	6.2-6.5	6.0-6.5

* Determinations made on each day (N = 11) that fish were sampled.

** Determinations made from one tank.

removed from tanks 3 and 4 respectively. To minimize the effects of circadian rhythms, all sampling was done at the same time of day (9:30 - 11:30 hrs.). Furthermore, fish were removed alternately from each of the two tanks being sampled from.

Each fish was netted and a blood sample was taken from the caudal artery with a syringe rinsed in 6% potassium oxalate. The fish were then stunned by a blow to the head and the liver and islet organ were removed. These tissues were immediately frozen in a slurry of dry ice and acetone and then stored in capped vials at -20° C. The blood was centrifuged, the plasma decanted and then stored also at -20° C.

Plasma glucose was assayed in duplicate for each fish on 10 µl aliquots of plasma, using the Hexokinase-Glucose-6-Phosphate Dehydrogenase assay kit (Sigma). A single plasma blank determination was also made for each sample to eliminate errors arising from the variable turbidity of the plasma. Glucose values were read from a standard curve (30 - 250 mg%) after the subtraction of the blank values. The data were analyzed by the Student-Newman-Keuls test and groups were considered to be significantly different if $P < 0.05$.

Liver glycogen was measured enzymatically in each fish from tanks 3 (0.352 ppm) and 4 (control) on days 1, 3, 5, 7 and 9, according to Murat and Serfaty (1974), with minor

modifications. These authors found it necessary to assay for endogenous glucose-6-phosphate in the liver homogenate before hydrolysis with amyloglucosidase. They then subtracted this amount from the final glucose determination to give a more accurate measurement of liver glycogen. In Salmo gairdneri, the concentration of glucose-6-phosphate in the homogenate before hydrolysis was found to be less than 0.1% and this step was therefore omitted. The glucose liberated by hydrolysis was assayed in duplicate as before. The data were interpreted by analysis of variance and groups were considered significantly different if $P < 0.05$.

Plasma insulin determinations were made for each fish from Tanks 3 (0.352 ppm) and 4 (control) on days 1, 2, 3, 5, 7 and 9. Insulin was measured by a charcoal separation radioimmunoassay using an antibody generated in guinea pigs against anglerfish (Lophius piscatorus) insulin (Johnson et al., 1977) and anglerfish insulin for the labelled antigen and unlabelled standards. Both components were supplied by Dr. G. E. Bauer, Dept. of Anatomy, University of Minnesota, Minneapolis, U. S. A.

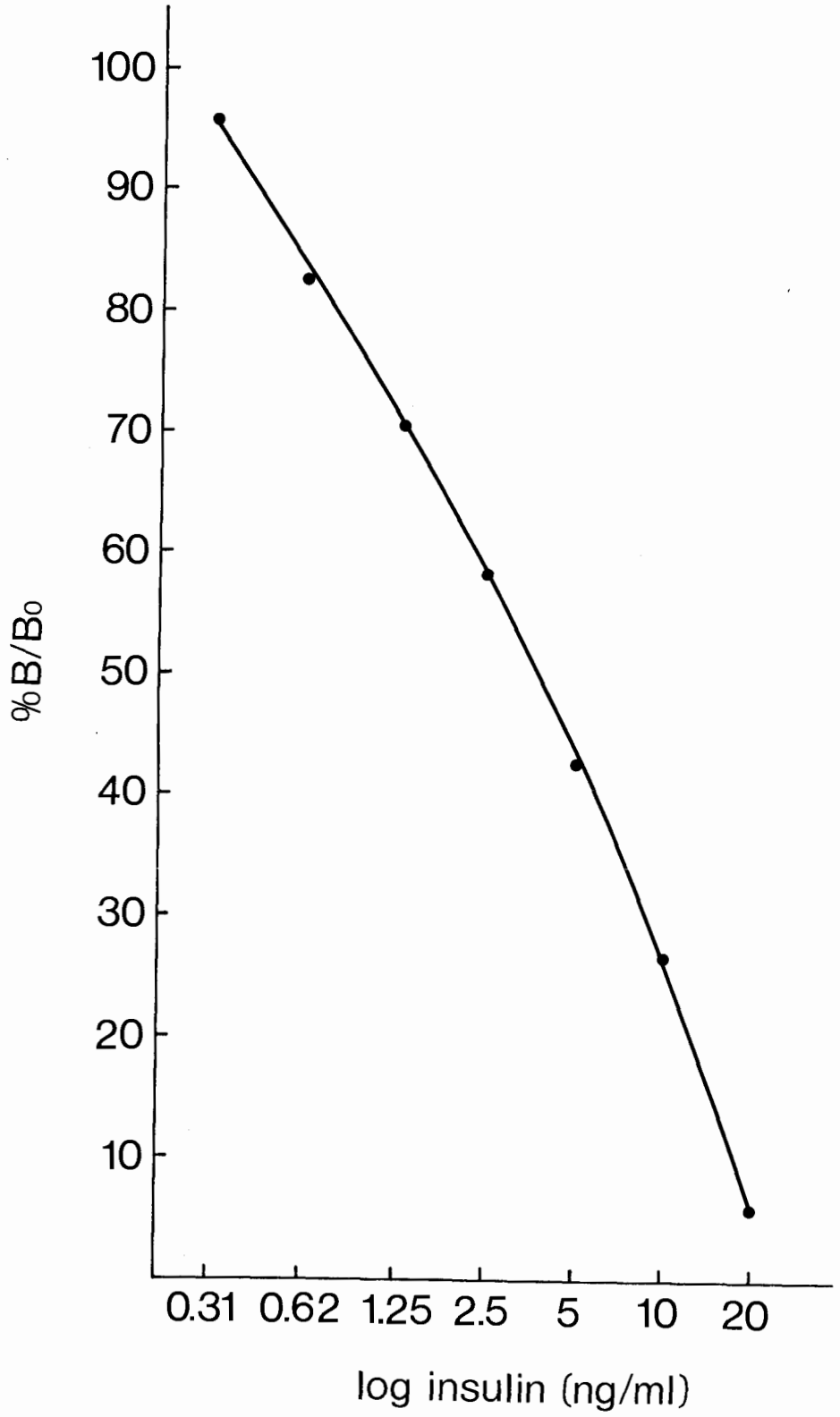
The insulin was iodinated (^{125}I, 11-17 mCi/μg, sp. act., Amersham) by the Chloramine-T procedure (Greenwood et al., 1963) except that the oxidation was allowed to proceed for only thirty seconds. The labelled insulin was separated from the free iodide with QUSO (microfine silica, G32,

Philadelphia Quartz Co.). The QUSO (20 mg) was added to the iodination mixture with 1.8 ml of 0.04 M phosphate buffer, pH 7.5, to give a total volume of approximately 2 ml. The mixture was vortexed for thirty seconds and then centrifuged at 1000 g for one minute. The supernatant was decanted, and the pellet was re-suspended and washed (twice) with 2 ml of phosphate buffer. The final pellet was then re-suspended in 5 ml of HCl-Ethanol (50 ml Ethanol, 17 ml H_2O, 1 ml conc. HCl) and vortexed for thirty seconds. Then 2 ml of distilled water were added and the mixture centrifuged at 1000 g for ten minutes. The supernatant containing the labelled insulin was decanted and stored at -20° C. The incorporation of ^{125}I was approximately 30%.

The conditions of the radioimmunoassay were similar to those described by Thorpe and Ince (1976). The diluent used was a 0.04 M phosphate buffer, pH 7.5, containing 1% bovine serum albumin (BSA). Standard curves were set up to determine the optimum antibody titre and label concentration. For the assay, 10,000 cpm per tube and an antibody dilution of 1:16,000 initial and 1:80,000 final were chosen. Under these conditions, 40% binding of the label was obtained in the absence of unlabelled insulin.

Disequilibrium conditions were chosen for the assay. To construct a standard curve (figure 6), 50 µl of each standard (0.31 - 20 ng/ml) and 25 µl of charcoal-extracted trout plasma were added to 12x75 mm glass test tubes along

Figure 6. Standard curve for measuring plasma insulin in rainbow trout using anglerfish insulin and antisera generated in guinea pigs against anglerfish insulin. The minimum detectable quantity of insulin is 0.31 ng/ml; at this concentration there is a significant ($P < 0.05$, Student's T-Test) displacement of the labelled antigen.


100
90
80
70
60
50
40
30
20
10
%B/B0
0.31
0.62
1.25
2.5
5
10
20
log insulin (ng/ml)

with 100 µl of antisera (1:16,000) and 225 µl of diluent buffer. These tubes were equilibrated for 24 hours at 4° C at which time 100 µl of labelled insulin (10,000 cpm/100 µl) were added. The tubes were allowed to equilibrate a further 18 hours at 4° C and then the antibody bound, labelled insulin was separated from the unbound, labelled insulin with dextran-coated charcoal. The charcoal was prepared by adding 5 g of activated charcoal (Norit A) to 100 ml of 0.04 M phosphate buffer, pH 7.5, containing 0.5% Dextran (Sigma). The solution was stirred overnight before use. Each tube received 200 µl of the dextran-charcoal, was vortexed and then allowed to sit for ten minutes at 4° C. They were then centrifuged to 1000 g for 30 minutes, the supernatant decanted and the tubes counted in a gamma well counter (Nuclear Chicago). The zero-standard tubes contained 50 µl of diluent buffer instead of an insulin standard. Similarly, for the non-specific binding (NSB) tubes, the antiserum and insulin standard aliquots were replaced by equivalent volumes of buffer. The limits of detection of the assay were 0.31 ng/ml.

For the determination of plasma insulin, 25 µl aliquots of plasma were added to test tubes containing 100 µl of antiserum (1:16,000) and 275 µl of diluent buffer. These tubes were then treated identically to the standard curve tubes. All points on the standard curve and all plasma insulin deter-

minations were done in duplicate. Plasma samples which contained less than 0.31 ng/ml of insulin were assigned concentrations according to their %B/Bo value. Samples which had a %B/Bo value between 100 and 97.5% were considered to contain 0.15 ng/ml, while samples between 97.5 and 95% contained 0.31 ng/ml. In this way, all samples could then be treated statistically.

Charcoal-extracted plasma was added to the standard curve tubes to equalize their protein concentration with that of the unknown tubes. This was prepared by mixing heparinized trout plasma with Norit A (1 g/ml) for one hour. The charcoal was then removed by centrifugation and filtration (Millipore (U. S. A.) Ltd., type HA, pore size 0.45 μ).

The specificity of the assay for plasma insulin in trout was determined by parallelism studies. Serial dilutions (1/1 to 1/8) of trout plasma and anglerfish insulin, made up in charcoal-extracted trout plasma, were assayed and the results were plotted on a log-logit plot. A regression line was then calculated for each set of points and the slopes were compared using the Student's t-test (Zar 1974). The slopes were considered significantly different if $P < 0.05$. Recovery studies, in which anglerfish insulin (1.25 ng/ml) was added to trout plasma, were also done.

Correlation analyses (Zar 1974) were performed between plasma glucose and liver glycogen, plasma glucose and insulin and plasma insulin and liver glycogen.

Results

$\underline{LC_{50}}$. The regression line was determined to be a good fit ($P < 0.01$) and 48-hour LC_{50} of 0.99 ppm of zinc was derived from this line (Fig. 7). The upper and lower confidence limits of the LC_{50} were 1.73 and 0.56 ppm respectively. No differences were found between the weights of the fish from each test tank ($P > 0.05$).

Long Term Exposure. No mortalities occurred in Tanks 1, 2 and 4 during the experiment. In Tank 3 (0.352 ppm zinc) mortalities commenced after twenty-four hours and continued until day 7. The fish in Tank 3 were depleted by day 9 because of sampling and mortalities.

The mean plasma glucose levels for each group of fish are shown in Figure 8. At 0.081 ppm of zinc, plasma glucose ranged from 57.5 (day 26) to 89.0 mg% (day 16) and was significantly higher than controls on day 1 only. At 0.96 ppm of zinc, plasma glucose ranged from 51 (day 3) to 123 mg% (day 21) and was significantly higher than control levels on days 1 and 16. At 0.352 ppm of zinc, glucose levels ranged from 81 (day 9) to 151 mg% (day 5) and were significantly higher than controls on days 1, 2, 3 and 5. In this group glucose levels rose from days 1 to 5. Thereafter, the levels declined until day 9. The plasma glucose response of the fish exposed to zinc at this concentration was extremely

Figure 7. The acute toxicity of zinc to rainbow trout after 48 hours. Ten fish were used at each concentration of zinc (0.40, 0.95 and 1.36 ppm). A 48-hour LC_{50} of 0.99 ppm of zinc was derived from the regression time. The upper and lower confidence limits of the LC_{50} were 1.73 and 0.56 ppm respectively.

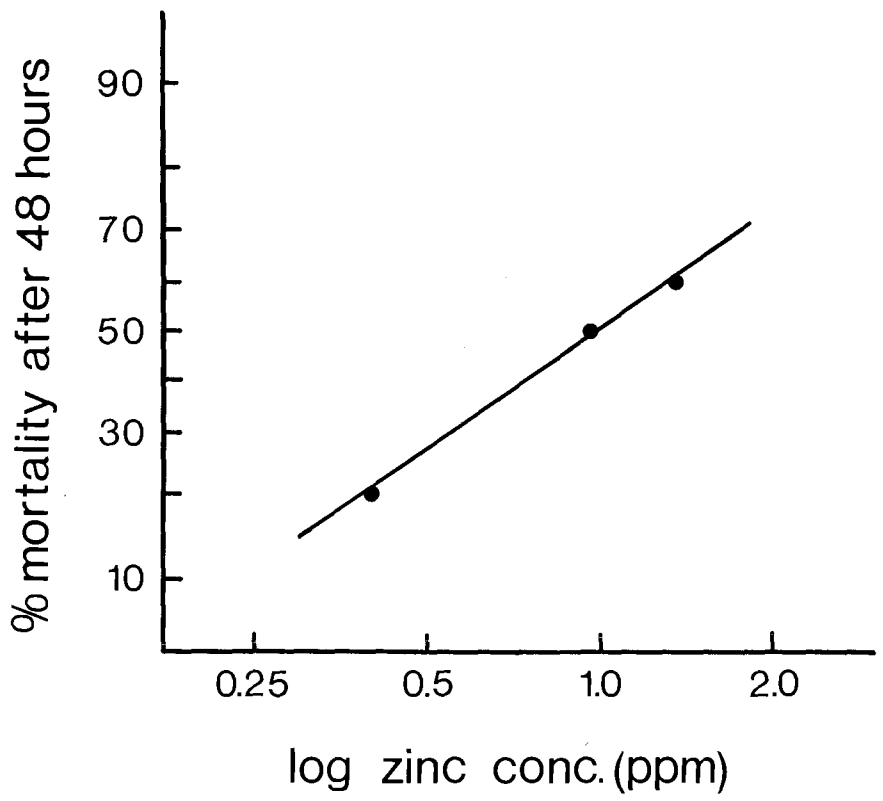
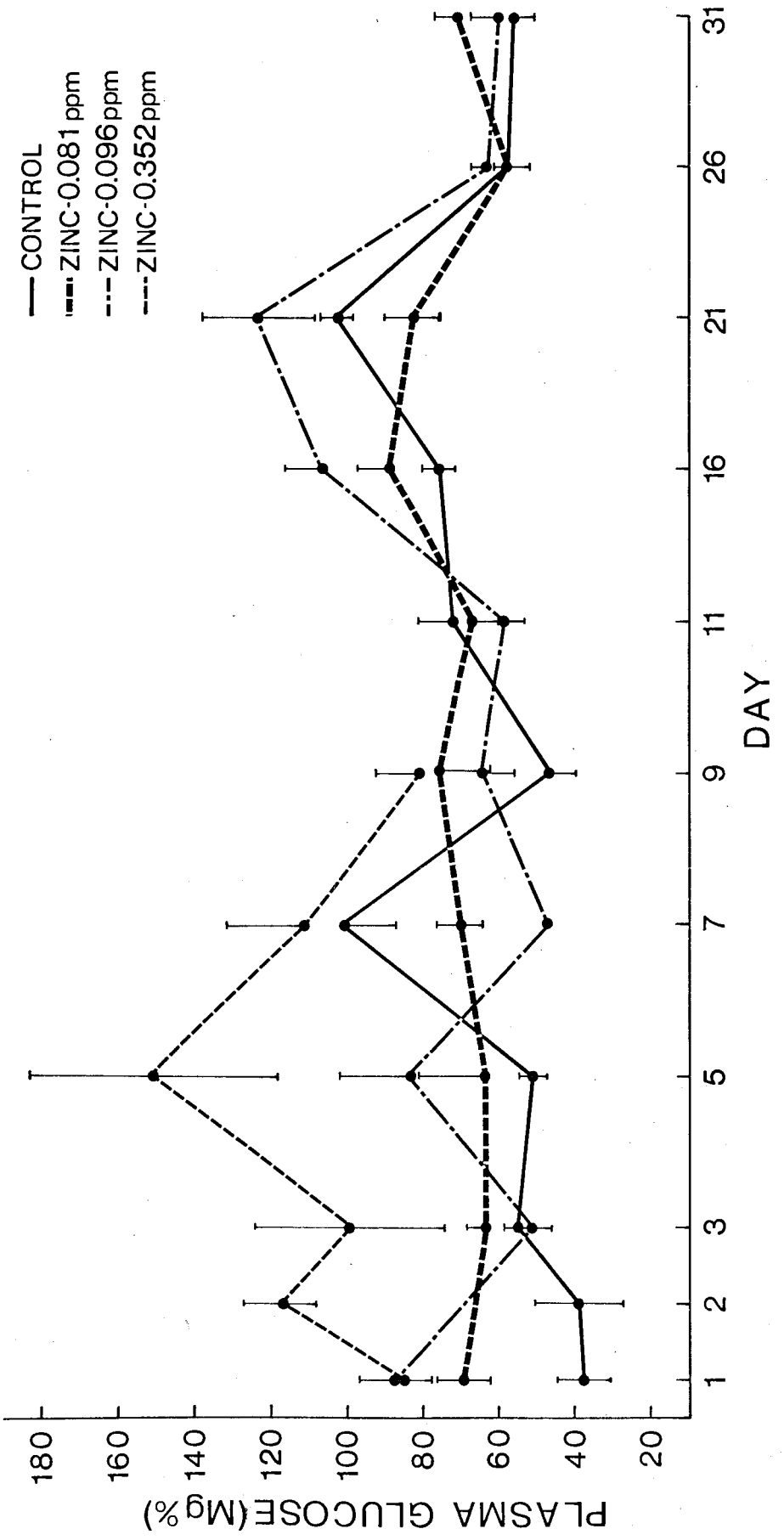

% mortality after 48 hours
90
70
50
30
10
0.25
0.5
1.0
2.0
log zinc conc. (ppm)

Figure 8. The mean plasma glucose levels (± standard error) for Salmo gairdneri exposed to different concentrations of zinc for up to 31 days. Significant differences from control levels are as follows: 0.081 ppm zinc group, day 1 (0.05); 0.096 ppm zinc group, day 1 (0.01), day 16 (0.05); 0.352 ppm zinc group, day 1, 2 and 5 (0.01), day 3 (0.05). Also, on days 3 and 5, the 0.352 ppm group was significantly different than the 0.081 and 0.096 ppm groups (0.05). On day 21, the 0.096 ppm group was significantly different than the 0.081 ppm group (0.05). Plasma glucose levels in the controls on day 7 were significantly higher than control levels on days 5 (0.001) and 9 (0.005). Similarly, on day 21, glucose levels in the controls were significantly higher (0.001) than control levels on days 16 and 25.
N = 7 or 8 in all cases except for groups on day 2 (N = 5), for the 0.352 ppm group on day 9 (N = 3) and for the 0.096 ppm group on day 7 (N = 1).

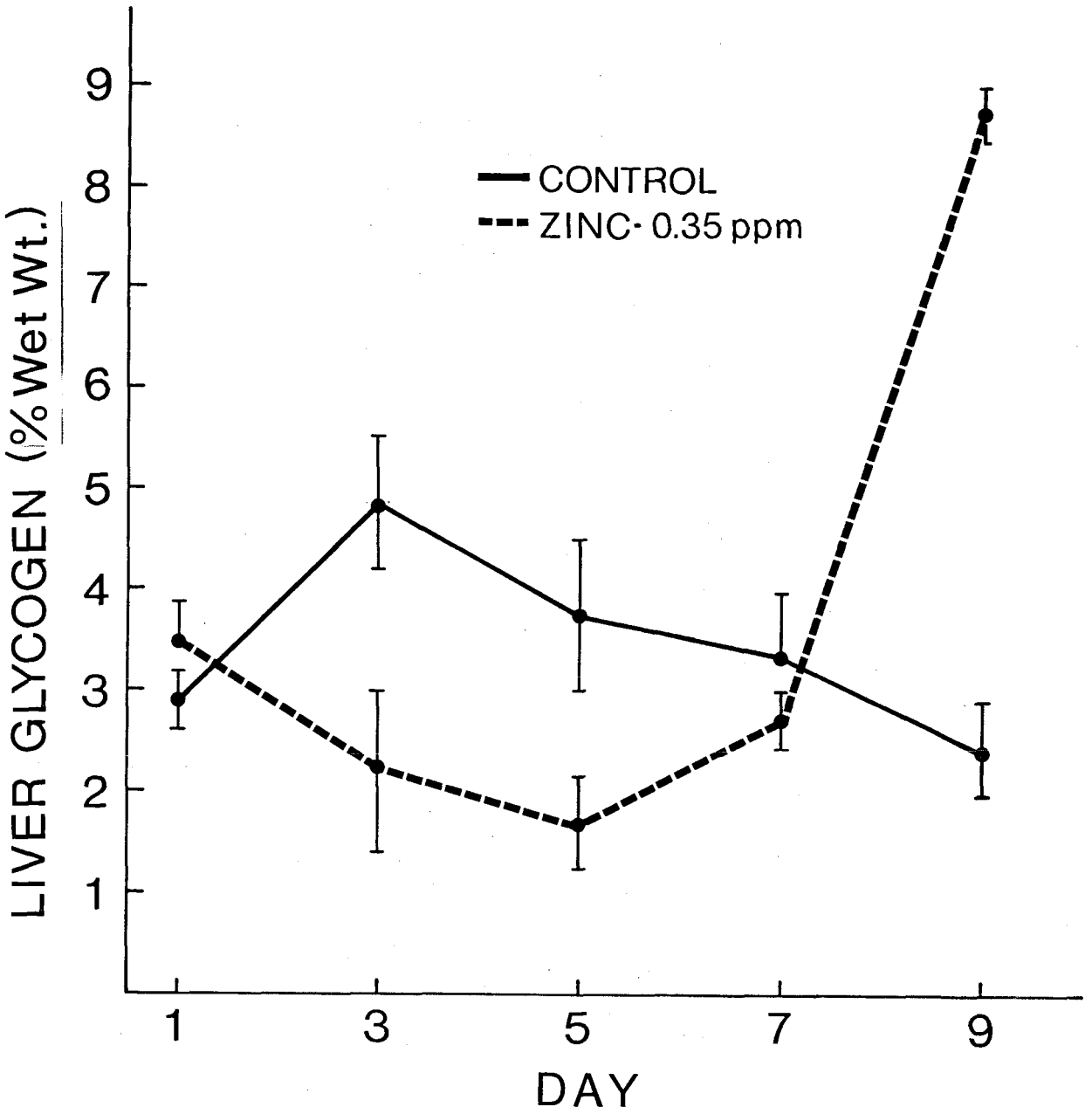
NOTE: After day 11, the scale changes to intervals of five days.


CONTROL
ZINC-0.081 ppm
ZINC-0.096 ppm
ZINC-0.352 ppm
PLASMA GLUCOSE (Mg%)
DAY
180
160
140
120
100
80
60
40
20
1
2
3
5
7
9
11
16
21
26
31

variable as indicated by the large standard errors on days 3 and 5. In the control group, plasma glucose ranged from 37 to 101 mg% and peaked twice during the experiment; on day 7 (March 19) and day 21 (April 2). These peaks were of equal height (101 and 102 mg% respectively) and were exactly fourteen days apart. Plasma glucose on day 7 was significantly higher than levels on days 5 ($p < 0.001$) and 9 ($p < 0.005$). Similarly, on day 21 glucose levels were significantly higher ($p < 0.001$) than on days 16 and 25. Plasma glucose increased in a stepwise manner preceding each peak and then dropped off sharply afterwards. These peaks may be part of a monthly glucose cycle in Salmo gairdneri. Further evidence of this is the similarity in glucose profiles between the experimental and control groups between days 11 and 31. There is a general rise in plasma glucose for all groups after day 11. In the controls, glucose peaks on day 21 (103 mg%), drops to 57 mg% on day 26 and remains stable until day 31. In the 0.096 ppm group, the rise in plasma glucose occurs earlier with levels significantly higher than controls on day 16 but the peak (123 mg%) also occurs on day 21. In the 0.081 ppm group, the peak (89 mg%) occurs on day 16 instead. For both zinc-treated groups, plasma glucose drops to control levels on day 26 and remains relatively stable until the termination of the experiment.

Liver glycogen levels for the controls and the 0.352 ppm zinc group are shown in Figure 9. Liver glycogen ranged

Figure 9. The mean liver glycogen levels (± standard error) for control and zinc-treated (0.352 ppm) rainbow trout over nine days. Glycogen levels in the zinc-treated fish as compared to control fish are significantly lower on days 3 and 5 ($p < 0.05$) and significantly higher on day 9 ($p < 0.005$). N = 8 in all cases except on day 9 (N = 3).


LIVER GLYCOGEN (% Wet Wt.)
9
8
7
6
5
4
3
2
1
CONTROL
ZINC- 0.35 ppm
1
3
5
7
9
DAY

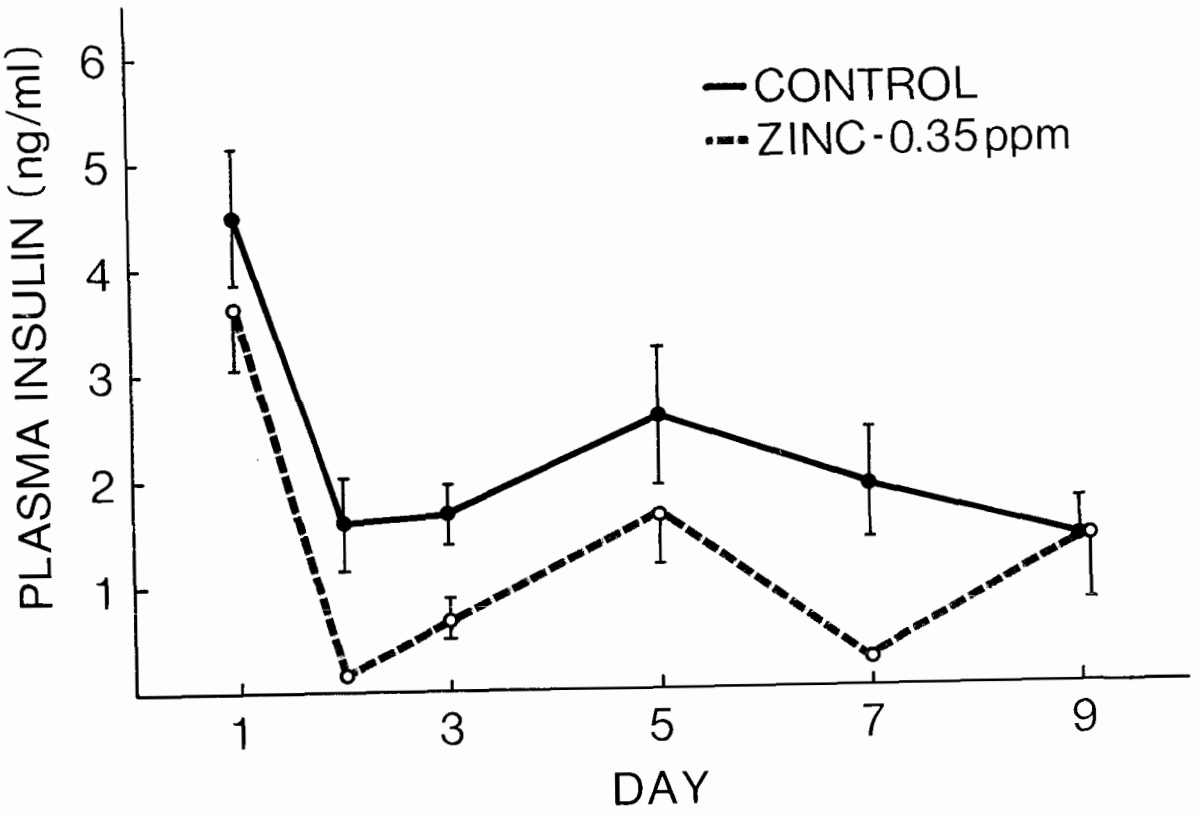
from 2.4 to 4.8% in the controls with the highest levels occurring on day 3. At 0.352 ppm of zinc, glycogen ranged from 1.7 to 8.7% and was significantly lower than controls on days 3 and 5. Glycogen levels dropped progressively from the first day of zinc exposure until day 5. Thereafter they rose to highly significant levels ($P < 0.005$) on day 9.

The radioimmunoassay was determined to be specific for insulin in Salmo gairdneri by parallelism studies. No significant differences ($P > 0.05$) were found between the slopes of serially diluted trout plasma and anglerfish insulin. Recovery of anglerfish insulin added to trout plasma was 88 - 94%.

Plasma insulin levels for the control and the 0.352 ppm zinc group over the first nine days of the experiment are shown in Figure 10. Insulin levels are lower in fish exposed to 0.35 ppm of zinc over the first seven days, with significant differences occurring on days 2, 3 and 7. On day 9, the levels are the same in both groups. In the control group, plasma insulin ranged from 1.4 to 4.5 ng/ml and in the zinc treated group from 0.17 to 3.6 ng/ml. The profiles of plasma insulin were very similar for both groups over the first seven days.

No significant correlations ($P > 0.05$) were found between any of the parameters tested.

Figure 10. The mean (± standard error) plasma insulin levels for control and zinc-treated (0.352 ppm) rainbow trout over nine days. Plasma insulin levels in the zinc-treated fish as compared to the controls are significantly lower on days 2 ($P < 0.05$), 3 ($P < 0.02$) and 7 ($P < 0.01$).
N = 7 or 8 in all cases except on day 2 (N = 5) and for the zinc-treated fish on day (N = 3).
Standard errors were too small to be included for the zinc group on days 2 and 7.


PLASMA INSULIN (ng/ml)
6
5
4
3
2
1
CONTROL
ZINC - 0.35 ppm
1
3
5
7
9
DAY

Discussion

The 48-hour LC_{50} of 0.99 ppm of zinc reported here is in agreement with previously reported studies with Salmo gairdneri under similar conditions of water temperature and hardness (Herbert and Sherben 1964, Sinley et al., 1974).

Many previous studies have demonstrated the deleterious effects of heavy metal pollution upon teleost fish (Brown and Dalton 1970, Gardner and Tevitch 1970, Greig et al., 1977, Nehring and Goettl 1974, McKim and Benoit 1971, McKim et al., 1970, Sprague et al., 1965, Sinley et al., 1974, Watson and McKeown 1976a,b). Zinc at sublethal concentrations, for example, caused significant elevations in plasma glucose in Salmo gairdneri after seven days (Watson and McKeown 1976a). This hyperglycemia was attributed to stress, due to the exposure to zinc, which causes increased corticosteroid production in teleosts (Donaldson and Dye 1975, Mazeud et al., 1977, Watson and McKeown 1976a,b). Corticosteroids are gluconeogenic in teleosts (Butler 1968, Butler 1973, Freeman and Idler 1973, Inui and Yokote 1975, Chan and Woo 1978) and elevated levels would therefore result in increased hepatic glucose output. In this study, there were significant elevations in plasma glucose of fish exposed to 0.081 ppm, 0.096 ppm and 0.352 ppm of zinc after one day. Increased plasma corticosteroids may therefore have contributed to the observed hyperglycemia.

Lactic acid is a major carbon source for gluconeogenesis in zinc-stressed fish. Stress and eventual death in Salmo gairdneri exposed to lethal concentrations of zinc have been attributed to tissue hypoxia, resulting from damage and mucous buildup on the gill epithelia. Concomitant with the development of hypoxia are increased tissue and plasma levels of lactic acid (Burton et al., 1972). In this study trout exposed to 0.352 ppm of zinc demonstrated the most severe hyperglycemia. As this zinc concentration proved to be lethal, it is therefore likely that the fish were hypoxic and that blood lactic acid levels were elevated. This lactic acid would have contributed substantially (through gluconeogenesis) to the observed hyperglycemia.

Plasma catecholamines are also elevated in teleosts subjected to various forms of stress (Mazeud et al., 1977, Nakano and Tomlinson 1967) and epinephrine, in particular, is a potent hyperglycemic agent. In Salmo gairdneri (Terrier and Perrier 1975), Esox lucius (Thorpe and Ince 1974) and Anguilla anguilla (Ince and Thorpe 1977a) for instance, epinephrine causes a rapid rise in plasma glucose. This occurs through the activation of hepatic glycogen phosphorylase, whereby glycogen is hydrolyzed and released into the bloodstream as glucose (Thorpe and Ince 1974, Umminger and Benziger 1975). Concomitant with the rise in plasma glucose in trout exposed to 0.352 ppm of zinc, liver glycogen levels

dropped and were significantly lower than controls on days 3 and 5. The observed glycogen depletion may consequently be due to increased plasma levels of epinephrine and the associated activation of glycogen phosphorylase.

Insulin potentiates glucose-induced glycogen synthesis in mammals and is also necessary for the maintenance of basal rates of synthesis (El-Refai and Bergman 1976). In fishes, insulin also has an important role in carbohydrate metabolism. Insulin lowers plasma glucose (Ince and Thorpe 1974, Thorpe and Ince 1974) and hyperglycemia and glycogen depletion occur during conditions of insulin lack (Plisteskaya et al., 1976, Thorpe 1976). In this study, trout exposed to 0.352 ppm of zinc had depressed plasma insulin levels for the first seven days, with significant differences on days 2, 3 and 7. This reduction in plasma insulin may have contributed to the observed hyperglycemia and glycogen depletion. In addition, epinephrine inhibits insulin secretion in teleosts, with resulting hyperglycemia (Ince and Thorpe 1977a). The depressed insulin levels may therefore have been due in part to raised plasma epinephrine levels.

In trout exposed to 0.081 and 0.096 ppm of zinc changes in plasma insulin and liver glycogen were not monitored. However, the observed hyperglycemia in these groups may also have been due to changes in plasma insulin and epinephrine, in addition to activation of the pituitary-interrenal axis.

On day 9 of the experiment, insulin levels in trout exposed to 0.352 ppm of zinc returned to control levels. Plasma glucose also dropped to non-significant levels while liver glycogen rose to highly significant levels ($P < 0.005$). Although the sample size for this group was small ($N = 3$), a trend towards acclimation is evident. Plasma insulin and glucose levels are normalizing and there is repletion of liver glycogen. However the overextent to which liver glycogen is restored on day 9 is not easily explained. In mammals, an increase in plasma glucose stimulates hepatic glycogen production (Dewulf and Hers 1967, El-Refai and Bergman 1976). If the same is true in teleosts, then the observed hyperglycemia in zinc-treated trout should have stimulated glycogen synthesis. In actual fact, however, no repletion was evident until day 7, and this was coincident with a decline in plasma glucose. It is therefore possible that elevated plasma epinephrine and lowered plasma insulin levels effectively prevented any synthesis up to this point.

The drop in plasma glucose on days 7 and 9 may have been coincident with a decline in catecholamine levels as the fish adjusted to the zinc concentration. This would then have allowed normal stimulation of glycogen synthesis by glucose. Furthermore, the rise in plasma insulin would probably have potentiated this synthesis.

Studies carried out on young dogs in which hypoxia was experimentally induced, demonstrated a catecholamine

induced suppression of insulin release coupled with severe hyperglycemia. This hyperglycemia was attributed to a decreased peripheral utilization of glucose (due to low insulin levels) and increased liver glycogenolysis by direct sympathetic stimulation and raised plasma epinephrine (Porte and Robertson 1973). If this response of dogs to hypoxia is compared to the response of Salmo gairdneri exposed to 0.352 ppm of zinc and presumed to be also hypoxic, there is a striking similarity in the physiological responses.

As zinc is an important trace element in insulin storage in mammals (Greider et al., 1969, Howell et al., 1969) and is found in appreciable quantities in the insulin cells of Salmo gairdneri (Chapter 2), a direct effect of the metal upon insulin secretion in zinc-exposed fish cannot be ignored. Zinc may enter the fish by way of the gills (Hodson 1974) and especially the intestine, as the food pellets invariably become contaminated with the test water. The islet organ of Salmo gairdneri has been shown to accumulate injected ^{65}Zn (Chapter 2) and therefore, it may also accumulate zinc in fish exposed to and ingesting higher than normal levels of the metal. While Watson (1978) found no increases in plasma zinc of Salmo gairdneri exposed to sub-lethal zinc concentrations over thirty days, other studies with teleosts and elasmobranchs have demonstrated significant levels of tissue accumulation over time (Flos et al., 1979, Nakatani 1966, Skidmore 1964).

It is possible that plasma levels of zinc are closely regulated and that any excess accumulation is simply shunted to organs, such as the liver, kidneys and pancreas, for storage or excretion. In this study an accumulation of zinc within the insulin cells may have contributed to the lowered plasma insulin levels.

Seasonal and circadian variations in carbohydrate metabolism have been investigated in several teleosts to date. While there is conflicting evidence in regard to a circadian rhythm for plasma glucose (Leatherland et al., 1974, Narasimhan and Sundararaj 1971, Shapiro and Hoffman 1975), seasonal variations in plasma glucose (Leach and Taylor 1977, McKay and Beatty 1968, Robertson et al., 1961) and liver and muscle glycogen (Foda 1974, Gordon and McLeay 1978, Valtonen 1974) have been well documented. However, monthly variations in carbohydrate metabolism have not been investigated to our knowledge. In this study, two peaks in plasma glucose of equal height and fourteen days apart occurred in the control group over 31 days. Each of these peaks was significantly higher than the mean plasma glucose levels on the sampling days preceding and following it. Seasonal changes in plasma glucose have been correlated with plasma cortisol in salmonids (Robertson et al., 1961) and with temperature and spawning in Fundulus heteroclitus (Leach and Taylor 1977). In Salmo gairdneri, changes in glycogen reserves occur

independently of photoperiod and have been attributed to either growth or innate rhythmicity (Gordon and McLeay 1978). As there were no significant fluctuations in temperature during this experiment and sampling was done at the same time each day, temperature and circadian effects may be ruled out. The changes in plasma glucose observed may therefore reflect an internal biological rhythm operating on a monthly cycle. However, further studies are necessary to confirm or deny this hypothesis.

Several radioimmunoassays employing teleost insulin components have been developed during the past decade (Patent and Foa 1971, Pilsetskaya et al., 1976, Thorpe and Ince 1976). In this study, a charcoal-separation radioimmunoassay, employing anglerfish insulin and anti-anglerfish insulin serum, was used to measure plasma insulin in Salmo gairdneri. While it is acceptable to use a radioimmunoassay in a heterologous fashion as such, the specificity of the assay for other species must be verified. Thorpe and Ince (1976) verified the specificity of their assay, employing codfish insulin components, by recovery and parallelism studies. In this study, 88 - 94% recovery was obtained of anglerfish insulin added to trout plasma. This indicates that there is minimal interference by the trout plasma in the measurement of insulin. Furthermore, the assay was determined to be specific for a rainbow trout insulin by parallelism studies.

GENERAL SUMMARY AND CONCLUSIONS

This study was undertaken to answer three basic questions in the area of trout pancreatic physiology:

1) What hormone-producing cells are present in the pancreatic islets of the rainbow trout?
2) Do the insulin cells contain zinc?
3) What effects do zinc-induced hyperglycemia have upon glycogen metabolism and insulin secretion in this species?

Using immunocytochemical staining methods, four different cell types were localized; insulin, glucagon/GIP, somatostatin and pancreatic polypeptide cells. A fifth cell type, the clear cell, was also found in large numbers. The function of this cell type is not known and it will therefore be the subject of further investigations.

Zinc was found in higher concentration (two-fold) in the insulin cells than in any other cell type. While the function of this metal is not known, previous studies in fish and mammals would indicate that it is important in the storage of insulin.

Zinc-induced hyperglycemia in trout is accompanied by hepatic glycogen depletion and depressed plasma insulin levels. The lowered insulin levels may be due to hormonal influences or to the action of zinc on the insulin cells. These observed

changes may be short-term survival-orientated adaptations to toxicant stress. On the long-term however, they may have serious consequences for fish in the natural environment.

BIBLIOGRAPHY

Bawden, J. W. and Hammarstrom, L. E. 1977. Autoradiography of ^{65}Zn in developing rat teeth and bones. Archs. oral. Biol. 22, 449-454.

Bensley, R. R. 1911. Studies on the pancreas of the Guinea Pig. Am. J. Anat. 12:297-388.

Brinn, J. E. 1973. The pancreatic islets of bony fishes. Amer. Zool. 13, 653-665.

Brinn, J. E. 1975. Pancreatic islet cytology of Ictaluridae (Teleostei). Cell Tiss. Res. 162, 357-365.

Brown, J. C., Dept. of Physiology, U. B. C.

Brown, J. C., Dryburgh, J. R., Ross, S. A., and Dupre, J. 1975. Identification and actions of gastric inhibitory polypeptide. Recent. Prog. Hormone Res. 31, 487-532.

Brown, V. M. and Dalton, R. A. 1970. The acute lethal toxicity to rainbow trout of mixtures of copper, phenol, zinc and nickel. J. Fish. Biol. 2, 211-216.

Brunk, U., Brun, A., and Skold, G. 1968. Histochemical demonstration of heavy metals with the sulphide-silver technique. A methodological study. Acta Histochem. (Jena) 31, 345-357.

Burton, D. T., Jones, A. H. and Cairns, J. 1972. Acute zinc toxicity to rainbow trout (Salmo gairdneri): confirmation of the hypothesis that death is related to tissue hypoxia. J. Fish. Res. Bd. Canada 29:1463-1466.

Butler, D. G. 1968. Hormonal control of gluconeogenesis in the North American eel, Anguilla rostrata. Gen. Comp. Endocrinol. 10, 85-71.

Butler, D. G. 1973. Structure and function of the adrenal gland of fishes. Amer. Zoo. 13:839-879.

Chan, D. K. O. and Woo, N. Y. S. 1978. Effect of glucagon on the metabolism of the eel, Anguilla japonica. Gen. Comp. Endocrinol. 35, 216-225.

Crandall, C. A. and Goodnight, C. J. 1962. Effects of sub-lethal concentrations of several toxicants on growth of the common guppy, Lebistes reticulataus. Trans. Amer. Mic. Soc. 82, 59-73.

Crandall, C. A. and Goodnight, C. J. 1962. Effects of sublethal concentrations of several toxicants on growth of the common guppy Lebistes reticulatus. Limnol. and Ocean. 7, 233-239.

De Wulf, H. and Hers, H. G. 1967.. The stimulation of glycogen synthesis and of glycogen synthetase in the liver by the administration of glucose. European J. Biochem. 2:50-56.

Donaldson, E. M. and Dye, H. M. 1975. Corticosteroid concentrations in sockeye salmon (Oncorhynchus nerka) exposed to low concentrations of copper. J. Fish. Res. Bd. Canada 32, 533-539.

El-Refai, M. and Bergman, R. N. 1976. Simulation study of control of hepatic glycogen synthesis by glucose and insulin. Am. J. Physiol. 231: 1608-1619.

Epple, A. and Brinn, J. E. 1975. Islet histophysiology: evolutionary correlations. Gen. Comp. Endocrinol. 27, 320-349.

Epple, A. and Lewis, T. L. 1973. Comparative histophysiology of the pancreatic islets. Am. Zool. 13, 567-590.

Falkmer, S. 1961. Experimental diabetes research in fish. Acta Endocrinol. 37, Suppl. 59, 1-122.

Falkmer, S., Havu, N. and Boquist, L. 1970. Role of sulphydryl compounds and heavy metals in the synthesis and storage of insulin. In "The Structure and Metabolism of the Pancreatic Islets", Falkmer, S., Hellman, B. and Taljedal, I.-B. (eds.), 203-212, Pergamon Press, Oxford.

Flos, R., Caritat, A. and Balasch, J. 1979. Zinc content in organs of dogfish (Scyliorhinus canalicula L.) subject to sublethal experimental aquatic zinc pollution. Comp. Biochem. Physiol. 64C, 77-81.

Floyd, J. C., Fajans, S. S., Pek, S. and Chance, R. E. 1978. A newly recognized pancreatic polypeptide; plasma levels in health and disease. Recent Prog. Hormone Res. 34, 519-570.

Foda, A. 1974. Seasonal variation in blood parameters and muscle and liver glycogen of hatchery-reared Atlantic salmon (Salmo salar). Technical report Series No. MAR/T-74-3. Resource Development Branch, Fisheries and Marine Service, Dept. of Environment, Canada.

Freeman, H. C. and Idler, D. R. 1973. Effects of corticosteroids on liver transaminases in two salmonids (Salmo gairdneri) and the brook trout (Salvelinus fontinalis). Gen. Comp. Endocrinol. 20, 69-75.

Fujimoto, W. J., Ensinck, J. W., Merchant, F. W., Williams, R. H., Smith, P. H. and Johnson, D. G. 1978. Stimulation by GIP of insulin and glucagon secretion by rat islet cultures. Proc. Soc. Expt. Biol. Med. 157, 89-93.

Fujita, T., Yanatori, Y. and Murakami, T. 1976. Insuloacinar axis, its vascular basis and functional and morphological changes caused by CCK-PZ and caerulin. In "Edocrine, Gut and Pancreas", ed. T. Fujita, pp. 347-357, Amsterdam: Elsevier.

Gairdner, B. M. 1969. Deteriorated paraformaldehyde: an insidious cause of failure of aldehyde fuchsin staining. Stain Tech. 44, 52-53.

Gardner, G. R. and Yevitch, P. P. 1970. Histological and hematological responses of an estuarine teleost to cadmium. J. Fish. Res. Bd. Canada 27, 2185-2196.

Gielink, A. J., Sauer, G., and Ringoet, A. 1966. Histoautoradiographic localization of calcium in oat plant tissues. Stain Technol. 41, 281-286.

Gordon, M. R. and McLeay, D. J. 1978. Effect of photoperiod on seasonal variations in glycogen reserves of juvenile rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. 60, 349-351.

Graham, B. C. and Karnovsky, M. J. 1966. The early stages of adsorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14, 291-302.

Grant, P. T., Coombs, T. L., Thomas, N. W. and Sargent, J. R. 1971. The conversion of [^{14}C] proinsulin to insulin in isolated subcellular fractions of fish islet preparations. In "Subcellular Organization and Function in Endocrine Tissue", Heller, H. and Lederis, K. (eds.), pp. 481-496, Cambridge University Press.

Grant, P. T. and Reid, K. B. M. 1968. Isolation and partial amino acid sequence of insulin from the islet tissue of the cod (Gadus callarias). J. Biochem. 106, 531-541.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114-123.

Greider, M. H., Howell, S. L. and Lacy, P. E. 1969. Isolation and properties of secretory granules from rat islets of Langerhans II. J. Cell Biol. 41, 162-166.

Greig, R. A., Adams, A. E. and Nelson, B. A. 1977. Physiological response of the Cunnter, Tautogolabrus adspersus, to cadmium. II. Uptake of cadmium by organs and tissues. Reprinted from NOAA Technical Report NMFS SSRF-681.

Grimelius, L. 1968. A silver nitrate stain for α-cells of the human pancreas. Acta Soc. Med. Uppsalla 73, 243-270.

Haug, F.-M.S. 1967. Electron microscopal localization of the zinc in hippocampal mossy fibre synapses by a modified sulphide-silver procedure. Histochemie 8, 355-368.

Hellman, C. and Hellerstrom, C. 1961. Some aspects of the silver impregnation of the islets of Langerhans in the rat. Acta Endocrinol. 35, 518-532.

Herbert, D. W. M. and Shurben, D. A. 1964. The toxicity of fish to mixtures of poisons. I. Salts of ammonia and zinc. Ann. Appl. Biol. 53, 33.

Hodson, P. V. 1974. The effect of temperature on the toxicity of zinc to fish of the genus Salmo. Ph.D. Thesis, University of Guelph.

Howell, S. L., Tyhurst, M., Duvefelt, H., Andersson, A. and Hellerstrom, C. 1978. Role of zinc and calcium in the formation and storage of insulin in the pancreatic β-cell. Cell Tiss. Res. 188, 107-108.

Howell, S. L., Young, D. A. and Lacy, P. E. 1969. Isolation and properties of secretory granules from rat islets of Langerhans III. J. Cell Biol. 41, 167-176.

Ince, B. W. 1979. Insulin secretion from the in situ perfused pancreas of the European silver eel, Anguilla anguilla L. Gen. Comp. Endocrinol. 37, 533-540.

Ince, B. W. and Thorpe, A. 1974. The effects of insulin and of metabolite loading on blood metabolites in the European silver eel (Anguilla anguilla L.). Gen. Comp. Endocrinol. 23, 460-471.

Ince, B. W. and Thorpe, A. 1975. Hormonal and metabolite effects upon free fatty acids in the northern pike, Esox lucius L. Gen. Comp. Endocrinol. 27, 144-152.

Ince, B. W. and Thorpe. A. 1977a. Plasma insulin and glucose responses to glucagon and catecholamines in the European silver eel (Anguilla anguilla). Gen. Comp. Endocrinol. 33, 453-459.

Ince, B. W. and Thorpe, A. 1977b. Glucose and amino acid-stimulated insulin release in vivo in the European silver eel (Anguilla anguilla L.). Gen. Comp. Endocrinol. 31, 249-256.

Inui, Y., and Yokote, M. 1975. Gluconeogenesis in the eel: IV, Gluconeogenesis in the hydrocortisone administered eel. Bull. Japan Soc. Sci. Fish. 41, 973-981.

Inui, Y. and Yokote, M. 1977. Effects of glucagon on amino acid metabolism in japanese eels, Anguilla japonica. Gen. Comp. Endocrinol. 33, 167-173.

Johnson, D. E., Torrence, J. L., Elde, R. P., Bauer, G. E., Noev, B. D. and Fletcher, D. J. 1976. Immunohistochemical localization of somatostain, insulin and glucagon in the principal islets of the anglerfish (Lophius piscatorus) and the channel catfish (Ictalurus punctata). Am. J. Anat. 147, 119-124.

Klein, C. and Lange, R. H. 1977. Principle cell types in the pancreatic islets of a teleost fish, Xiphophorus helleri H. Cell Tiss. Res. 176, 529-551.

Klein, C. and Van Noorden, S. 1978. Use of immunocytochemical staining of somatostain for correlative light and electron microscopic investivation of D cells in the pancreatic islets of Xiphophorus helleri H. Cell Tiss. Res. 194, 399-404.

Kobayashi, K., Shibasaki, S. and Takahashi, Y. 1976. Light and electron microscopic study on the endocrine cells of the pancreas in a marine teleost, Fugu rubripes rubipres. Cell Tiss. Res. 1974, 161-182.

Lange, R. H., Ali, S. S., Klein, C. and Trandaburu, T. 1975. Immunohistological demonstration of insulin and glucagon in islet tissue of reptiles, amphibians and teleosts using epoxy-embedded material and antiporcine hormone sera. Acta Histochem. 52, 71-78.

Larsson, A. and Lewander, K. 1972. Effects of glucagon administration to eels (Anguilla anguilla L.). Comp. Biochem. Physiol. 43A, 831-836.

Leach, G. J. and Taylor, M. H. 1977. Seasonal measurement of serum glucose and serum cortisol in a natural population of Fundulus heteroclitus L. Comp. Biochem. Physiol. 56A, 217-223.

Leatherland, J. F., McKeown, B. A. and John, T. M. 1974. Circadian rhythm of plasma prolactin, growth hormone, glucose and free fatty acid in juvenile kokanee salmon, Oncorynchus nerka. Comp. Biochem. Physiol. 47A, 821-828.

Litchfield, J. T. and Wilcoxon, F. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96, 99-113.

MacKay, W. C. and Beatty, D. D. 1968. Plasma glucose levels of the white sucker Catostomus commersonii and the northern pike Esox lucius. Can. J. Zoo. 46, 797-803.

Mager, M., McNary, W. F. and Lionetti, F. 1953. The histochemical detection of zinc. J. Histochem. Cytochem. 1, 493-504.

Maske, H. 1957. Interaction between insulin and zinc in the pancreatic islets. Diabetes 6, 335.

Mayor, H. D., Hampton, J. C., and Rosario, B. 1961. A simple method for removing the resin from epoxy-embedded tissue. J. Biophysio. Biochem. Cytol. 9, 901-910.

Mazeaud, M. M., Mazeaud, F. and Donaldson, E. M. 1977. Primary and secondary effects of stress in fish: some new data with a general review. Trans. Amer. Fish. Soc. 106, 201-212.

McIsaac, R. J. 1955. The distribution of zinc^{65} in the rat pancreas. Endocrinology, 57, 571.

McKim, J. M. and Benoit, D. A. 1971. Effects of long-term exposures to copper on survival, growth and reproduction of brook trout, Salvelinus fontinalis. J. Fish. Res. Bd. Canada 28, 655-662.

McKim, J. M., Christensen, G. M. and Haut, E. P. 1970. Changes in the blood of brook trout, (Salvelinus fontinalis) after short-term and long-term exposure to copper. J. Fish. Res. Bd. Canada 27:1883-1889.

McNary, W. F. 1954. Zinc-dithizone reaction of pancreatic islets. J. Histochem. Cytochem. 2, 185-195.

Murnt, J. C. and Serfaty, A. 1974. Simple enzymatic determination of polysaccharide (glycogen) content of animal tissues. Clin. Chem. 20:1576-1577.

Nakano, T. and Tomlinson, N. 1967. Catecholamine and carbohydrate concentrations in rainbow trout (Salmo gairdneri) in relation to physical disturbance. J. Fish. Res. Bd. Canada 24:1701-1715.

Nakatani, R. E. 1966. Proceedings series Biology response of rainbow trout (Salmo gairdneri) ingesting zinc-65. Disposal of radioactive wastes into seas, oceans and surface waters. Proceedings of the symposium on Disposal Waters held by the International Atomic Energy Agency at Vienna.

Narasimhan, P. V. and Sundararaj, B. I. 1971. Circadian variation in carbohydrate parameters in two teleosts, Notopterus notopterus (Pallas) and Colisa fasciata (Block and Schneider). Comp. Biochem. Physiol. 39A, 89-99.

Nehring, B. R. and Goettl, J. P. 1974. Acute toxicity of a zinc-polluted stream to four species of salmonids. Bull. Environ. Contam. Toxicol. 12, 464-469.

Neumann, P. A. and Humbel, R. E. 1969. Isolation of a single component of fish insulin from a bonito-tuna-swordfish mixture and its complete amino acid sequence. Intern. J. Protein Res. 1, 125-140.

Neumann, P. A., Koldenhof, M. and Humbel, R. E. 1969. Amino acid sequence of insulin from the anglerfish (Lophius piscatorus). Z. Physiol. Chem. 350, 1286-1288.

Noe, B. D., Baste, C. A. and Bauer, G. E. 1977. Studies on proinsulin and proglucagon biosynthesis and conversion at the subcellular level. J. Cell Biol. 74, 578-588.

Okamoto, K. and Kawanashi, H. 1966. Submicroscopic histochemical demonstration of intracellular reactive zinc in cells of pancreatic islets. Endocrinol. jap. 13, 305-318.

Ontario Ministry of Environment. 1974. Guidelines and criteria for water quality management in Ontario. Supervised for publication by the Hon. William G. Newman, Minister, and Everett Biggs, Depty Minister, 135 St. Clair Avenue, West, Toronto, Ontario.

Orci, L. and Unger, R. H. 1975. Hypothesis: functional subdivisions of the islets of Langerhans and the possible role of the insular D-cells. Lancet 2, 1243-1244.

Patent, G. J. and Foa, P. P. 1971. Radioimmunoassay of insulin in fishes, experiments in vivo and in vitro. Gen. Comp. Endocrinol. 16, 41-46.

Pelletier, G. 1977. Identification of four cell types in the human endocrine pancreas by immunoelectron microscopy. Diabetes 26, 749-756.

Petkov, P. 1970. Comparative histochemical studies of mammalian pancreatic islets. In "The structure and Metabolism of the Pancreatic Islets", Falkmer, S., Hellman, B. and Taljedal, I.-B. (eds.), pp. 213-224, Pergamon Press, Oxford.

Petkov, P. and Galabowa, R. 1968. Sur le role biologique du zinc dans les ilots de Langerhans, Ann. Histochim. 13, 141.

Phil, E. 1967. Ultrastructural localization of heavy metals by a modified sulphide-silver method. Histochemie 10, 126-139.

Phil, E. and Falkmer, S. 1967. Trials to modify the sulphide-silver method for ultrastructural tissue localization of heavy metals. Acta Histochem. 27, 34-41.

Pilsetskaya, E., Leibush, B. N., and Bondareva, V. 1976. The secretion of insulin and its role in cyclostomes and fishes. In "The Evolution of Pancreatic Islets" (T. A. Grillo, L. Leibson and A. Epple, eds.). pp. 251-270. Pergamon Press, Oxford.

Popham, D. J. and Webster, W. S. 1976. The ultrastructural localization of cadmium. Histochemistry 46, 249-259.

Porte, D. and Robertson, R. P. 1973. Control of insulin secretion by catecholamines, stress and the sympathetic nervous system. Fed. Proc. 32:1792-1796.

Randle, D. J. 1969. The Circulatory System. In "Fish Physiology", Hoar, W. S. and Randle, D. J. (eds.), Vol. IV, pp. 133-172, Academic Press, New York.

Robertson, O. H., Krupp, M. A., Favor, C. B., Jane, S. and Thomas, S. F. 1961. Physiological changes occurring in the blood of the Pacific salmon, Oncorhynchus tshawytscha accompanying sexual maturation and spawning. Endocrinology 68, 733-746.

Rogers, A. W. 1973. "Techniques of Autoradiography", 2nd ed., Elsevier Scientific Publishing Co., Amsterdam, pp. 372.

Rixon, R. H. and Whitfield, J. F. 1958. The histochemical localization of zinc in the dorsolateral prostate of the rat. J. Histochem. Cytochem. 7, 262-266.

Scott, H. R. 1952. Rapid staining of beta granules in pancreatic islets. Stain Technol. 27, 267-268.

Sechrist, J. W. and Upson, R. H. 1974. Prevention of chemography in prestained epoxy autoradiographs by an intervening carbon film. Stain Technol. 49, 297-300.

Shapiro, S. A. and Hoffman, D. L. 1975. Effects of photoperiod on serum glucose levels in goldfish (Carrasius auratus). Comp. Biochem. Physiol. 52A, 253-254.

Silbergeld, E. R. 1974. Blood glucose: a sensitive indicator of environmental stress in fish. Bull. Environ. Contam. Toxicol. 11, 20-25.

Sinley, J. R., Goettl, J. P., and Davies, P. H. 1974. The effects of zinc on rainbow trout (Salmo gairdneri) in hard and soft water. Bull. Environ. Contam. Toxicol. 12, 193-201.

Smith, P. H., Dept. of Anatomy, University of Washington, Seattle, Wash., U. S. A.

Smith, P. H., Merchant, F. W., Johnson, D. G., Fujimoto, W. Y., and Williams, R. H. 1977. Immunocytochemical localization of a gastric inhibitory polypeptide-like material within the A-cells of the endocrine pancreas. Am. J. Anat. 149, 585-590.

Sprague, J. B., Elson, P. F. and Saunders, R. L. 1965. Sublethal copper-zinc pollution in a salmon river: a field and laboratory study. Air Water Pollution 9, 531-543.

Sternberger, L. A. 1974. "Immunocytochemistry", Prentice-Hall, Englewood Cliffs, pp. 246.

Stewart, J. K., Goodner, C. J., Koerker, D. J., Gorbman, A., Ensinck, J. and Kaufman, M. 1978. Evidence for a biological role of somatostatin in the Pacific hagfish, Eptatretus stouti. Gen. Comp. Endocrinol. 36, 408-414.

Szeto, S. Y., Dept. of Biological Sciences, Simon Fraser University.

Taras, M. J., Greenburg, A. E., Hoak, R. D. and Rand, M. C. (eds.), 1971. "Standard Methods for the Examination of Water and Wastewater". 13th Edition, published jointly by American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 874 pp.

Terrier, M. and Perrier, H. 1975. Cyclic 3',5' adenosine monophosphate level in the plasma of the rainbow trout (Salmo gairdneri Richardson) following adrenaline administration and constrained exercise. Experimentia 31, 196.

Thomas, N. W. 1970. Morphology of endocrine cells in the islet tissue of the cod, Gadus callarias. Acta Endocrinol. 63, 679-695.

Thomas, N. W. 1975. Observations of the cell types present in the principle islet of the dab, Limanda limanda. Gen. Comp. Endo. 26, 496-503.

Thorpe, A. 1976. Studies on the role of insulin in teleost metabolism. In "The Evolution of Pancreatic Islets" (T. A. I. Grillo, L. Leibson, and A. Epple, eds.) pp. 271-284. Pergamon Press, Oxford.

Thorpe, A. and Ince, B. W. 1974. The effects of pancreatic hormones, catecholamines and glucose loading on blood metabolites in the northern pike (Esox lucius L.). Gen. Comp. Endocrinol. 23, 29-44.

Thorpe, A. and Ince, B. W. 1976. Plasma insulin levels in teleosts determined by a charcoal-separation radio-immunoassay technique. Gen. Comp. Endocrinol. 30, 332-339.

Timm, F. 1958. Zur histochemie der schwermetalle. Das sulfid-silberverfahren. Drsch. Z. ges. gerichtl. Med. 46, 706-711.

Umminger, B. L. and Benziger, D. 1975. In vitro stimulation of heaptic glycogen phosphorylase activity by epinephrine and glucagon in the brown bullhead, Ictalurus nebulosus. Gen. Comp. Endocrinol. 25, 96-104.

Unger, R. H., Dobbs, R. E. and Orci, L. 1978. Insulin, glucagon and somatostatatin secretion in the regulation of metabolism. Ann. Rev. Physiol. 40, 307-343.

Vale, W., Brazeau, P., Rivier, C., Brown, M., Boss, B., Rivier, J., Burgus, R., Ling, N. and Guilleman, R. 1975. Somatostatatin. Recent Prog. Hormone Res. 31, 365-397.

Valtonen, T. 1974. Seasonal and sex bound variations in the carbohydrate metabolism of the liver of the whitefish. Comp. Biochem. Physiol. 47A, 713-727.

Van Noorden, S. and Patent, J. 1978. Localization of pancreatic polypeptide (PP)-like immunoreactivity in the pancreatic islets of some teleost fishes. Cell Tiss. Res. 188, 521-525.

Van Noorden, S. and Pearse, A. G. E. 1974. Immunoreactive polypeptide hormones in the pancreas and gut of the lamprey. Gen. Comp. Endocrinol. 23, 311-324.

Vassallo, G., Capella, L. and Solcia, E. 1971. Grimelius' silver stain for endocrine cell granules as shown by electron microscope. Stain Technol. 46, 7-13.

Voigt, G. E. 1958. Histochemical investigation on the distribution of zinc in the pancreas in experimental zinc poisoning. Acta path. microbiol. scand. 44, 146.

Watson, T. A. 1975. The effects of sublethal concentrations of zinc on histological, histochemical, growth and blood parameters in rainbow trout (Salmo gairdneri Richardson). M.Sc. Thesis, University of Guelph, Ontario, Canada.

Watson, T. A. 1978. Effects of zinc on branchial adenosine triphosphatases and blood constitituents of Rainbow Trout, Salmo gairdneri. Ph.D. Thesis, University of Guelph, Ontario, Canada.

Watson, T. A. and McKeown, B. A. 1976a. The effect of sublethal concentrations of zinc on growth and plasma glucose levels in rainbow trout, Salmo gairdneri (Richardson). J. Wildl. Dis. 12, 263-270.

Watson, T. A. and McKeown, B. A. 1976b. The activity of $\Delta 5$-3β hydroxysteroid dehydrogenase enzyme in the interrenal tissue of rainbow trout (Salmo gairdneri Richardson) exposed to sublethal concentrations of zinc. Bull. Environ. Contam. Toxicol. 16, 173-181.

Wilske, K. R. and Ross, R. 1965. Autoradiographic localization of lipid- and water-soluble compounds: a new approach. J. Histochem. Cytochem. 13, 38-43.

Zar, J. H. 1974. "Biostatistical Analysis", Prentice-Hall, Inc., Englewood Cliffs, N. J., U. S. A. 620 pp.

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UNIVERSITY EDUCATION

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1) Canadian Conference for Fisheries Research; Fredericton (1977)
2) Canadian Society of Zoologists' Conference; Victoria (1977)
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Papers Presented -

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PROFESSIONAL TEACHING EXPERIENCE

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