



National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

AVIS

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

The effect of calcitonin on the efflux of calcium from the
North American eel,
Anquilla rostrata,
with emphasis on the gill.

by

Eric K.S. Leung

A Thesis
presented to the University of Ottawa
in partial fulfillment of the
requirements for the degree of
Master of Science
in
Department of Biology

Ottawa, Ontario, 1979

(c) Eric K.S. Leung, 1979

May, 1979

© Eric K.S. Leung, Ottawa, Canada, 1979.

I hereby declare that I am the sole author of this thesis.

I authorize the University of Ottawa to lend this thesis to other institutions or individuals for the purpose of scholarly research.

Eric K.S. Leung

I further authorize the University of Ottawa to reproduce this thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Eric K.S. Leung

Whooppee!

The University of Ottawa requires the signatures of all persons using or photocopying this thesis. Please sign below, and give address and date.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. J.C. Fenwick, for his guidance and assistance both during the research period and the preparation of this thesis.

I would also like to thank Dr. T.W. Moon and Dr. F.E. Engelhardt for their invaluable discussions.

I would like to gratefully acknowledge Dr. M. Alvo of the Department of Mathematics, University of Ottawa, for his interest and guidance in the statistical analysis of the data and Dr. J. Carlson of the Department of Education, University of Ottawa, for additional advice.

I am indebted to Elizabeth Sullivan at the Department of Applications for her immense help in running programs on the IBM 360 computer and Mrs. A. Hurd for additional help.

I would like to thank Nino Cavacciuti for his help in the construction of the experimental apparatus.

I extend my thanks to the personnel at Chat's Falls and Cornwall Hydro dams who made possible the procurement of the eels used for this study.

Drs. M. Komourdjian, W. Hulbert, and J. Bailey, as well as, Ying-Peng So, E. McCauley and J. Ferguson deserve thanks for their advice and support.

I would like to thank A. M. Pajor for her remarkable dedication in the typing and final preparation of this manuscript which was made possible through the kind permission of Dr. R. Strickler for the full use of the computer terminals and lab space.

Finally, I would like to express my gratitude to members of my family and friends who made possible the completion of this thesis through their understanding and forbearance during the more critical periods.

This work was supported by a grant-in-aid of research (No. A-6246) from the National Science and Engineering Research Council (NSERC) of Canada to Dr. J.C. Penwick.



ABSTRACT

The calcium regulating role of calcitonin, a hypocalcemic hormone produced by the ultimobranchial bodies, was examined in the North American eel, Anquilla rostrata L.

Exogenous calcitonin elicited an increased gill calcium efflux in the intact yellow eel exposed to a calcium-challenge. But, changes in total plasma calcium were not observed. However, a trend towards a decrease in plasma calcium-45 was noted. Isolated gills taken from freshwater- or seawater-adapted eels showed no change in calcium efflux when perfused or incubated with calcitonin. Calcitonin counteracted the effects of corpuscles of Stannius extract on gills of freshwater eels. In the seawater eel, however, this effect of calcitonin was not observed.

The present study on the eel showed that the calcium regulatory role of calcitonin is manifested in times of calcium stress, as in the other animal groups, and, that under "normal" conditions, secretion(s) from the the corpuscles of Stannius would be the major hypocalcemic factor(s).

RESUME

Le rôle de régulation du calcium par la calcitonine, une hormone hypocalcémique produit par les corps ultimobranchielles, a été étudié dans l'anguille d'Amérique du Nord, Anquilla rostrata L.

La calcitonine exogène élicite une augmentation du flux de sortie du calcium par les branchies dans l'anguille jaune intacte exposée à un 'challenge' de calcium. Mais un changement complet du calcium dans le plasma n'a pas été observé. Toutefois, une tendance vers une diminution du calcium-45 du plasma a été remarquée. Des branchies isolées prises d'anguilles adaptées à l'eau douce ou à l'eau de mer n'ont montrées aucun changement dans le flux de sortie du calcium quand elles ont été perfusées ou incubées avec la calcitonine. La calcitonine a reversé les effets des homogènes des corps de Stannius sur les branchies des anguilles d'eau douce. Pourtant, dans l'anguille d'eau de mer, cet effet de la calcitonine n'a pas été observé.

Cette étude sur l'anguille démontre que le rôle de régulation du calcium par la calcitonine se manifeste pendant le stress de calcium comme dans les autres groupes d'animaux et, que, durant les conditions normales, les secrétions des corps de Stannius sont les facteurs hypocalcémiques principaux.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iv
ABSTRACT	vi
RESUME	vii

Chapter	page
I. INTRODUCTION	1
II. MATERIALS AND METHODS	28
Experimental Animals	28
Solutions	30
Radio-calcium	30
Tritiated-Inulin	30
Double-isotope Standards	31
Calcitonins	31
Bovine	31
Salmon	32
Corpuscles of Stannius extracts	32
Perfusates	33
Bathing Medium	34
TECHNIQUES	34
Blood Sampling	34
Intraperitoneal Injections	35
Gill Perfusion	35
Handling of Samples	38
Tissue Samples	38
Determination of Isotopic Activity in Samples	39
EXPERIMENTAL	42
Exogenous calcitonin in the intact eel	43
Effects of calcitonin on eels transferred to seawater	43
BCT, Ca distribution and efflux, intact eel in SW.	45
Effect of calcitonin on gill	48
Single isotope gill perfusion - constant- flow system	50
Single Isotope Gill Perfusion - constant pressure system	52
Double Isotope Gill Perfusion - modified constant-flow system using small eels.	54

Double Isotope Gill Perfusion - modified constant-flow system using large eels.	56
Double Isotope Gill Perfusion - static system using small eels.	57
III. RESULTS	61
In vivo studies	61
Total Plasma Calcium	61
Plasma calcium-45	61
Distribution of calcium-45 within the eel	63
Radio-calcium clearance	66
GILL PERFUSION STUDIES	69
Effect of SCT on net gill Ca efflux	69
Effect of salmon calcitonin perfusion on net gill calcium and inulin efflux	69
Effect of SCT, CS on incubated gills	72
IV. DISCUSSION	77
Appendix	page
A. THE EFFECT OF CORPUSCLES OF STANNIUS EXTRACTS IN RATS	101
BIBLIOGRAPHY	106

LIST OF TABLES

Table	page
I. Effect of Calcitonin in Mammals	4
II. Calcitonin and Blood Calcium in Mammals	7
III. Exogenous CT and calcium in fish	20
IV. BCT and Plasma Calcium : intact eels	62
V. BCT and Plasma Calcium-45 : intact eels	64
VI. BCT and Internal Calcium Distribution	65
VII. Efflux in Calcium-challenged eels	67
VIII. SCT and Calcium efflux : constant-pressure system .	70
IX. SCT and calcium efflux : large and small eel gills .	71
X. Incubated FW gill Calcium Efflux	73
XI. Incubated SW gill Calcium Efflux	74

LIST OF FIGURES

Figure	page
1. Apparatus for intact eel efflux studies	47
2. Gill perfusion apparatus	51
3. Modifications to gill perfusion apparatus	53
4. Time-course study of calcium-45 efflux	68

Chapter I

INTRODUCTION

The divalent cation calcium participates in many diverse cellular functions and, as a result, is closely controlled within the intracellular compartment (Rasmussen and Goodman, 1977.) Further, most organisms also exert some degree of regulation over the level of calcium in the extracellular compartment with the degree of regulation being observed somewhat dependent on the complexity of the organism studied. (Prosser, 1973). Terrestrial vertebrates, as compared to the aquatic species, are essentially isolated from their calcium environment. Their source of calcium is dependent on uptake in the gastrointestinal tract, a site where regulation is observed. The main regulating mechanisms for extracellular calcium within these organisms lies in the shuttling of calcium between bone calcium stores and blood and the rate of clearance by the kidney. Conversely, aquatic animals live in a calcium environment coexistent with internal calcium. Surfaces exposed to this external medium, such as the gills, skin/scales and fins, are additional sites possible for calcium fluxes and regulation. Of the different animals studied, the regulation of calcium is best understood in mammals.

The regulation of calcium in mammals involves three hormones: parathyroid hormone, the active metabolites of vitamin D and particularly 1,25-dihydroxycholecalciferol, and calcitonin. The tissues that secrete these substances are responsive to changes in extracellular calcium concentration. The parathyroids secrete parathyroid hormone when the circulating calcium falls below a certain level (in mammals, 2.5 mM total calcium) (Cohn *et al.*, 1978). Parathyroid hormone exerts its hypercalcemic action through its effect on bone, where it causes resorption of bone salts (Raisz, 1970; Raisz and Bingham, 1972); on the kidney, where it increases the reabsorption of calcium (Massry *et al.*, 1973; Nordin *et al.*, 1967); and by increasing the intestinal absorption of calcium indirectly by stimulating the conversion of vitamin D to 1,25-dihydroxycholecalciferol in the kidney (Rasmussen *et al.*, 1973). Parathyroid hormone is believed to act on these target organs with 3',5'-cyclic adenosine monophosphate (cAMP) as the secondary messenger (Borle, 1974; DeLuca, 1975; MacIntyre *et al.*, 1976). Vitamin D-3 (1,25-dihydroxycholecalciferol) is also a hypercalcemic agent with the main target organ being the intestinal epithelium (Russell, 1976) but also increases calcium reabsorption in the kidney (MacIntyre *et al.*, 1977). It is proposed that 1,25-dihydroxycholecalciferol acts on these tissues through the induction of calcium-binding proteins, which then facilitate calcium transport (Raisz and Trummel, 1971), as well

as operating synergistically with parathyroid hormone in osteolysis (DeLuca, 1975). The parafollicular or "C" cells of the mammalian thyroid respond to elevated calcium levels with a secretion of calcitonin. Calcitonin increases kidney clearance of calcium and phosphate; inhibits bone resorption and decreases intestinal absorption of calcium and phosphate, thereby lowering circulating calcium, in mammals other than adult man (where it is thought to be a "vestigial" hormone) (Table I; and also Potts, 1976). The overall effect of calcitonin in mammals is to regulate blood calcium during periods of high calcium fluxes, such as after oral ingestion, during pregnancy and lactation, and in young growing animals where there is a high turnover of bone salts (Table II). At present, the generally accepted picture of calcium regulation in animals is that major roles are played by parathyroid hormone and 1,25-dihydroxycholecalciferol (DeLuca, 1975; Care et al., 1975). The role of calcitonin is important in times of elevated calcium stress (Copp and Ma, 1978).

TABLE I

Effect of Calcitonin in Mammals

EFFECT OF CALCITONIN ON THE PRINCIPAL SITES FOR CALCIUM REGULATION IN MAMMALS.

Target Organ	Response	References
1) Bone	inhibit PTH induced osteoclast proliferation	Friedman & Raisz, 1965
	inhibit bone resorption	Poster et al., 1966; Sturtridge & Kumar, 1967
	inhibit entire process of bone metabolism	Klein & Talmage, 1968
	CT action on bone decreases with increasing age	Copp & Kuczespa, 1968
	inhibit formation of new osteoclasts and action of those already present	Reynolds & Dingle, 1970
	inhibit release of calcium-45 from prelabelled calvaria.	Minkin et al., 1971
	decrease osteoclast ruffle borders	Kallio et al., 1972
	osteocytes decrease in size after i.v injection of CT	Matthews et al., 1972
	inhibit cell Ca efflux	Harell et al., 1973
	SCt more effective than HCT or PCT in prevention of bone resorption in Paget's disease	Avramides et al., 1974
	activate adenylate cyclase	Heersche et al., 1974
	inhibition of bone resorption possibly	Nagata et al., 1975

cAMP mediated

protects female ruminants from excessive bone resorption in pregnancy and lactation

2) Kidney

kidney & gut not target organ

PCT no effect on renal Ca excretion

no effect

natriuresis observed

PCT, SCT no effect on electrolytes but prevented effect of DHCC on kidney

SCT in parathyroidectomized animals no effect

inhibit kidney cell Ca efflux

SCT increase urinary Ca & P with hypocalcemia; PCT only hypocalcemia

inhibit D-1-hydroxylation in kidney

activate kidney adenylate cyclase

increase clearance of phosphate, Ca & Na

PCT increase urinary Ca, Mg, Na, phosphate and cAMP

Barlet & Garel, 1973

Kenny & Heistell, 1965

Clark et al., 1968

Clark & Kenny, 1969

Bijvoet et al., 1971

Puschett et al., 1974

Sammon et al., 1969

Borle, 1969

Aldred et al., 1970

Rasmussen et al., 1972

Marx et al., 1972;
Dousa, 1974

Haas et al., 1971

Adachi et al., 1974

3) Intestine

CT for protection against hypercalcemia from oral ingestion of Ca

Munson & Gray, 1970

Jejunum - marked secretion of water
electrolytes, Ca not affected
Ileum - decrease Ca absorption

GI hormones stimulation by ingested Ca
increase secretion of CT by thyroid
inhibit Ca & phosphate absorption

Gray et al., 1973,
1975

Hirsch et al., 1975

Olson et al., 1972

Tanzer & Navia, 1973

TABLE II

Calcitonin and Blood Calcium in Mammals

Calcitonin and Blood Calcium in Mammals

<u>Animal</u>	<u>Response</u>	<u>Reference</u>
rat	epinephrine induced hypercalcemia increase CT secretion from thyroid	Hsu & Cooper, 1975
rat	PCT & SCT prevent calcium-45 entry into plasma; CTS act differently on (32)P & (45)Ca?	Talmage et al., 1972
rat	SCT prevented hypercalcemia in TX animals	Talmage et al., 1973
rat	CT had no effect when blood Ca is low	Bell & Stern, 1970
rat	lower blood Ca of vit.D deficient animals, PTH had no effect	Norri & DeLuca, 1967
rat	hypercalcemia in TX animals with Ca thru stomach tube	Munson & Gray, 1969
pig	CT secretion control by -ve feedback mechanism from plasma Ca; acts as a fine regulator of Ca	Care et al., 1967
pig	CT needed in hypercalcemia; with Ca challenge, plasma Ca increased 10 fold	Care et al., 1968
pig	TX increase plasma Ca, CT infusion restores Ca levels; plasma Ca increase again after cessation of infusion	Svaminathan et al., 1972

pig	CaCl infusion caused slight increase in plasma Ca before TX; much larger increase after TX	Cooper (in Hirsch et al., 1975)
pig	CaCl infusion elicit CT secretion from thyroid	Care et al., 1975
pig	previous hypocalcemic state results in exaggerated CT secretion from hypercalcaemic stimulus	West et al., 1973 Heynen & Franchimont, 1974
man	"	Heynen & Franchimont, 1974
fetal sheep	C cells respond to infused CaCl	Garel et al., 1974
rabbit	PCT had no effect on serum Ca, urinary Ca, urinary P	Salako et al., 1971
comparative	CT plays protective role in rat & pig but not in man and hamster	Hirsch et al., 1975

To a certain extent, calcium regulation in other vertebrates (with the exception of the fishes) seems to reflect the mechanisms found in mammals (Copp and Ma, 1978). Parathyroid hormone has been demonstrated to act in birds, reptiles, and amphibians in a hypercalcemic fashion as in mammals (Copp and Ma, 1978). A hypercalcemic action of 1,25-dihydroxycholecalciferol has also been shown in amphibians and birds but little is known about its action in reptiles (Copp and Ma, 1978). Calcitonin plays a more important role in young reptiles and birds during the first few months after hatching than in the adults. Chronic administration of salmon calcitonin to turtles (Bélanger *et al.*, 1973) and cockerels (Bélanger and Copp, 1972) during early growth resulted in animals with much denser bone. However, the hypocalcemic action of calcitonin in older reptiles and birds has not been demonstrated (Clark, 1971; Bélanger and Copp, 1972). The ultimobranchial bodies¹ have been reported to hypertrophy in laying hens, presumably as a result of the calcium stress associated with egg production (Urist, 1967).

¹These are structures found in all vertebrates other than mammals and lampreys (Watzka, cited in Copp, 1970). The glandular tissues are the source of calcitonin in these animals. It is now known that the parafollicular "C" cells in mammals are ultimobranchial body tissue cells that have been incorporated into the thyroid, and to a lesser extent, the parathyroids and thymus. Embryological evidence showed that ultimobranchial body cells (thus the "C" cells) are of neural crest origin. During development, these cells migrate to form the last branchial pouch, which finally gives rise to the glandular tissues (Copp, 1970; Copp and Ma, 1978).

In amphibians, the effect of calcitonin on the intestine and kidney is similar to that found in mammals (Robertson, 1972). Because a more readily available source of calcium (calcium carbonate) is stored in the paravertebral lime sacs² than bone in these animals, the effect of calcitonin is seen as the prevention of the mobilization of calcium from the lime sacs (Robertson, 1972). Thus, in birds, reptiles, and amphibians, the mechanisms for calcium regulation is similar to those found in mammalian systems.

The regulation of calcium is also observed in the teleosts. For example, euryhaline species such as the eel and salmon are able to maintain normocalcemia in the face of the widely fluctuating environmental conditions encountered during their migratory journeys between seawater (10 mM Ca^{2+}) and freshwater (0.45 mM Ca^{2+}). As a result, attempts have been made to show that the same, or similar, endocrine factors are involved in calcium regulation in teleosts as previously described in the other vertebrates.

²These are extensions of the endolymphatic sacs which run along the vertebral canal and emerge between the vertebrae (Whiteside, cited in Robertson, 1969).

Vitamin D was shown to be present in fish liver oils in 1936 by Brockman (cited by MacIntyre et al., 1976). However, very little information is available on vitamin D and calcium metabolism in this group of animals. Peignoux-Deville (1977) recorded that injected 1,25-dihydroxycholecalciferol had no effect on plasma calcium in the European eel, Anquilla anquilla. MacIntyre et al (1976) reported that 1,25-dihydroxycholecalciferol elevated plasma phosphate levels in the European eel and postulated that non-mammalian vertebrates employ vitamin D as a phosphate-conserving agent. More work needed on the aspect of 1,25-dihydroxycholecalciferol and calcium regulation in the fishes.

Parathyroid tissue appears to be a tetrapod invention and does not appear in the Pisces (Krishnamurthy, 1976; Pang, pers. comm., cited in Copp and Ma, 1978) and experimental support for the action of mammalian parathyroid hormone in this group has yet to be demonstrated (Pang, 1973).

More concrete evidence is available on the role played by the pituitary gland, the corpuscles of Stannius and the ultimobranchial bodies.

A hypercalcemic factor from the pituitary gland has been demonstrated with experiments involving the surgical removal of the gland, replacement therapy with tissue homogenates and transplantation of the tissue in the killifish, Fundulus heteroclitus (Pang, 1973; Pang et al., 1978). This hypercalcemic factor has recently been identified conclusively to be prolactin, secreted by the anterior pituitary, in the killifish (Pang et al., 1978) and the stickleback, Gasterosteus aculeatus (Wendelaar Bonga and Greven, 1978). Prolactin is essential for calcium homeostasis when the animal is in a low calcium environment, that is, in freshwater (Pang, 1973).

The corpuscles of Stannius are encapsulated structures that are found in close association with the kidney and are unique to the ray-finned fishes (Krishnamurthy, 1976). Increased cellular activity is observed in goldfish (Carassius auratus) corpuscular tissue after transfer to 30% seawater (Ogawa, 1963). Similar observations were reported in the silver and yellow eels (Olivereau, 1964; Hanke et al., 1967), the Atlantic salmon, Salmo salar, and the eel, Anquilla vulgaris (Fontaine and Lopez, 1965) on adaptation to seawater. Surgical removal of the corpuscles of Stannius, or stanniectomy, results in a transient hypercalcemia in the goldfish (Ogawa, 1968), the eel, Anquilla an-

quilla (Fontaine, 1964; Chester Jones and Henderson, 1965; Chan et al., 1967; Chan and Chester Jones, 1968; Fenwick and Forster, 1972); the North American eel, Anquilla rostrata (Butler, 1969; Fenwick, 1974), the Japanese eel, Anquilla japonica (Chan, 1972) and the killifish, Fundulus heteroclitus (Pang, 1971a; Pang et al., 1973). Prevention of hypercalcemia after stanniectomy can be achieved by injection of corpuscles of Stannius homogenates (Fontaine et al., 1967; Chan et al., 1969) and autotransplantation of the corpuscles of Stannius (Fenwick and Forster, 1972; Pang et al., 1973). These observations all point to a hypocalcemic substance or substances secreted by the corpuscles of Stannius. Attempts to identify the chemical nature of the secretions pointed to a proteinaceous substance (Oguri, 1966; Ogawa, 1967). Pang et al. (1974), based on the results of their injection studies with crude corpuscles of Stannius homogenates, proposed that the factor(s) be termed "hypocalcin". Ogawa et al. (1978) identified hypocalcin as the renin-like substance that has been demonstrated in the corpuscles of Stannius in earlier reports (Chester Jones and Henderson, 1965; Chester Jones et al., 1966; Chan et al., 1969). This would link the corpuscles of Stannius with the renin-angiotensin system which has a pressor effect in vertebrates (Sokabe, 1974). Recently, a purified hypocalcemic glycoprotein with a molecular weight of 3000 (approx.) has been obtained from salmon corpuscles of Stannius and was named "teleocalcin" by the

authors (Ma and Copp, 1978). These authors suggest that it is different from hypocalcin. Thus, the active principles from the corpuscles of Stannius that are involved in calcium regulation in fish known at present are "teleocalcin" and "hypocalcin"; though the distinction of these substances as separate identities awaits further confirmation. The sites of hypocalcin/teleocalcin action have been looked at. Initial investigations relied on the location of the source of the elevated calcium apparent in stanniectomized animals. One possible source was thought to be bone (Butler, 1969) but the absence of osteoclasts (Lopez, 1970, cited in Krishnamurthy, 1976), the absence of calcium-45 deposition (Fontaine et al., 1972, cited in Krishnamurthy, 1976) and the absence of calcium-45 release from pre-labelled bone (Lwowski, 1978) following stanniectomy would tend to rule out bone as a major target organ. A similar conclusion is reached when one looks at intestinal calcium absorption, based on the fact that hypercalcemia is also observed in starved animals (Fontaine, 1964; Chester Jones and Henderson, 1965; Chan et al., 1967; Fenwick and Forster, 1972) and even in the fed killifish, ingested calcium contributed minimally to the observed hypercalcemia, whereas these same animals held in acalcemic water did not develop hypercalcemia (Pang et al., 1973). Another possible site responsible for elevating plasma would be the kidney, where a decrease in calcium clearance after stanniectomy has been observed (Chester

Jones et al., 1967, 1969 ; Chan et al., 1969b ; Fontaine et al., 1970 ; Chan, 1972). Also, when hypercalcemic stanniectomized fish were treated with corpuscles of Stannius extract, the level of hypercalcemia was reduced and this coincided with increased urinary calcium (Fontaine, 1964; Rankin et al., 1967 ; Chan et al., 1969b). However, more recent findings showed a high urinary calcium in stanniectomized eels (Butler, 1969; Fontaine et al., 1972 ; Fenwick, 1974, 1978). Further, in the killifish, calcium deposits were found in the kidney ducts (Pang, 1971a). One can conclude from these data that increased calcium resorption from bone, decreased kidney calcium clearance, and increased intestinal absorption contribute minimally to the hypercalcemia observed after stanniectomy. This points to the environment as the source of calcium (Fontaine et al., 1972). Indeed, hypercalcemia was observed in stanniectomized eels kept in tap water but absent from those kept in acalcemic water (Fenwick, 1974). Various reports have pointed to the gills as the site of entry of exogenous calcium (Fontaine et al., 1972 ; Fenwick, 1974;; Pang et al., 1974) and the demonstration with a perfused gill preparation lent support to this hypothesis (Fenwick and So, 1974). Further support was obtained when the development of hypercalcemia was seen to parallel the increase in calcium influx in the perfused hemibranch (So and Fenwick, 1977). Thus, the gills are important target sites for the hypocalcemic factors from the cor-

puscles of Stannius, and for calcium regulation. In the absence of hypocalcin, the gills in stanniectomized animals could take up excess calcium and this might account for the transient hypercalcemia observed. But the question of the mechanism(s) for the subsequent return to normocalcemia remains unresolved. This points to the presence of another hypocalcemic factor(s). At present, the only other hypocalcemic factor that has been demonstrated in mammals and is found in teleosts is calcitonin.

It is now well documented that calcitonin has a hypocalcemic action in many of the tetrapods (see foregoing section on vertebrates), but the question of whether calcitonin plays a similar role in fishes remains unanswered. Certainly fish possess calcitonin (Watts et al., 1970 ; Copp et al., 1967, 1968a ; Pang et al., 1971b ; Orimo et al., 1971 ; Deftos et al., 1974) which is secreted by the ultimobranchial body (Orimo et al., 1971 ; Copp et al., 1972 ; van Noorden and Pearse, 1972;; Tisseerand-Jochen et al., 1976). Also, the successful in vitro induction of calcitonin production by monolayer cultures of trout ultimobranchial body cells can be taken as suggestive evidence that the ultimobranchial body is a source of calcitonin in all fishes (Roos et al., 1974 ; Roos and Deftos, 1976).

If this endogenous calcitonin is involved in calcium regulation, then perturbations in plasma calcium levels might be reflected in the secretory activity of the ultimobranchial body. Indeed, histological examinations have demonstrated increased secretory activity in the ultimobranchial body during hypercalcemia experimentally induced by injections of calcium chloride (Lopez *et al.*, 1968 ; Dacke *et al.*, 1972 ; Deftos *et al.*, 1972 ; Suryawanshi and Mahajan, 1976) or through stanniectomy with its resultant hypercalcemia (Chan, 1972).

Based on histological evidence, one would then postulate that the surgical removal of the ultimobranchial body, or ultimobranchialectomy, would result in an increase in plasma calcium levels. This has been verified in the Japanese eel, where ultimobranchialectomy elicited a significant hypercalcemia (Chan, 1968; Chan, 1972); in the goldfish with partial ultimobranchialectomy and acute transfer to 30% seawater (Fenwick, 1975); in the European eel, where ultimobranchialectomy increased plasma calcium in fish held in calcium-rich tap water (Lopez *et al.*, 1976) and in the North American eel, where partial ultimobranchialectomy was shown to have a transient hypercalcemic effect which was more marked in animals held in seawater than freshwater (Fenwick, 1978).

The secretory activity of the ultimobranchial body is also affected by the circulating levels of calcitonin. In Anquilla anquilla, injections of exogenous salmon calcitonin altered plasma calcitonin levels during hypercalcemia. A marked atrophy of the gland was observed. Further, hypercalcemia induced by injections of carp pituitary extract was followed by an increase in ultimobranchial body secretory activity (Peignoux-Deville et al., 1975 ; Lopez et al., 1976).

It is apparent that a change in circulating calcitonin levels is also effected by a change in the external calcium concentration. Radioimmunoassay of plasma calcitonin in the migrating male sockeye salmon showed that there is a three-fold decrease as they migrated into freshwater; whereas, in the females, a two-fold drop is observed only after spawning (Deftos et al., 1972). The discrepancy observed between males and females was attributed to a hypercalcemia in the females which was, in turn, related to ovarian maturation (see also Deftos et al., 1974). This explanation is supported by observations from the goldfish where hyperplasia and hypertrophy of the ultimobranchial body were observed in the sexually mature females (Oguri, 1973) and in migrating Atlantic salmon where hyperplasia was also observed (Deville and Lopez, 1970). Also, there is a concomi-

tant drop in calcitonin with a drop in plasma calcium in the male salmon as they enter freshwater (Watts et al., 1975). In other words, a high circulating calcitonin level is observed when the animal is in seawater (high calcium), and as the animal migrates into freshwater (low calcium) and the calcium-challenge is removed, the animal responds with a drop in plasma calcitonin concentration.

Observations based on in vitro and in vivo experiments, point to a role played by calcitonin in the maintenance of normocalcemia in the fishes studied thus far. To further support this hypothesis, administration of exogenous calcitonin to fishes should elicit a hypocalcemic response; and this increase in circulating calcitonin would also help in the localization and identification of the target organs for this hormone. However, studies involving the administration of exogenous calcitonin in fish have given results inconsistent with each other and with the hypothesis that calcitonin causes a marked hypocalcemia (Table III). The inconsistent findings in the Ictaluridae (the catfishes) and the Anquillidae (the eels) could be attributed to fluctuating endogenous calcium which in turn could affect the circulating calcitonin levels, as was shown in the killifish, Fundulus kansae, where in both males and females, plasma calcium showed seasonal changes; higher in the summer months than in the winter months (Fleming et al., 1973). A similar

TABLE VII

Exogenous CT and calcium in fish

Species	Calcitonin	Blood parameter	Response	Reference
<i>Poroderma africanum</i> (the lazy shark)	porcine	serum	nil	Louv et al., 1969
<i>Prionace glauca</i> (blue shark)	shark	serum	nil	Urist, 1967
<i>H. francisci</i> (horn shark)	"	"	"	"
<i>Squalus suckleyi</i> (dogfish)	salmon	"	"	Copp et al., 1970
<i>Squalus acanthias</i> (dogfish)	salmon	"	"	Hayslett et al., 1971 " , 1972
<i>Fundulus heteroclitus</i> (killifish)	porcine	"	"	Pang & Pickford, 1967
<i>Lepidosiren paradoxa</i> (S. Amer. lungfish)	salmon	plasma	"	Urist et al., 1972
"	"	serum	"	Pang & Sawyer, 1975
<i>Salmo gairdneri</i> (Rainbow trout)	porcine (*thyroxine)	serum	hypocalcemia	Lopez et al., 1971
<i>Onchorynchus kisutch</i> (coho salmon)	salmon (O. nerka)	serum	nil	Pang & Grant, in Pang, 1971b
<i>O. nerka</i>	salmon	plasma	nil	Copp et al., 1970

observation was made in the brown bullhead by Bradshaw and Sutton (1970). Fleming et al (1973) also reported that varying responses to injected calcitonin were obtained from their animals depending on the time of year when the study was carried out. Chan (1972) reported that, calcitonin had no effect in the stanniectomized eel, and he postulated that the already high endogenous calcium elicited a maximal calcitonin secretion from the ultimobranchial body which resulted in a masking of the effect of the injected hormone. Copp et al (1972) also attributed the lack of effect in the spawning female sockeye salmon used for the study to a high endogenous calcitonin level in these fishes. Other hypercalcemic endocrine factors such as ACTH, cortisol, estrogens, and prolactin (Pang, 1973; Fleming et al., 1973) could also mask the effect of injected calcitonin. Although ACTH and cortisol do not have as pronounced effects as prolactin on circulating calcium levels, and estrogens affect females during spawning, these factors still have to be taken into consideration. Indeed, Peignoux-deville et al (1975) reported that injected calcitonin did not prevent carp pituitary induced hypercalcemia in the eel. Thus, in view of these factors, results based on circulating calcium levels are poor estimations of the effect of injected calcitonin. A measurement of the calcium fluxes between the animal and the external medium would be a better approach (Fleming et al., 1973).

Calcium flux studies in whole animals with injected calcitonin showed an increase in calcium influx from the environment in Fundulus kansae during the summer months (Fleming et al., 1973) and a decrease in calcium efflux in Anquilla anquilla (Dacke, 1975). However, the former authors also reported that in Fundulus kansae, an increase in plasma calcium is related to the spawning season (the summer months), therefore the effect observed could be secondary, that is, the injected calcitonin elicited a compensatory response from the other endocrine factors (estrogens in the females, possibly; in the males, as yet unidentified -- Fleming et al., 1973) so that the hypercalcemic response due to the other factors is magnified. The decreased efflux reported by Dacke (1975) seems to support the above findings but since the time of year that the experiment was carried out was not stated, perhaps the same explanation could hold, that is, the effect of injected calcitonin is masked by compensatory factors. Thus, a more specific approach would be to look at the handling of calcium at the target organs.

As stated earlier, fishes, because of their environment, have additional sites for ionic exchanges besides the gut and kidney, as in mammals, namely, the gills, fins and skin (Mashiko and Jozuka, 1964). Internal calcium can be controlled by exchanges between bone and blood. The more

important sites in preventing drastic changes in internal calcium when the animal migrates between a hyperosmotic and a hypoosmotic environment are the ones that are in direct contact with the environment.

In tetrapods, as mentioned earlier, one of the target organs for calcitonin is bone. In fishes, there are two types of bone: acellular (no enclosed osteocytes) and cellular (enclosed osteocytes) (Moss, 1965). The lack of effect of exogenous calcitonin in the dogfishes, the sharks and killifish (Table III) could be explained by the absence of cellular bone (Louw et al., 1969 ; Hayslett et al., 1971, 1972 ; Pang, 1971c; Urist et al., 1972). The lungfishes (Urist et al., 1972) and the salmonids (Moss, 1965) have a mixture of both types of bone perhaps this, together with the other endocrine factors mentioned earlier, could account for the lack of effect in these fishes. In the European eel, with cellular bone, however, chronic injections of salmon calcitonin showed a decrease in osteoclast activity with an increase in osteoblastic apposition (Lopez et al., 1967). The foregoing explanation is based on the premise that acellular bone is metabolically inactive (Moss, 1963). However, findings from the killifish, Fundulus kansae, showed that calcium can be mobilized from acellular bone (Fleming, 1971; Fleming et al., 1973) but this can be regarded as a special

case as these animals can tolerate much higher fluctuations in salinity than other euryhaline species. Thus it would seem that cellular bone is one of the target organs.

The effect of calcitonin on the kidney has been examined in the dogfish and the eel (Hayslett et al., 1971, 1972) and the sockeye salmon (Copp et al., 1972). Negative findings were reported with intravenous and intraperitoneal injections and infusions of salmon calcitonin into intact animals. However, a distinct diuresis with no increase in urinary calcium was observed in the South American lungfish (Pang and Sawyer, 1975). Chan (1972) reported an increase in urinary calcium in ultimobranchialectomized eels and a decrease in stannioectomized ones, thus implying that the ultimobranchial body secretions (calcitonin) affect the kidney by decreasing calcium excretion. Unfortunately, urinary calcium data from animals in which both the corpuscles of Stannius and the ultimobranchial body were removed, but with apparent normocalcemia, were not included. A good demonstration of the teleost kidney as another target organ has recently been obtained with experiments involving stannioectomy, partial ultimobranchialectomy or both procedures and salmon calcitonin replacement in the eel (Penwick, 1978). The decreased kidney calcium clearance found in the placebo-injected fish returned to sham-operated levels with salmon

calcitonin injections. This would reflect the observations reported in the tetrapods, in that calcitonin causes an increase in calcium excretion by the kidney. Whether calcitonin has a hyperphosphaturic effect in fish is still unknown. Also, data from the stanniectomized and partial ultimobranchialectomized animals suggests that the active principles from the corpuscles of Stannius, hypocalcin/teleocalcin, act antagonistically to calcitonin at the kidney to achieve normocalcemia in the intact animal (Fenwick, 1978).

The question of whether the gills are sites for the action of calcitonin has been examined by several researchers, using an isolated perfused gill preparation, with conflicting results. Ma (1976) reported that gill arches from the intact freshwater eel showed an increase in calcium-45 in samples collected from the efferent gill arteries when salmon calcitonin was perfused through the afferent arteries, thus indicating that salmon calcitonin caused an increase in influx of calcium through the gill epithelium. However, Milhaud et al (1977) found the opposite effect with their salmon preparation, that is, a decreased influx. Peignoux-Deville et al (1978) lent support to the decreased influx effect of salmon calcitonin with their perfusion studies using the European eel. They also reported an increase in efflux with salmon calcitonin, thus concluding that the

hypocalcemic effect of calcitonin is mainly in increasing calcium extrusion at the gills. However, Milhaud et al (1977) are of the opinion that calcitonin controls mainly the influx of calcium.

The purpose of the present investigation is to take two experimental approaches to try to elucidate the role of calcitonin in a euryhaline teleost, the North American eel, Anquilla rostrata Lesueur. The first approach utilizes the intact eel in which the effect of injected calcitonin on various parameters are measured. The second approach involves the isolated gill perfusion system to demonstrate the effect of calcitonin on one site where calcium is known to be regulated.

Chapter II

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

North American eels, Anquilla rostrata, were used for the experiments. Immature female eels of two different size groups were used: the larger group had body weights ranging between 726 and 934 grams.; the other group was between 32 and 112 grams.

The larger animals were purchased from a local dealer. Upon arrival in the laboratory, they were put into large tanks with about 200 liters of dechlorinated Ottawa City tap water for one to two days for the removal of dried mucous and other debris. They were then transferred to holding tanks at 10°C fed with running dechlorinated tap water and were allowed to acclimate for at least two weeks prior to use. Animals used for seawater-adapted studies were then transferred to recirculating seawater-Living Streams (Frigid Units Inc., Toledo, Ohio) systems containing 100% seawater. The seawater used was purchased in granular form (Instant Ocean, Aquarium Systems Inc., Calif.) and

made up according to the manufacturer's specifications with tap water. Diurnal photo-periods were not controlled but were approximately 12 hours of incandescent light alternating with 12 hours of darkness. The eels were not fed.

The smaller eels were captured with nets from the Ottawa River near Chat's Falls, Ontario, or were taken from the St. Lawrence Seaway system at an eel ladder at the Hydro dam at Cornwall, Ontario. They were kept in 400 liter holding tanks supplied with running dechlorinated tap water at about 10°C. Those used for the experiments were transferred to a Living Stream and acclimated for at least two weeks before use. For one experiment, they were fed thawed beef liver and held at 15°C. The animals used for other experiments were unfed and were held at 10°C. A 0.1% malachite green solution was added periodically to the water to inhibit fungal and bacterial growth. Sick eels were transferred to a "hospital" tank where they were treated with NaCl (97.5 gm.), sulfathiazine (1.25 gm.) and sulfadiazole (1.25 gm.) in 100 liters of dechlorinated tap water until they appeared healthy.

2.2 SOLUTIONS

2.2.1 Radio-calcium

Radioactive-calcium (calcium-45) was purchased from Amersham/ Searle in solution as calcium chloride. Upon receipt, the isotopic solution was diluted in its shipping vial with deionized water to give a solution (solution A) containing 200 microcuries/ml ($\mu\text{Ci/ml}$). An aliquot of 0.1 ml of this solution was then diluted to 10 ml with deionized water ($2 \mu\text{Ci/ml}$; solution B) in a volumetric flask and stored in a 10 ml non-heparinized vacutainer. Solution B was then used as a counting standard with each experimental run. Aliquots of solution A were used for making up the experimental solutions.

2.2.2 Tritiated-Inulin

Tritiated-inulin (M.W.=5000) was purchased as a freeze-dried powder from Amersham/Searle. As in the case of calcium-45, two solutions ($200 \mu\text{Ci/ml}$ and $2 \mu\text{Ci/ml}$) were made up. The diluent used was freshwater teleost Ringer's (Shuttleworth, 1972) with 0.1% bovine serum albumin (Fisher Scientific Co. Ltd., Canada). The $2 \mu\text{Ci/ml}$ solution was used as counting standard and the other solution for making up experimental solutions.

2.2.3 Double-isotope Standards

A solution of calcium-45 (2 $\mu\text{Ci/ml}$) and tritiated-inulin (2 $\mu\text{Ci/ml}$) was made up and used as a counting standard for the double-isotope labelling experiments.

All solutions containing isotopes were stored in a refrigerator.

2.2.4 Calcitonins

2.2.4.1 Bovine

Bovine calcitonin was purchased from Calbiochemicals as a freeze-dried powder (60 MRCmU/gm).³ On the day of the experiment, the powder was weighed on a Mettler analytical balance (model H64) and dissolved in 0.9% saline. This solution was made up fresh each day.

³An MRCmU (Medical Research Council milli-Unit) is equivalent to the biological activity of 5 nanograms of porcine calcitonin as established by the Division of Biological Standards of the National Institute for Medical Research, Mill Hill, England (Copp, 1970).

2.2.4.2 Salmon

Salmon calcitonin was purchased as a freeze-dried powder (23 MRC Units) from Calbiochemicals. The powder was reconstituted in 1 ml of freshwater teleost Ringer's with 0.1% bovine serum albumin. This solution was further diluted and stored frozen in 10 ml aliquots of 230, 345, 460, and 1150 MRCmU/ml. On the day of the experiment, the appropriate aliquot was thawed and diluted for making up the experimental solutions. All stock solutions were only thawed once for dilution.

2.2.5 Corpuscles of Stannius extracts

The preparation of corpuscles of Stannius extract, for the study of the hypocalcemic substance(s) in the corpuscles of Stannius on gill calcium efflux, was essentially the one referenced by So and Fenwick (1977). Stock solutions (25mg/ml approx.) were prepared by homogenizing preweighed glands, collected from immature female eels and stored frozen, in freshwater teleost Ringer's on a Brinkmann Polytron (Brinkmann Instr. Can. Ltd.) tissue homogenizer for 15 seconds. The solutions were then centrifuged in a Sorvall refrigerated centrifuge (model RC2-B) at 4,000g (18,000 rpm) for 30 minutes. The supernatants were stored in 10 ml vacutainers in a refrigerator and were used the following day. Aliquots of the stock solutions were used

for the preparation of perfusates 8 and 9 (see following section).

2.2.6 Perfusates

In vitro studies on gill calcium efflux were carried out by perfusing the isolated gills with the following solutions:

Perfusate 1 : Teleost Ringer's + 0.1% bovine serum albumin + heparin (100 units/ml).

Perfusate 2 : Perfusate 1 + calcium-45 (20 μ Ci) to give a solution with 4 μ Ci/ml in a total volume of 5 ml.

Perfusate 3 : Perfusate 2 + salmon calcitonin (2-57.5 MRCmU/ml) in a total volume of 5 ml.

Perfusate 4 : Perfusate 2 + tritiated-inulin (0.4 μ Ci) + epinephrine (10.92 μ M) to give a solution with 0.08 μ Ci (tritiated-inulin)/ml in a total volume of 5 ml.

Perfusate 5 : Perfusate 4 + salmon calcitonin (50-100 MRCmU/ml) in a total volume of 5 ml.

Perfusate 6 : Perfusate 1 + calcium-45 (40 μ Ci) + tritiated-inulin (40 μ Ci) + epinephrine (10.92 μ M) to give a solution with 13.3 μ Ci (calcium-45)/ml and 13.3 μ Ci (tritiated-inulin)/ml in a total volume of 3 ml.

Perfusate 7 : Perfusate 6 + salmon calcitonin (383.3 MRCmU/ml) in a total volume of 3 ml.

Perfusate 8 : Perfusate 1 + calcium-45 (40 μ Ci) + tritiated-inulin (40 μ Ci) + corpuscles of Stannius homogenate (4.5 mg/ml) to give a solution with 13.3 μ Ci (calcium-45)/ml and 13.3 μ Ci (tritiated-inulin)/ml in a total volume of 3ml.

Perfusate 9 : Perfusate 8 + salmon calcitonin (383.3 MRCmU/ml) in a total volume of 3 ml.

2.2.7 Bathing Medium

The holding medium for the isolated-perfused gills of freshwater-adapted eels was made up with 1% teleost Ringer's and calcium chloride to give a calcium concentration of 0.45 mM/L (Fenwick and So, 1974) which is equivalent to the average freshwater calcium levels in freshwater-holding conditions. In studies that used seawater-adapted eels, the gill arches were bathed in reconstituted 100% seawater.

2.3 TECHNIQUES

2.3.1 Blood Sampling

Blood samples were obtained from the caudal vein with 23-16 needles fitted to heparinized 1 ml disposable syringes. The point of entry of the needle was about 3 cm caudad to the anterior end of the ventral caudal fin. The needle was passed through the hypaxial muscles until the

obstruction from the groove in the vertebrae was found. The needle was lifted slightly and the caudal vein was located when blood was seen to enter the syringe through the application of a slight pressure on the plunger. Blood was then drawn slowly into the syringe. The collected blood was then transferred to a heparinized centrifuge tube and centrifuged at 770xg for 10 minutes to obtain plasma for later analysis.

2.3.2 Intraperitoneal Injections

Injections of experimental solutions into the peritoneal cavity were carried out with 26-1/2G needles fitted to 1 ml disposable syringes. The needle entered the animal at a 45-degree angle, at a point about 2-3 cm anterior to the urogenital opening. The injected volume did not exceed 1 ml at a time.

2.3.3 Gill Perfusion

A modification of the technique described by Fenwick and So (1974) was used. The eel was decapitated at a point just in front of the pectoral fins. Movement of the head segment was prevented by pithing with a piece of wire. A ball of cotton was inserted into the buccal cavity to sepa-

rate the afferent branchial artery from the efferent branchial artery. The head segment was placed ventral surface up and the integument was reflected to expose the gills, the ventral aorta and the afferent branchial arteries. The two afferent arteries going to the first left and right gill arches were dissected free of the connective tissue and a cannula was inserted into the right artery and tied with a piece of No.10 cotton thread with the tip of the cannula going about 2-3 mm into the arch. The left arch was then cannulated in the same manner. Perfusion fluid was pumped continuously through the afferent cannula, before and after its insertion into the afferent artery. The point of attachment of the right arch to the ventral surface was then cut to allow access to the efferent arteries located on the dorsal surface of the buccal cavity. The efferent arteries were then cut just prior to the point where they join the efferent arteries from the second set of branchial arches to facilitate the flushing of the cannulated arches. The right efferent artery was then cannulated, the whole arch dissected free and removed to a sampling jar holding the bathing medium. The same manipulations were carried out with the left efferent artery and arch. The above procedure took about 15-20 minutes from the time of decapitation. Both arches were perfused in 50 ml of bathing medium until a satisfactory flow rate had been established and the arches were colourless. The arches were then transferred to their

own individual bath and the baths were covered with Parafilm (Fisher Scientific). The two arches were used alternately as the control and the experimental preparation; that is, in one run, the left arch would be used as the control while the other arch would be perfused with salmon calcitonin, and on the next run, the right arch would be the control and the left perfused with salmon calcitonin. All bathing media were bubbled vigorously with 100% oxygen. The temperature of the bathing media was maintained, in a water bath at 10°C, with a Lauda refrigerated circulator (model K-2/R). The temperature of the water bath was monitored with a Yellow Springs Tele-Thermometer (model 46TUC). The flow rate was checked again after transferring to the individual jars. The effluent flow rate was checked by collecting the fluid in 0.1 ml pipettes graduated in 0.01 ml divisions. Preparations with flow rates less than 90% of the perfusion rate (0.8-1.0 ml/hr) were discarded. After a satisfactory flow rate was established, the perfusion solutions were switched to the experimental solutions containing the isotope(s), or isotope(s) and salmon calcitonin. The flow rate was monitored constantly throughout the experimental period and the sampling of the collected effluent and the bathing media was done every 15 minutes. Sampling of the bathing media was done with a Gilson (0-200 µl) adjustable pipettor set at 100 µl; the collected effluent was sampled with either the Gilson, a 50 or 20 µl Eppendorf pipettor, depending on the flow



rate. At the termination of the experiment, the perfusion was stopped and the perfused filaments were blotted on absorbent paper and removed for weighing.

2.3.4 Handling of Samples

2.3.4.1 Tissue Samples

All tissue samples for the in vivo study were left in a muffle furnace at 100°C overnight (18-20 hr) for the dry weight. Then the tissues were digested in 3 ml of 3N HCl and then ashed in a muffle furnace at 500°C overnight. The ashed tissues were then redissolved in 0.1N HCl (5 ml). One milliliter of the ashed solution was then counted in 4 ml of Aquasol (NEN) on a Beckman LS 233 liquid scintillation counter.

For the in vitro studies, the activity of the perfused gill filaments was determined first by digestion with NCS (1 ml) tissue solubilizer (NEN) in a 50°C oven overnight, neutralized with 0.4 ml glacial acetic acid and counted in 16 ml of Aquasol-2 (NEN).

2.3.4.2 Determination of Isotopic Activity in Samples

1. Quench Corrections

Due to the phenomenon of the shifting of the energy spectrum by various agents (quenching) inherent in a liquid scintillation system, quench correction graphs were established with graded amounts of Sudan IV (colour), chloroform (chemical), HCl (acidic), KOH (basic) and water (volume) (see Wang and Willis, 1965). A known amount of the isotope(s) (0.2 μ Ci) was counted with the above quenching agents and the channel ratios were plotted with the efficiency to give the graphs. Appropriate calculations were done on the Wang 600 programmable calculator (Wang Laboratories) with the channel ratios obtained from the unknown samples to give the corrected activity, for single isotope determinations. The two methods for double-isotope labelling have the quench corrections incorporated into the equations and the graphs.

2. Single Isotope - calcium-45

Determination of the calcium-45 activity in the samples was carried out by counting the samples in 4 ml of Aquasol in the Beckman LS 233 liquid scintillation counter. The counting windows used were the full tritium Isofet module (Beckman) and the variable window Isofet module set at 0-550. The samples were counted three times with a counting time of two minutes each. Quench corrections and sample activity were obtained with the use of a program on the Wang 600 programmable calculator.

3. Double Isotopes - calcium-45 and tritiated-inulin

Method I - 2 channel determinations

Samples were counted in 8 ml of Aquasol in the Beckman LS 233 liquid scintillation counter. Determination of the activity of each isotope in the samples was carried out using the discriminator-ratio method (Okita et al., 1957), with the full tritium Isofet as Channel 1 and the variable Isofet (0-550) as Channel 2. The activity of tritiated-inulin and calcium-45 were calculated with the following equations:

TABLE

Calculation of activity of tritiated-inulin (3HIn)
and Ca-45

3HIn dpm = $(bN_1 - N_2/b - a)$ / channel 1 3HIn efficiency

Ca-45 dpm = $(b(N_2 - aN_1)/b - a)$ / channel 2 Ca-45 efficiency

where: N_1 = net cpm of channel 1
 N_2 = net cpm of channel 2
 H_1 = net cpm of 3HIn in channel 1
 H_2 = net cpm of 3HIn in channel 2
 C_1 = net cpm of Ca-45 in channel 1
 C_2 = net cpm of Ca-45 in channel 2
 $a = H_2/H_1$
 $b = C_2/C_1$

and: $N_1 = H_1 + C_1$
 $N_2 = H_2 + C_2$

The above calculations were carried out with a program written for the Wang 600 programmable calculator. This method is good for tritiated-inulin/calcium-45 ratios ranging from 1-15 (Okita et al., 1957).

4. Method II - 3 channel determinations

The procedure employed is the one proposed by Hendler (1964) but adapted for the Beckman LS 3133 liquid scintillation counter. It entails the use of three counting channels with the windows used being the half-tritium Isoset (channel A), the variable Isoset (300-360; channel B) and another variable Isoset (300-550; channel C). Standard graphs were obtained with ratios of the counts from the three channels and the activity in unknown samples were determined from these graphs. The ratio of tritium/calcium-45 in the samples is not critical with this method. The efficiency of each sample is determined, thus giving a better estimation of the activity for both isotopes than the 2 channel method. Each sample was counted three times at 2 minutes each and the means of these values were used for the final calculations. All calculations were done with the Wang 600.

2.4 EXPERIMENTAL

Two sets of experiments were carried out with the use of the intact eel in the first set and the isolated, perfused gill in the second.

2.4.1 Exogenous calcitonin in the intact eel

In vivo study of the effect of exogenous calcitonin on the distribution of calcium in various compartments and on calcium efflux in the intact eel.

2.4.1.1 Effects of calcitonin on eels transferred to seawater

To study the effect of exogenous calcitonin (bovine) on total plasma calcium and plasma calcium-45 activity in eels with acute transfer to 100% seawater.

Twenty-one feeding yellow eels (Cornwall) with body weights ranging between 52-112 grams were used to study the effect of intraperitoneal injections of bovine calcitonin, calcium chloride, and calcium-45 (30 μ Ci/fish) on total plasma calcium and calcium-45 activity in the freshwater-acclimated eel transferred directly into 100% seawater. The eels were divided into four groups: the control group, injected with only calcium-45; the second group was injected with bovine calcitonin (50 mU/100 gm body weight) and calcium-45; the third group was injected with calcium-45 and calcium chloride (9.19 mg/100 gm body weight); and the last group was injected with all three substances. All solutions were made up to a final volume of 0.5 ml with 0.9% saline. The amount of calcium chloride injected was calculated to increase ex-

tracellular fluid concentration by 2.5 mM, by taking the extracellular fluid volume to be 25% body weight. Hexamethonium bromide (5 mg/100 gm body wt.) was used to immobilize the animals for the duration of the experimental period of six hours.

At the start of the experiment, the eels were anesthetized with tricainemethanesulphonate (MS222; Sigma) at 1 part/ 2000 for the blood sample at 15 minutes prior to the injection of the experimental solutions. After the injections were given, the animals were put into a plexiglass chamber containing 2 liters of 100% seawater. Blood sampling was done 15 minutes, 3 hours and 6 hours after the initial injections with heparinized syringes. This procedure involved the removal of the animal from the chamber for a brief period of time (1-3 min.) during which a volume of 0.3 ml of blood was taken. No replacement solution was given. Total plasma calcium was determined fluorometrically with the Turner fluorometer (model 111; G.K. Turner Associates, Calif.). Plasma calcium-45 activity was determined on the Beckman liquid scintillation counter (LS 233). Total plasma calcium was expressed as mM/l and plasma calcium-45 activity as relative activity; that is, sample dpm/injected dpm per 100 gm body wt. A 2-way analysis of variance (ANOVA) was

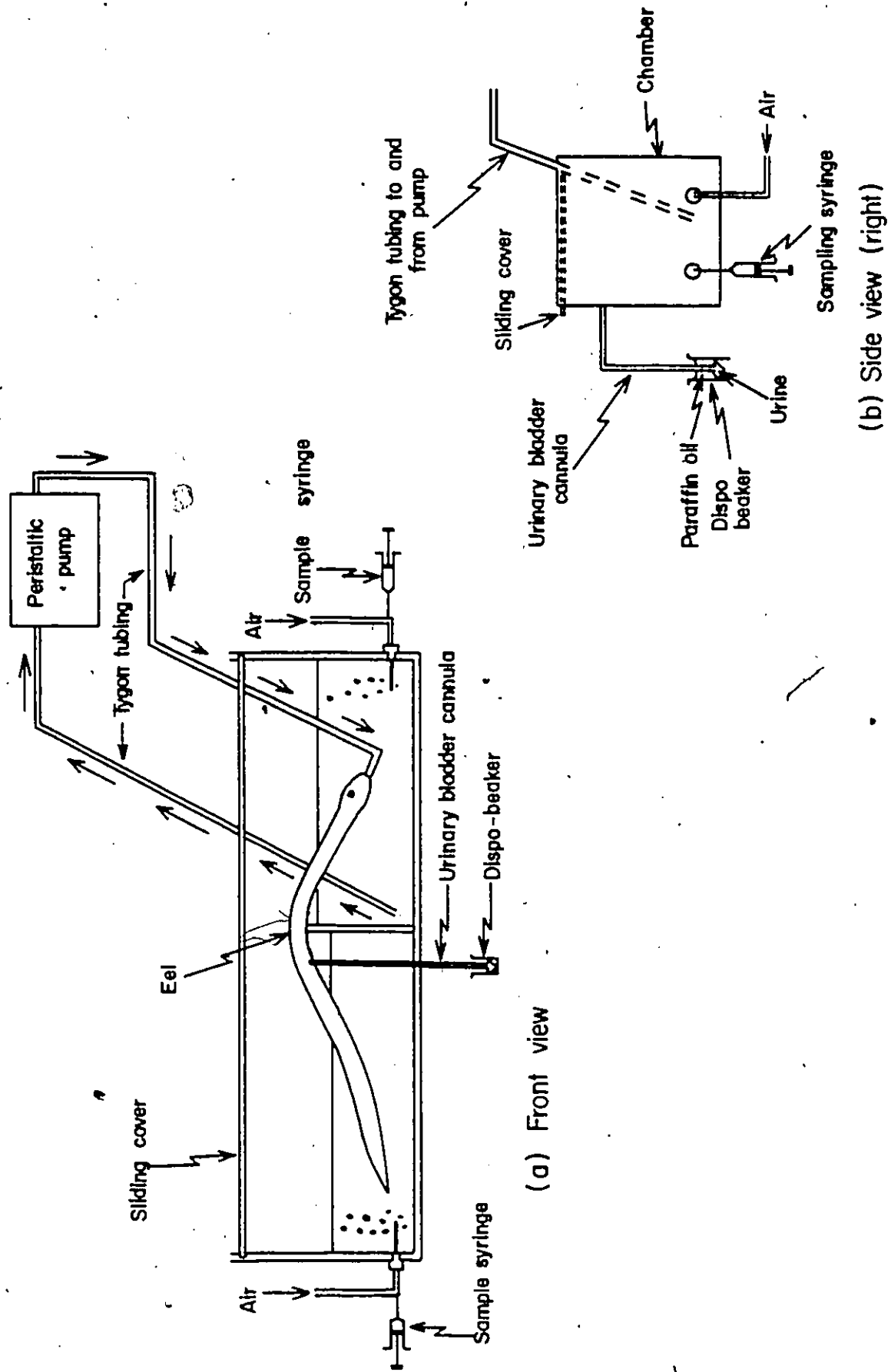
used to test for significant differences among the four treatment groups and the different sampling times. The lsd multiple-range test for unequal replicates was used to locate values that differ significantly (see Steel and Torrie, 1960).

2.4.1.2 BCT, Ca distribution and efflux, intact eel in SW.

To study the effect of exogenous calcitonin (bovine) on calcium distribution in various tissue compartments and on calcium efflux from the intact eel following acute transfer to 100% seawater.

This experiment was carried out using a divided chamber in which the head and tail region of the immobilized eel is partitioned. The method is a modification of the one proposed by Bellamy and Chester Jones (1961). Eleven feeding yellow eels (Chat's Falls) with body weights ranging between 38-101 grams were used. The eels were anesthetized (as described in the previous experiment) for the implantation of the urinary bladder cannula and the attachment of the Manostat cassette pump (Manostat, N.Y.; John's Scient., Ont. distr.) for gill irrigation (Fig. 1a and 1b). Injection solutions were made up as in the previous experiment. Hexamethonium bromide was used to immobilize the eel for the duration of the experimental period (6 hours). Control ani-

mals were injected with calcium-45 and calcium chloride. The experimental group was injected with a similar solution but with bovine calcitonin added. The injections were given intraperitoneally. After the injection of the solutions, the eel was then introduced into a plexiglass chamber, divided into a head chamber (200 ml seawater) and a tail chamber (400 ml seawater) by a plexiglass partition with a sliding cover (Fig. 1a). The urinary cannula was passed through a small opening on the side of the chamber to a conical bottom (1 ml) Dispo-beaker (Fisher Scientific). Urine was collected under paraffin oil (Fig. 1b). Irrigation of the gills was set at 5 ml/min. Air was bubbled vigorously through both chambers for aeration and to ensure that the media were well mixed. One milliliter samples were taken from the bottom of both chambers through openings stoppered with vacutainer caps with 23-16 needles attached to 1 ml disposable syringes every 30 minutes. At the end of six hours, a blood sample was taken from the caudal vein and the spinal cord was cut just behind the cranium with a No. 22 scalpel blade. Tissue samples were taken from the liver, epaxial muscle, posterior kidney, skin, gill filaments and the vertebrae. These samples were handled as described (section III) and results were expressed as tissue cpm/injected cpm per mg dry tissue wt. $\times 100\%$. Bathing media, blood and urine samples were counted in 4 ml Aquasol. Results for the bathing media were expressed as total cpm



(a) Front view

(b) Side view (right)

Diagrammatic (not drawn to scale) representation of the divided plexiglass chamber used for the study of calcium-45 efflux from the intact eel: (a) front view of entire apparatus; (b) side view showing positions of air and sampling ports.

Figure 1: Apparatus for intact eel efflux studies

(corrected for volume)/injected cpm per 100 gm body wt. Blood and urine calcium-45 activity were expressed as sample cpm/ injected cpm per 0.1 ml sample x 100%. Student's "t" test was used to determine differences in the various parameters between the control and the calcitonin-injected group.

2.4.2 Effect of calcitonin on gill

In vitro studies of the effect of exogenous calcitonin (salmon) on calcium efflux from the isolated, perfused gill of intact eels.

The following experiments were carried out with the gill perfusion system proposed by Shuttleworth (1972) and Fenwick and So (1974) with modifications. In general, the experiments involved the perfusion of isolated gill arches (taken from large and small eels) with isotopic calcium (calcium-45; single-isotope perfusion) or isotopic calcium and tritiated-inulin (double-isotope perfusion). The bathing media in which the gills were bathed during perfusion was not labelled with the tracer(s) so that the efflux rate of the tracer(s) could be calculated. This was done by sampling the bathing media at different times during the perfusion period. The rate of efflux was calculated by using the following equations:

For total calcium efflux:

$$\frac{\text{BMdpm} \times \text{BMvol}}{\text{P(dpm)}} \times \frac{\text{P(Ca}^{2+})}{\text{MW}} \times \frac{1}{\text{P(time)}} \times \frac{1}{\text{tissue wt.}}$$

= $\mu\text{M/hr}$ per gm tissue wt.

where: BMdpm = bathing medium calcium-45 activity/ml sample
 P(dpm) = perfusate calcium-45 activity/ml sample
 BMvol = total volume (ml) of bathing medium at end of experiment
 P(Ca²⁺) = total hot and cold Ca²⁺/ml of perfusate
 MW = molecular weight of calcium (40.08)
 P(time) = duration of perfusion period in hours
 tissue wt. = weight of perfused gill filaments (gm)

For tritiated-inulin (3HIn) efflux:

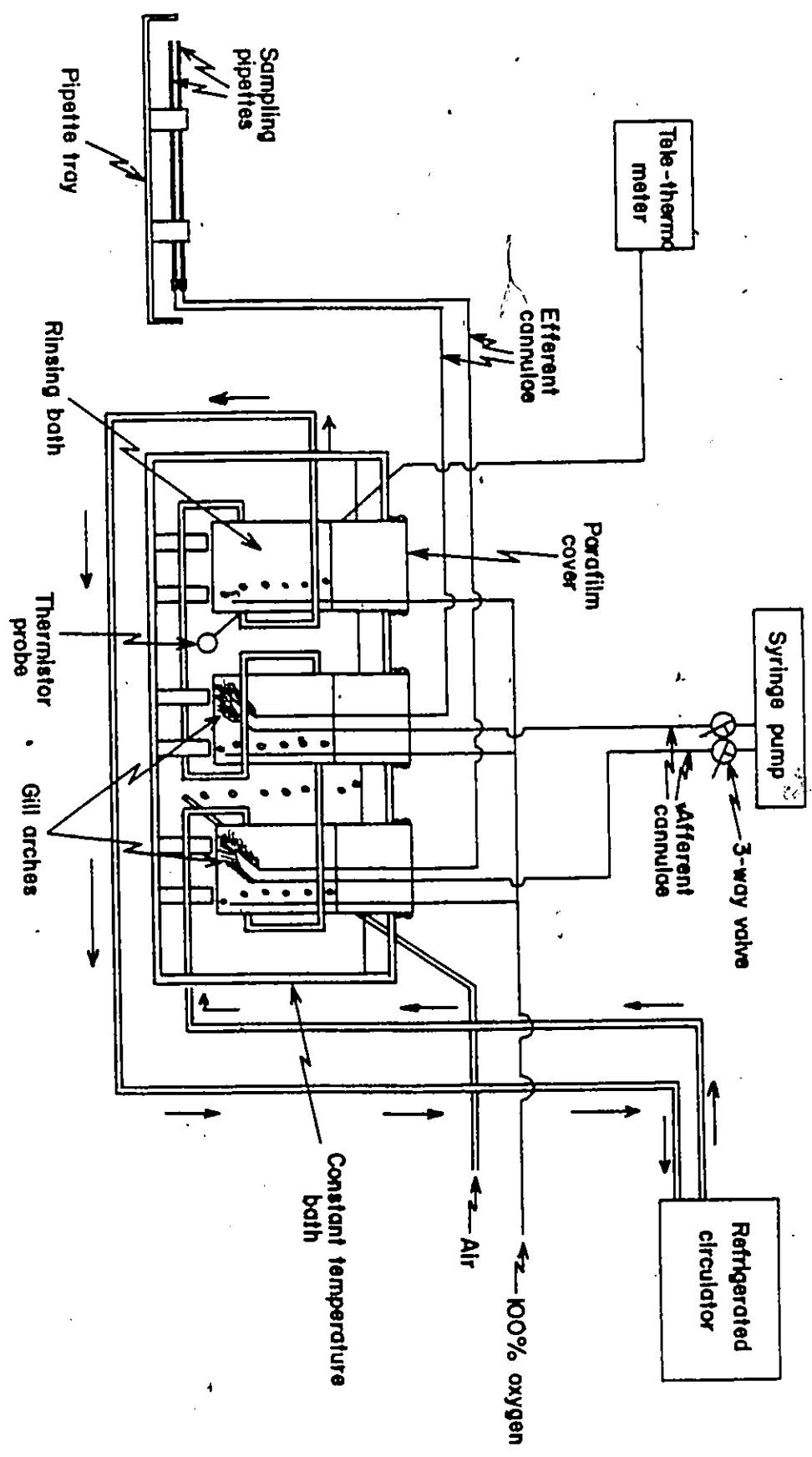
$$\frac{\text{BMdpm} \times \text{BMvol}}{\text{3HIn(dpm}/\mu\text{g})} \times \frac{1}{\text{MW}} \times \frac{1}{\text{P(time)}} \times \frac{1}{\text{tissue wt.}}$$

= $\mu\text{M/hr}$ per gm tissue wt.

where: BMdpm = bathing medium tritiated-inulin activity/ml sample
 3HIn(dpm/ μg) = tritiated-inulin activity/ μg inulin
 BMvol = total volume (ml) of bathing medium at end of experiment
 MW = molecular weight of inulin (5000)
 P(time) = duration of perfusion period (hours)
 tissue wt. = weight of perfused gill filaments (gm)

2.4.2.1 Single isotope gill perfusion - constant-flow system

Five unfed yellow eels (Cornwall) with body weights ranging between 39-75 grams were used. The first two branchial arches were cannulated as described (section IIIC) under a Zeiss dissecting microscope at 60x magnification. Polyethylene tubing (PE 10) was used for cannulation, with the afferent cannulae connected to 20 ml disposable syringes (containing perfusate 1) through 26-1/2G needles attached to 3-way clear plastic valves. A perfusion rate of 0.8 ml/hr was achieved with a Sage syringe pump (model S-352) (Fig. 2). After transfer to their own individual baths and establishment of a satisfactory flow rate, perfusate 1 was replaced by perfusate 2 (with calcium-45), in the control; by perfusate 3 (with calcium-45 and salmon calcitonin) in the other arch. After switching over to the labelled solutions, a 3 ml disposable syringe, with the plunger removed, was attached to the free connection on the 3-way valve. About 0.3-0.5 ml of the labelled solution was allowed to collect in this syringe (with the pumping rate set at 30 ml/hr) before the valve was turned to the position for the perfusion of the arches. This procedure minimized the occurrence of bubbles in the afferent cannulae. Twenty microliters of this collected solution was counted



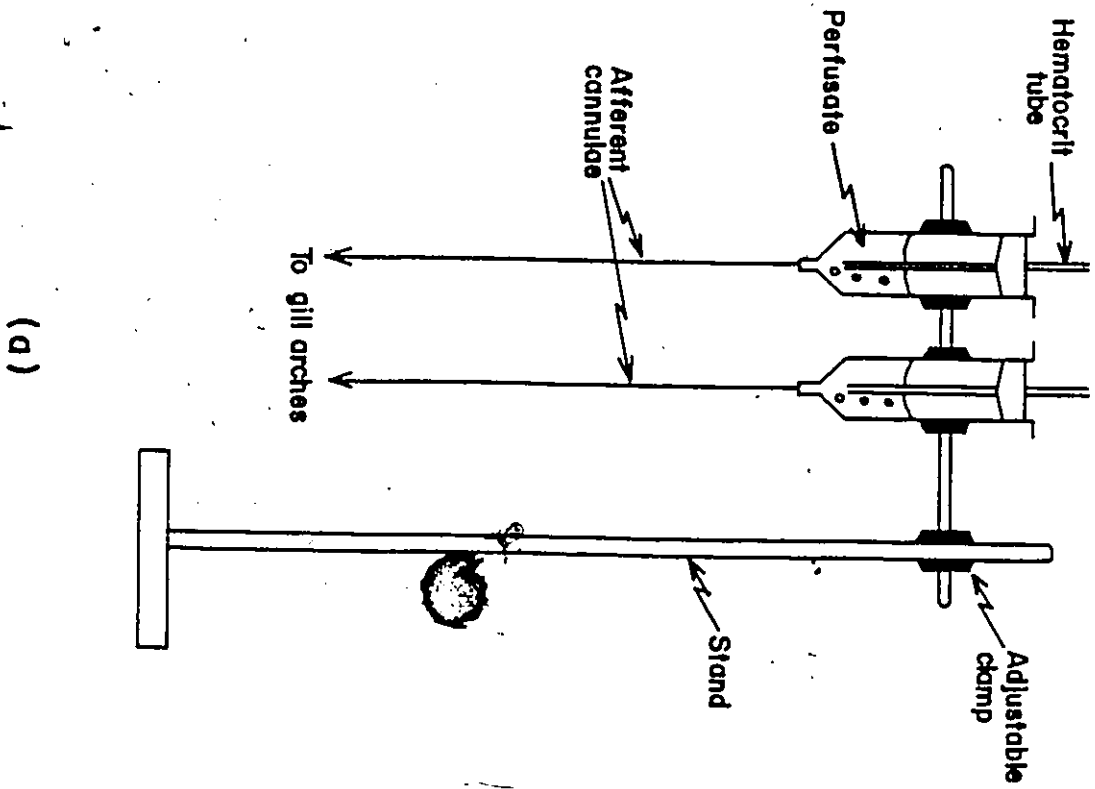
Diagrammatic (not drawn to scale) representation of the experimental set-up for a constant-flow perfusion of isolated gill preparations.

Figure 2: Gill perfusion apparatus

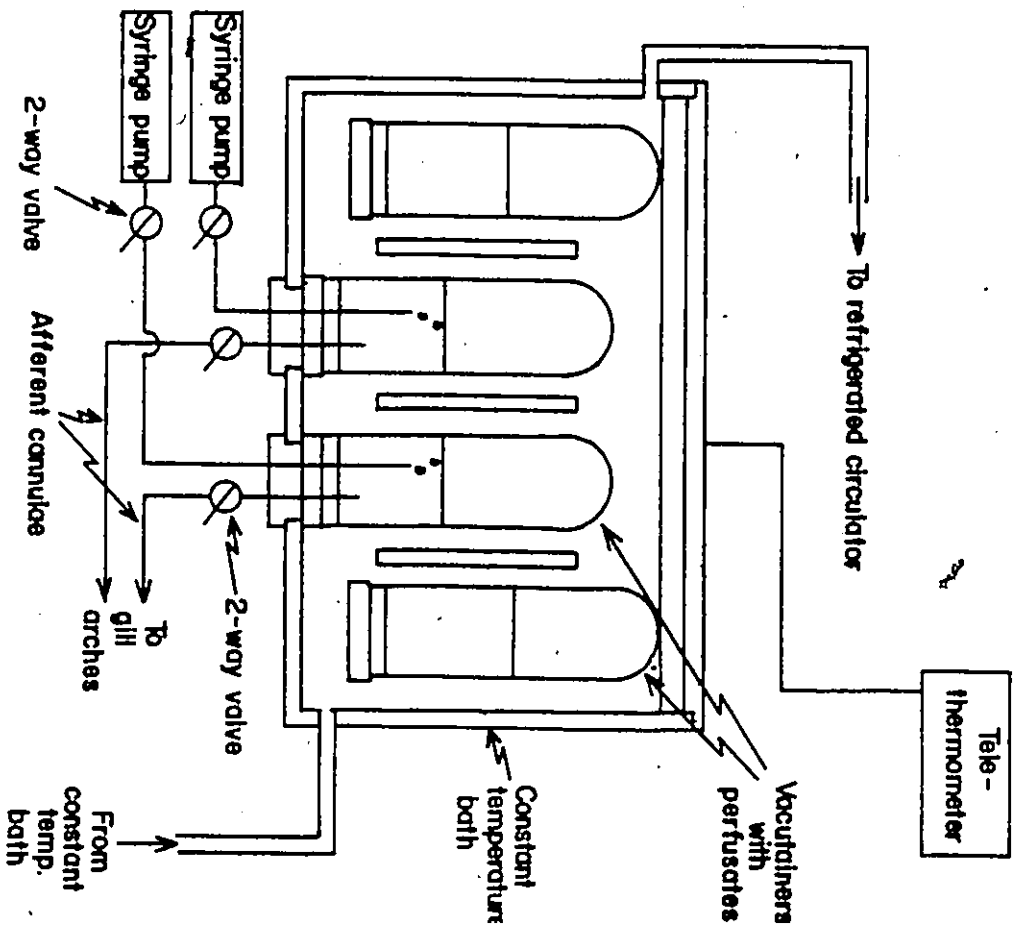
for the calcium-45 activity in the perfusate. The arches were perfused for two hours with the labelled-solutions. The filaments of both arches were removed for the determination of tissue dry weight at the end of the experiment. All samples were handled as described earlier. Calcium efflux was expressed as $\mu\text{M/hr}$ per gm tissue dry weight.

2.4.2.2 Single Isotope Gill Perfusion - constant pressure system

This experiment was carried out essentially the same way as the one just described with the exception that the Sage pump was replaced with a constant pressure perfusion system (Fig. 3a). This system utilized Mariotte flasks constructed from 20 ml disposable syringes and hematocrit tubes (Fig. 3a). This system was tried in the hopes of obtaining a more sensitive and accurate measure of the flow rate, which is a reflection of the leakage through the perfused arches. Two unfed yellow eels (Cornwall) were used. Results were expressed as $\mu\text{M/hr}$ per gm tissue dry weight.



(a)



(b)

Diagrammatic (not drawn to scale) representation of units for changing the constant-flow perfusion system (Fig. 2) to:
(a) a constant pressure perfusion system; and,
(b) a modified constant flow perfusion system.

Figure 3: Modifications to gill perfusion apparatus

2.4.2.3 Double Isotope Gill Perfusion - modified constant-flow system using small eels.

The experimental protocol was the same as experiment 1 with the exception of the addition of tritiated-inulin as a double-check on the leakage problem and a modified constant-flow perfusion system to minimize fluctuations in the pumping rate. Epinephrine ($10.92 \mu\text{M}$) was also added to the perfusate to counteract the increase in resistance in the arches with time (see results from constant pressure perfusion experiment). The modified constant-flow system involved the addition of a "bubble trap", that also contained the perfusate (see Fig. 3b). An additional water bath was hooked up to the constant temperature circuit maintained by the Lauda refrigerated circulator (see also Fig. 2). Air was pumped into the inverted vacutainers inside the added water bath from 20 ml disposable syringes on the Sage syringe pump (model S-352). The vacutainers with the perfusion solutions were first allowed to acclimate in the water bath (at least 1 hour) before they were hooked up to the syringe pump with vacutainer needles and PE 90 tubing. 2-way valves with 22-16 needles at both ends connected the afferent cannulae (PE 50 tubing) to the vacutainers with the appropriate perfusates. When set up properly, this system eliminated the occurrence of bubbles when the per-

fusates were changed. Changing the perfusates required the shutting off of the valves on the syringes and the afferent cannulae. The needles were removed from the original vacutainers and the ones with experimental solutions were put in their place. After the needles were inserted into the new vacutainers, the valves were opened and the perfusion resumed. The whole transferring process for the two arches took about three minutes.

Four unfed yellow eels (Cornwall) with body weight ranging between 38-87 grams were used for the experiment. Perfusate 1 was used for flushing and perfusates 4 and 5 were used as the control and experimental solutions respectively. The gills were perfused for two hours. Sampling was done as described previously. The activity of each isotope (calcium-45 and tritiated-inulin) was determined with Method I (section III). Calcium-45 and tritiated-inulin efflux were expressed as $\mu\text{M/hr}$ per gm tissue dry wt. The mean perfusion rate was expressed as % change in rate; that is, the rate after changing perfusates minus the rate before the change divided by the rate before the change multiplied by 100%. Student's "t" test was used to detect significant differences between the control and the calcitonin perfused gills.

2.4.2.4 Double Isotope Gill Perfusion - modified constant-flow system using large eels.

This experiment involved an experimental period of four hours. The first two hours consisted of perfusing both arches with the double-labelled control solution (perfusate 6). The second two hour period entailed the switching over to other perfusates: perfusate 6 for the control arch and perfusate 7 (with salmon calcitonin) for the experimental arch. The initial perfusion with the "control" perfusate was to load the arches with the isotopes (observations made from the previous experiment (3) indicated that the activity in the effluent collected was just reaching a maximum at the end of two hours). Thus, a loading time of two hours was used before the salmon calcitonin was applied. At the end of four hours, tissue samples were taken as before. All samples were counted in 16 ml of Aquasol-2 in a Beckman LS 3133 (3 channel) liquid scintillation counter. Activity of each isotope was determined with Method II (section III). Perfusion rates, calcium and inulin efflux were expressed as in the previous experiment (3). Statistical analyses were done using the Student's "t" test at a P level of 0.05. Animals used for this experiment were the immature female eels (Quebec City, P.Q.) ranging between 726-1134 grams.

2.4.2.5 Double Isotope Gill Perfusion - static system using small eels.

A "static" system was used to demonstrate the effect of calcitonin and corpuscles of Stannius extract on calcium efflux from the isolated gill. The procedure was a modification of the one proposed by Shuttleworth and Freeman (1974) which involved the perfusion of the head segment (all 8 gill arches) with a perfusate prior to the removal of the arches. The isolated arches were then placed in separate baths for monitoring tracer movement into the bathing media.

Both freshwater-adapted (n=16) and seawater-adapted (n=16) unfed yellow eels (Cornwall) with body weights ranging between 57-134 grams were used. These two groups were each divided into four treatment groups: the control group was "loaded" with perfusate 6 (without epinephrine); the second group with perfusate 7 (salmon calcitonin without epinephrine); the third group with perfusate 8 (corpuscles of Stannius extract without epinephrine) and the last group with perfusate 9 (both salmon calcitonin and corpuscles of Stannius extract without epinephrine).

Each eel was treated according to the following protocol. After decapitation, the ventral aorta was cannulated through the sinus venosus with PE 50 tubing attached to a 22-1G needle on a 20 ml disposable syringe containing perfusate 1. Blanching of the arches was achieved after perfusion with 5 ml of perfusate 1 at a rate of 5 ml/min. The 20 ml syringe was then replaced with a 3 ml disposable syringe containing the labelled solution. The arches were then perfused with 3 ml of the labelled solution at a rate of 3 ml/min. After the arches were "loaded" with the perfusate, the first arch on the right side of the animal was removed and the afferent and efferent ends were clamped with a hemostat. Then, by manipulating the isolated arch with the hemostat, the preparation was rinsed by dipping twice in each of 3 separate 10 ml aliquots of deionized water. The preparation was immersed immediately after the rinses in 2 ml of bathing medium. Oxygen (100%) was bubbled vigorously through the bathing medium to ensure mixing and aeration. The bathing medium was left at room temperature (20°C). The time taken from the removal of the arch to its immersion in the bathing medium was about 30 seconds. The rest of the arches were handled in the same way, going from the second to fourth arch on the right side, then the first to the fourth on the left side of the eel. After incubation in the bathing medium for 6 minutes, the arches were re-

removed, blotted on absorbent paper and the filaments removed for weighing and digestion for counting. Determination of tracer activity was carried out and the results were expressed as in the foregoing experiment (4).

Statistical analyses of the results were carried out with a multivariate analysis of covariance (MANCOVA) program in the IBM 360 computer (see Timm, 1975). A 3-way MANCOVA was used to test for variations among: the freshwater- and seawater-acclimated eels, the 4 treatment groups (control, salmon calcitonin, corpuscles of Stan-
nius extract, and salmon calcitonin + corpuscles of Stan-
nius extract) and the 8 gill arches within each fish. A 2-way MANCOVA (nested design) was then used to test for variations among the 4 groups within the freshwater- and seawater-acclimated eels. A 1-way MANCOVA was employed for testing variations among the 4 treatment groups in the freshwater- and seawater-acclimated eels independently. Gill calcium-45 and tritiated-inulin were taken as the independent variables and calcium and inulin efflux values were the dependent variables. Statistical significance was tested using the Bonferroni-Dunn "t" procedure for calculating confidence intervals with alpha levels at 0.05 and 0.01. The procedure involves the following:

$$\hat{\psi} \pm \hat{\sigma}_{\hat{\psi}}(t_{\alpha})$$

where: $\hat{\psi}$ = the least-square estimates adjusted for the
covariate term
 $\hat{\sigma}_{\hat{\psi}}$ = the standard errors of contrasts adjusted for
the covariate term
 t_{α} = Bonferroni-Dunn "t" with alpha levels at 0.05
or 0.01.

Comparisons of gill calcium-45 in the treatment groups were done using the least-square estimates and the standard errors of contrasts that had not been adjusted for the covariate term in the above calculations for the confidence intervals. Gill tritiated-inulin comparisons were carried out in the same manner.

Chapter III

RESULTS

3.1 IN VIVO STUDIES

Effect of bovine calcitonin (50 MRCmU/100 gm body wt.) and calcium-loading (9.19 mg/100 gm body wt) on various parameters in the intact freshwater-acclimated eel following acute transfer to 100% seawater.

3.1.1 Total Plasma Calcium

Table IV shows that bovine calcitonin did not significantly ($P > 0.05$) affect the total plasma calcium of the control group but a significant ($P < 0.01$) increase was observed in the calcium-loaded group at 15 minutes. Calcium loading did produce a significant ($P < 0.01$) hypercalcemia and there was no apparent recovery by 6 hours.

3.1.2 Plasma calcium-45

TABLE IV

BCT and Plasma Calcium : intact eels

Effect of a single intraperitoneal injection, at 0 time, of BCT (50MU/100 gm body wt), and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (9.19, mg/100 gm body wt)^a on total plasma calcium^b in intact FW-acclimated eels transferred to 100% SW.

Group	Sampling time		
	-15 min.	15 min.	3 hrs. 6 hrs
control (c) (n=4)	2.66 ± 0.39(b)	2.74 ± 0.33	2.69 ± 0.42 2.77 ± 0.24
control (c) + BCT (n=5)	3.09 ± 0.58	3.07 ± 0.38	3.08 ± 0.35 3.29 ± 0.37
Ca-loaded (c,d) (n=6)	2.71 ± 0.45	4.00 ± 0.42(e)	4.26 ± 0.54(e) 4.11 ± 0.42(e)
Ca-loaded (c,d) + BCT ^f (n=6)	2.98 ± 0.13	4.77 ± 0.10(ef)	4.28 ± 0.14(e) 4.41 ± 0.13(e)

a = amount calculated to increase plasma Ca^{2+} 2x approx. based on body wt.

b = mM/l (mean ± standard deviation)

c = injected with calcium-45 (30 μCi /fish)

d = injected with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (see a)

e = significantly different from pre-injection value (-15min) at $P < 0.01$ (2-way ANOVA)

f = significantly different from calcium-loaded (15 min) at $P < 0.01$ (2-way ANOVA)

The results of the effect of bovine calcitonin on the relative calcium-45 activity in the plasma of control and calcium-loaded eels are summarized in Table V. The injection of 30 uCi/fish of calcium-45 produced measurable plasma levels of calcium-45 in all groups and the level obtained was not affected by concomitant calcium-loading. Bovine calcitonin caused a significant ($P < 0.05$) reduction in plasma relative calcium-45 activity after 6 hours in the group which had not been loaded with calcium. The other three groups also showed reduced calcium-45 levels after 6 hours but these were not significantly lower than the 15-minute reading.

3.1.3 Distribution of calcium-45 within the eel

The distribution of calcium-45 in various tissues and body fluid compartments in calcium-loaded eel with or without bovine calcitonin injections is summarized in Table VI. Exogenous calcitonin caused an increase in skin calcium-45 ($P < 0.05$). Due to the small sample size and the large variations (standard deviations), no significant difference was observed in the other compartments between the control and the calcitonin treated group. However, within the control group, the distribution of calcium-45 appeared to be highest in blood, followed by the gills and urine. The levels of calcium-45 found in bone and muscle

TABLE V

BCT and Plasma Calcium-45 : intact eels

Effect of a single intraperitoneal injection, at 0 time, of BCT (50mU/100 gm body wt), and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (9.19 mg/100 gm body wt)^a on the relative percent^b of injected calcium-45 in the plasma of intact FW acclimated eels transferred to 100%SW.

Group	Time following injection	
	15 min.	3 hrs. 6 hrs.
control (c) (n=4)	0.524 ± 0.565 (b)	0.451 ± 0.262 0.361 ± 0.195
control (c) + BCT (n=5)	0.874 ± 0.503	0.716 ± 0.297 0.375 ± 0.208 (e)
Ca-loaded (c,d) (n=6)	0.641 ± 0.471	0.501 ± 0.293 0.396 ± 0.228
Ca-loaded (c,d) + BCT (n=6)	0.710 ± 0.360	0.517 ± 0.263 0.436 ± 0.212

- a = amount calculated to increase plasma Ca (2+) 2x approx. based on body wt
 b = sample cpm/injected cpm/100 gm body wt x 100% (mean ± standard deviation)
 c = injected with calcium-45 (30 $\mu\text{Ci}/\text{fish}$)
 d = injected with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (see a)
 e = significantly different from control + BCT at 15 min. with $P < 0.05$ (2-way ANOVA)

TABLE VI

BCT and Internal Calcium Distribution

Effect of BCT on the relative percent of injected ^{45}Ca appearing in different tissues and fluids in intact FW acclimated eels 6 hours after transfer into 100% SW^a.

<u>Compartment</u>	<u>Control</u>	<u>BCT injected</u>
Liver (b)	0.006 ± 0.005 (3)	0.010 ± 0.006 (4)
Muscle (b)	0.004 ± 0.003 (3)	0.006 ± 0.002 (4)
Bone (b)	0.007 ± 0.005 (3)	0.014 ± 0.008 (4)
Kidney (b)	0.018 ± 0.008 (3)	0.025 ± 0.010 (4)
Gill (b)	0.094 ± 0.140 (3)	0.034 ± 0.010 (4)
Skin (b)	0.014 ± 0.005 (3)	0.029 ± 0.008 (4) (d)
Urine (c)	0.115 ± 0.131 (3)	0.054 ± 0.055 (4)
Blood (c)	0.145 ± 0.132 (5)	0.301 ± 0.235 (4)

a = values given are mean ± standard deviation (no. replicates)

b = tissue cpm/injected cpm.mg dry wt x 100%

c = fluid cpm/injected cpm.0.1 ml sample x 100%

d = significantly different from control (P<0.05)

was very close to those found in the liver samples. In the calcitonin-injected group, the trend appeared to be towards an elevation of calcium-45 in the different compartments, with the exception of muscle, where the value was about the same as the control; and, in gill tissue and urine, where a decrease appears to be the case.

3.1.4 Radio-calcium clearance

The effect of bovine calcitonin on calcium-45 clearance from intact calcium-loaded eels following acute transfer to 100% seawater are shown in Table VII, and Fig. 4a and b. Overall, calcitonin caused an increase ($P < 0.01$) in total calcium-45 clearance by six hours. This increase, however, was entirely due to a stimulation of calcium clearance into the head chamber ($P < 0.01$) (Table VII). The observation that calcitonin effected calcium-45 clearance in the head chamber than the tail chamber is supported by the data shown Fig. 4a and 4b. These figures also show that the effect of calcitonin on calcium-45 clearance into the head chamber was already evident ($P < 0.05$) after 1 hour. Calcitonin did not appear to affect the rate of calcium-45 clearance from the tail region of the fish.

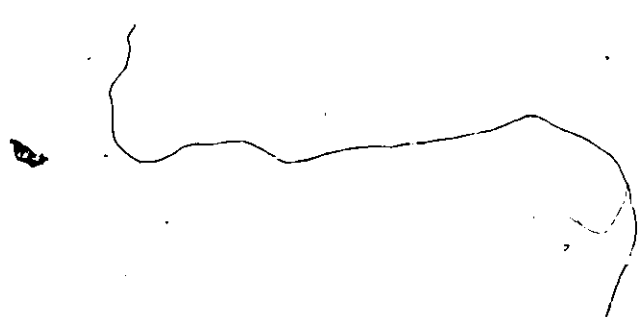


TABLE VII

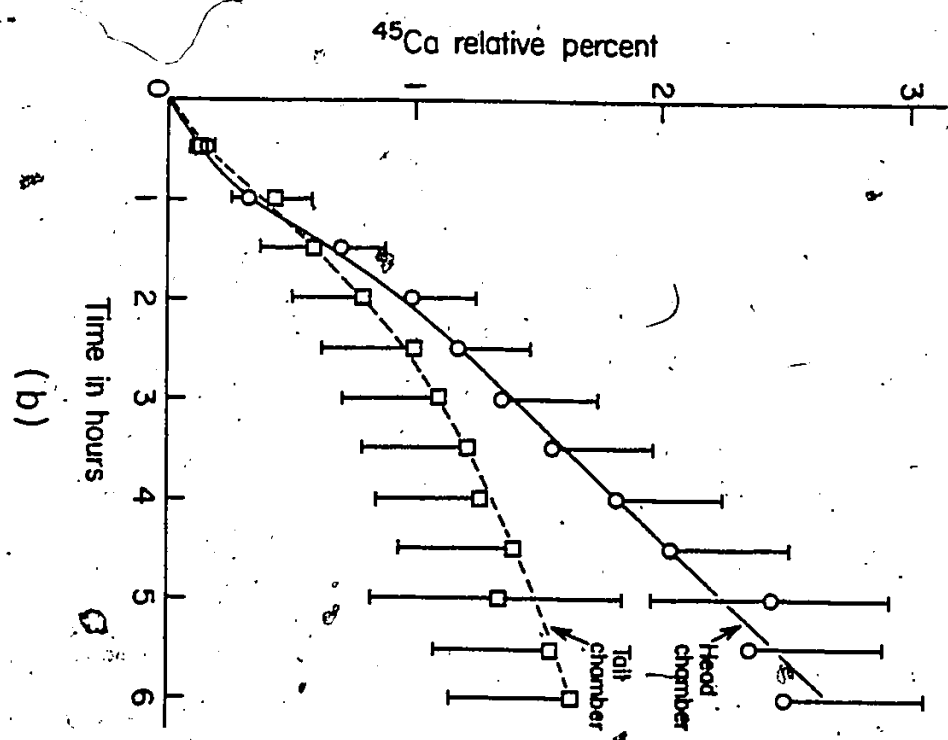
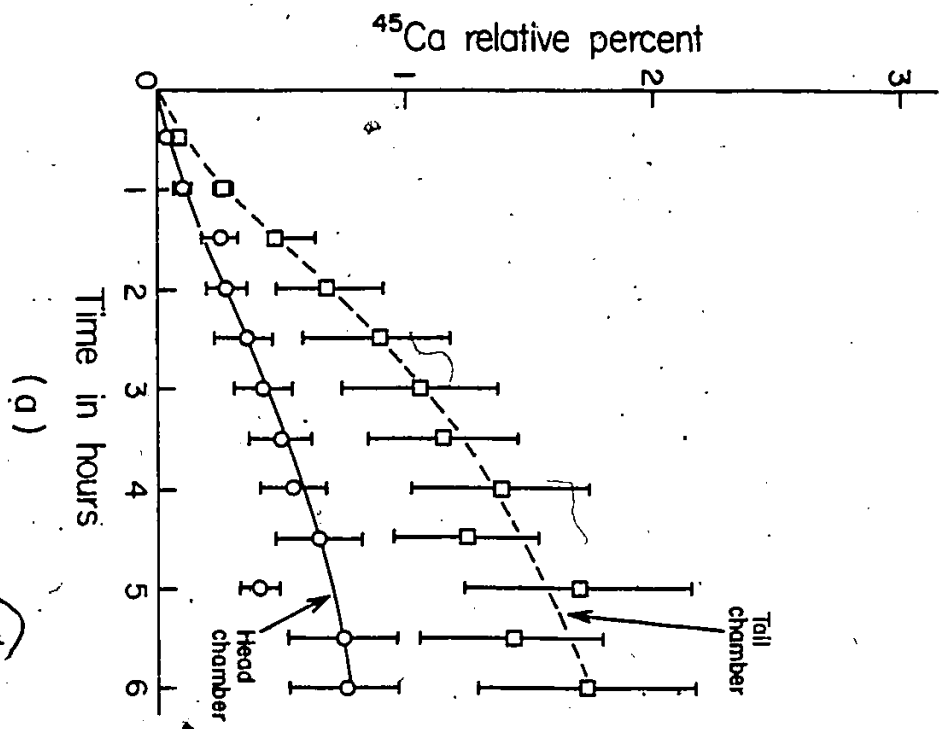
Efflux in Calcium⁴⁵-challenged eels

Effect of BCT on the relative percent of total calcium-45 appearing in the head and tail chambers in intact FW-acclimated eels 6 hours after transfer into 100% SW^a.

<u>Group</u>	<u>Control</u>	<u>BCT injected</u>
Head	0.85 ± 0.52 (6)	2.65 ± 1.0 (5) (d)
Tail (b)	1.77 ± 1.10 (6)	1.68 ± 1.1 (5)
Total (b)	2.62 ± 1.45 (6)	4.33 ± 2.1 (5) (e)
Total (c)	2.89 ± 2.55 (3)	4.97 ± 2.2 (4)

- a = values given are mean ± standard deviation (no. replicates)
 as total cpm extruded/injected cpm/100 gm body wt x 100%
 b = excluding renal excretion
 c = renal excretion included
 d = significantly different from control (P<0.01)
 e = P<0.10 when compared with control

67a



Time-course study of calcium-45 efflux into the head and tail chambers (Fig. 1) from calcium-challenged intact yellow eels: (a) controls (n=6); and, (b) calcitonin-injected (n=5). Values are mean $\pm$ s.d. (volume corrected) as relative percent: sample activity/injected activity multiplied by 100%.

Figure 4: Time-course study of calcium-45 efflux

3.2 GILL PERFUSION STUDIES

3.2.1 Effect of SCT on net gill Ca efflux from gills of eels perfused with the constant pressure system

The effect of salmon calcitonin (50 MRCMU/ml) on net calcium efflux is presented in Table VIII. Results from the four arches taken from two eels (both 41 gm in wt.) indicated a non-significant increase in net calcium efflux with salmon calcitonin. However, the efferent flow dropped in the control and salmon calcitonin-perfused groups. This observation suggests that either perfusion fluid was leaking out into the bathing medium or there was an increase in vascular resistance in the gill arches with time.

3.2.2 Effect of salmon calcitonin perfusion on net gill calcium and inulin efflux

Table IX shows that gills from control eels had considerable individual variation in net calcium efflux and that the small eels had a much higher rate than the large eels ($P < 0.01$). In the small eels, calcitonin appeared to reduce net calcium efflux but this reduction was not significant ($P > 0.05$). In the large eels, calcitonin had no obvious effect on net calcium efflux. However, in all three,

TABLE VIII.

SCT and Calcium efflux : constant-pressure system

Effect of SCT on calcium efflux and perfusion rate in gills of
 FW acclimated eels perfused at constant pressure.

<u>Fish</u>	<u>Calcium efflux (a)</u>		<u>%change in effluent rate (b)</u>	
	<u>Control</u>	<u>SCT-perf.</u>	<u>Control</u>	<u>SCT-perf.</u>
1	7.05	10.60	-21.3	-35.8
2	10.75	15.61	-53.0	-36.4

a = $\mu\text{M/hr}$ per gm tissue dry wt.

b = difference in rate before and after changing perfusates
 $\times 100\%$

c = 50 MRCmU/ml

TABLE IX

SCT and calcium efflux : large and small eel gills

Effect of SCT on net calcium and net inulin efflux and on effluent rate in perfused gills taken from small and large FW-acclimated eels (a).

Group	Net Calcium Efflux (b)	Net Inulin Efflux (b) (x 0.001)	% Change in effluent rate (c)
<u>Small eels</u>			
control (d)	3.60 ± 3.58 (4)	-	-4.3 ± 3.9 (4)
SCT-perfused (d)	2.38 ± 1.15 (5)	-	+7.9 ± 11.8 (5)
control (e)	3.19 ± 3.23 (4)	0.70 ± 0.44 (4)	-19.0 ± 31.0 (4)
SCT-perfused (e)	1.63 ± 0.37 (4)	0.36 ± 0.12 (4)	-20.0 ± 26.6 (4)
<u>Large eels</u>			
control (f)	1.33 ± 1.35 (5)	2.89 ± 2.18 (4)	+5.7 ± 7.4 (5)
SCT-perfused (f)	1.36 ± 1.00 (5)	2.61 ± 1.46 (4)	+1.4 ± 4.0 (5)

a = values given are mean ± standard deviation (number of replicates)

b = $\mu\text{M/hr}$ per gm tissue dry wt.

c = difference in rate before and after changing perfusates x 100%

d = constant flow perfusion at 0.8 ml/hr for 2 hrs; SCT = 3.5 MRCMU/ml

e = modified constant flow perfusion at 1 ml/hr for 2 hrs; SCT = 60 MRCMU/ml

f = modified constant flow perfusion at 1 ml/hr for 4 hrs; SCT = 383.3 MRCMU/ml

groups, the perfusion of the gills with calcitonin greatly reduced the individual variations in net calcium efflux seen in the untreated control groups. Calcitonin also reduced the net efflux of inulin in the small eels and this was not due to the decrease in perfusion rate as the perfusion rate decreased to the same extent as the controls. There appeared to be a higher net inulin efflux in the large eels irrespective of salmon calcitonin treatment.

3.2.3 Effect of SCT, CS on incubated gills

Effect of Salmon calcitonin and corpuscle of Stannius extract on calcium and inulin efflux from incubated gills from freshwater and seawater-acclimated eels.

Tables X and XI summarize the effects of salmon calcitonin and corpuscles of Stannius extract on calcium and inulin efflux from perfused gills taken from freshwater- and seawater-acclimated eels. Using a 3-way multivariate analysis of covariance (MANCOVA), it was judged that the various gill arches (8) in spite of their differences in size and sequential location, did not handle calcium or inulin differently. Therefore, the gill arches from each fish were treated as replicates. Some significant differences ($P < 0.01$) were, however, found between the gills from freshwater- and seawater-acclimated eels as well as among treat-

TABLE X

Incubated PW gill Calcium Efflux

Effect of SCT and CS homogenate on calcium and inulin efflux and tissue activity in the incubated gills of PW-acclimated eels.

Group	Calcium Efflux (a)	Gill Calcium- 45 (b)	Inulin Efflux (a) (x .001)	Gill 3HIn (b)
Control (n=24)	0.261 ± 0.084	5541 ± 2370	3.252 ± 1.754	5154 ± 3690
SCT (n=24)	0.361 ± 0.167	8848 ± 1575	3.769 ± 2.192	6756 ± 5942
CS (n=32)	0.457 ± 0.284 (d)	7918 ± 2744	5.853 ± 3.834 (c,d)	9220 ± 5700 (c)
SCT + CS (n=32)	0.282 ± 0.150	11485 ± 7664 (c)	3.236 ± 1.648	13037 ± 9691 (c)

a = $\mu\text{M/hr}$ per gm tissue wet wt. (mean ± standard deviation)

b = cpm/mg gill filament wet wt. (mean ± standard deviation)

c = significantly different from control at $P < 0.05$ (1-way MANCOVA)

d = significantly different from SCT + CS at $P < 0.05$ (1-way MANCOVA)

TABLE XI

Incubated SW gill Calcium Efflux

Effect of SCT and CS homogenate on calcium and inulin efflux and tissue activity in the incubated gills of SW-acclimated eels

Group	Calcium Efflux (a)	Gill Calcium- 45 (b)	Inulin Efflux (a) (x 0.001)	Gill 3HIn (b)
Control (n=32)	0.245 ± 0.093	1824 ± 1454	1.611 ± 0.749	1179 ± 586
SCT (n=32)	0.472 ± 0.243	5023 ± 2926 (c)	2.892 ± 1.191	1767 ± 893 (c)
CS (n=32)	1.257 ± 1.128 (cd)	10840 ± 20184 (c)	10.038 ± 8.007 (cd)	2763 ± 1029 (cd)
SCT + CS (n=32)	1.185 ± 0.486 (cd)	10472 ± 3461 (cd)	10.181 ± 4.217 (cd)	3839 ± 1142 (cd)

a = $\mu\text{M/hr per gm tissue wet wt.}$ (mean ± standard deviation)

b = $\text{cpm/mg gill filament wet wt}$ (mean ± standard deviation)

c = significantly different from control at $P < 0.01$ (1-way MANCOVA)

d = significantly different from SCT at $P < 0.01$ (1-way MANCOVA)

ment groups. A 2-way MANCOVA (nested-design) showed that calcium and inulin efflux in the control and salmon calcitonin-treated gills were not different in the freshwater and seawater eels. Gill calcium-45 and gill tritiated-inulin concentration were higher ($P < 0.05$) in the freshwater eels than in the seawater ones in all four treatment groups. Conversely, calcium and inulin efflux rates were higher ($P < 0.05$) in the corpuscles of Stannius extract and salmon calcitonin + corpuscles of Stannius extract-treated gills in seawater.

In the freshwater-acclimated eels (Table X), with a 1-way MANCOVA analysis, no effect on calcium efflux was found with either the salmon calcitonin or corpuscles of Stannius extract alone. Although gill calcium-45 was higher ($P < 0.05$) in the salmon calcitonin + corpuscles of Stannius extract group, no change in efflux was detected. But a significant decrease ($P < 0.05$) is apparent when this group is compared with the corpuscles of Stannius extract-perfused group. Higher gill tritiated-inulin was observed in the corpuscles of Stannius extract and salmon calcitonin + corpuscles of Stannius extract groups ($P < 0.05$), while salmon calcitonin alone had no effect. In view of this, inulin efflux rates did not change with salmon calcitonin or salmon calcitonin + corpuscles of Stannius extract, however, cor-

puscles of Stannius extract gave an increased rate ($P < 0.05$), which was also significantly higher ($P < 0.05$) than that of the salmon calcitonin or control group.

In the seawater-acclimated eels (Table XI), salmon calcitonin alone had no effect on calcium or inulin efflux but it did elevate gill calcium-45 and tritiated-inulin ($P < 0.01$). However, treatment with corpuscles of Stannius extract or salmon calcitonin + corpuscles of Stannius extract both gave significant increases ($P < 0.01$), in the efflux of both parameters. The same effect was also seen in the gill calcium-45 and tritiated-inulin measurements. Calcium and inulin efflux were also higher ($P < 0.01$) in the corpuscles of Stannius extract, and salmon calcitonin + corpuscles of Stannius extract-treated gills than in the salmon calcitonin-treated gills. However, while gill tritiated-inulin showed a similar increase ($P < 0.01$) in all these groups, gill calcium-45 was higher ($P < 0.01$) only in the salmon calcitonin + corpuscles of Stannius extract-treated gills when compared to the salmon calcitonin-treated group. In other words, the higher calcium efflux did not correspond to a higher gill calcium-45 in the corpuscles of Stannius extract-treated group when compared to the salmon calcitonin-perfused groups.

Chapter IV

DISCUSSION

Findings from the present study confirmed that plasma total calcium measurements are poor indicators of the effect of exogenous calcitonin. The skin and/or the fins of the yellow eel did participate in calcium exchanges. In the freshwater- or seawater-acclimated eel, calcitonin had no effect on gill calcium efflux; however, when the eel underwent a calcium-challenge, the gills did respond to injected calcitonin with an increased calcium efflux.

The North American eel, Anquilla rostrata, LeSueur, used for this study is a euryhaline species that spawns in the Sargasso Sea (near the Bermuda Islands). The larval eel is carried by the Gulf Stream to the mouth of the St. Lawrence River where they stay for a brief period of time in brackish water before starting their migratory journey into freshwater. The males stay in brackish water while the females migrate a considerable distance inland. The farthest point reached by the females is Lake Ontario. After spending their growth period in freshwater, they start on a sea-

ward-spawning migration (see Tesch, 1977). Thus, throughout the life history of this species, the animal is faced with widely fluctuating environmental conditions. Yet internal calcium concentrations is regulated to within very narrow limits. Indeed, the eel can be transferred acutely between freshwater and seawater with no apparent physiological stress (Kirsch, 1972; Tesch, 1977). No change is reflected in the total plasma calcium in these artificially manipulated fish (see Table IV). Therefore, very efficient mechanisms for the regulation of calcium must be present in the eel, which can also be extrapolated to other euryhaline species. However, attempts to elucidate the mechanisms involved showed a very complex and perplexing situation.

Calcium regulation in euryhaline species appear to involve the interplay of several endocrine factors. In freshwater, prolactin from the anterior pituitary is responsible for the maintenance of serum calcium in face of low calcium in the environment (Pang, 1973; Wendelaar Bonga, 1978). The hypocalcemic action of the secretions from the corpuscles of Stannius is well defined in the fishes (Chan, 1972; Fenwick and Forster, 1972; Pang, 1973; Fenwick, 1978; Wendelaar Bonga et al., 1978 ; Copp and Ma, 1978). Vitamin D injections into the eel produced a hyperphosphatemia but did not show a hypercalcemic effect that is apparent in man-

mals (MacIntyre et al., 1976 ; Lopez et al., 1977). Further work is required on the aspect of vitamin D and calcium regulation in fishes. Calcitonin, whose hypocalcemic effect is observed in times of calcium stress or when there is a high calcium turnover in young growing animals (Introduction, Table II) in the birds (Urist, 1967), the reptiles (Bélanger et al., 1973), and in amphibians (Robertson, 1972); and also appears to apply to the eel in the present investigation.

The initial report of the effect of calcitonin on the freshwater-adapted eel was by Chan et al (1968), where the injection of 10 and 50 mU/fish of porcine calcitonin elicited a hypocalcemic response. This finding was substantiated in subsequent reports (Pang and Griffith, cited in Pang, 1971; Peignoux-Deville et al., 1975 ; Lopez et al., 1976). However, contradictory results that questioned the hypocalcemic action of calcitonin in the intact eel have also been documented (Orimo et al., 1971 ; Dacke, cited by Kenny, 1972; Yamauchi et al., 1978). Also a variation in the plasma calcium response of the killifish, Fundulus kansae, to exogenous calcitonin with seasonal changes was reported by Fleming et al (1973). These authors also proposed that measuring calcium fluxes between the fish and the environment would be a better approach to address the calcitonin question.

In the present investigation, the freshwater-adapted eel, exposed to a calcium-challenge (calcium-loading with calcium chloride and acute transfer to 100% seawater) is employed to determine the role of exogenous calcitonin on calcium regulation in these animals. This approach was taken to circumvent the seasonal internal calcium variations; and based on the fact that in the other classes of animals studied where the effect of calcitonin is shown in times of calcium stress. The effect of exogenous calcitonin in these eels is one of an increased calcium-45 efflux from the animal (Table VII), with a major contribution coming from the head region, namely the gills, (Table VII, Fig. 4a and 4b). A decrease in calcium-45 efflux, however, was reported by Dacke (1975) in the European eel injected with a high calcitonin dose of 500 ng/100 gm fish (about 1000 mU/100 gm fish). Recently, McWhinnie and Scopelliti (1978) reported that injections of salmon calcitonin with doses ranging from 0.5 μ U - 20 mU/gm body weight in frogs caused hypocalcemia at the 1 μ U dose only. The higher doses resulted in a hypercalcemic response. The reversal of the effects observed with the high and low doses of salmon calcitonin in the frog might also be applied to the eel, where the decreased efflux observed by Dacke (1975) could be due to the high dose of salmon calcitonin administered. In the present study, with a bovine calcitonin dose of 50 mU/100 gm fish (Chan, 1968, 1972) (together with a calcium-challenge), an increased ef-

flux, principally by the gills, was demonstrated. This would mean that under adverse conditions, calcitonin does exert its effect at the gills.

The appearance of calcium-45 in the tail chamber would tend to support the findings by Mashiko and Jozuka (1964) in that the fins and/or the skin do play a role in calcium fluxes. Since the fins are more vascularized than the skin, it would seem probable that the calcium found in the tail chamber would be from efflux through the fins, rather than the skin. Also, a time course study of the efflux (Fig. 4) did not show an increase in calcium efflux with bovine calcitonin in the tail chamber. Thus, bovine calcitonin does not affect calcium efflux at the fins and/or skin.

A hypocalcemic effect was not reflected in the total plasma calcium determinations (Table IV). However, a decrease in plasma calcium-45 was observed in the group without calcium-loading but injected with bovine calcitonin (Table V). The apparently higher values of plasma calcium-45 in this group at 15 minutes though not statistically significant might imply that bovine calcitonin could cause an increase of the tracer entering the circulation from the in-

jected solution. The drop at the 6 hour sample could be an increase of the tracer going to the internal calcium stores: muscle and bone (Chan, 1972); or an increase in efflux through the gills as in the calcium-loaded group. Unfortunately, efflux studies were not carried out on these unloaded eels. A study of the calcium efflux would help[?] clarify the situation.

The significant increase in total plasma calcium at 15 minutes after the initial injection ($P < 0.01$; Table IV) which is not reflected in plasma calcium-45 (Table V) might be an effect of bovine calcitonin on the mobilization of calcium from the internal calcium stores, an increased influx from the environment or the increasing entry of the injected calcium into the circulation. The muscle compartment in the eel has been shown to be a storage depot for calcium (Chan, 1972). Thus, one source for the elevation of calcium observed would be the release of calcium from muscle, under the influence of bovine calcitonin. Bone is another site where calcium can be stored. However, observations made on the European eel (Lopez *et al.*, 1976), the frog (Robertson, 1967), and the perfused fetal rat fibula (Liskova-Kiar, 1978) all demonstrated a decrease in osteoclasts with an increase in osteoblast cells. Thus, it seemed unlikely that bone would contribute to the transient hypercalcemia ob-

served. The influx of calcium from the environment could be through the skin, fins, and gastrointestinal tract. The permeability of fish skins to water and ions has been studied in the trout, the killifish and the silver European eel (Fromm, 1968; Kirsch, 1972). In these animals, the skin was found to be impermeable to both water and ionic fluxes. However, the impermeability of the skin of yellow eels has not been established and because these eels have a thinner skin than the silver eels (Kirsch, 1972), it would seem possible that calcium influx may take place. The fins of the carp were shown to be a site for calcium exchanges (Mashiko and Jozuka, 1964). Therefore, the fins could contribute to the increased plasma calcium observed. The gastrointestinal tract as a possible source of calcium influx is based on the fact that the freshwater European eel, upon direct transfer to seawater, the drinking reflex is initiated (Kirsch, 1972). But, in the present study, hexamethonium bromide, used for immobilizing the eels, is a ganglionic blocking agent, which differs from decamethonium in that only the skeletal muscles are affected leaving the breathing reflexes and possibly the drinking reflex intact (Bellamy and Chester Jones, 1961). Thus, though the gills were irrigated with a pulsating pump, very little drinking would be expected due to the blocking action of hexamethonium bromide. Therefore, contributions of calcium from the gastrointestinal tract could be taken as minimal. In general, then, the increased

plasma calcium observed at 15 minutes in the calcium-loaded + bovine calcitonin eels would be due to an increased mobilization of calcium from the muscle compartment, internally, and an influx of external calcium through the fins and/or the skin. The return to control levels (calcium-loaded without bovine calcitonin) at 3 hours could imply a transient hypocalcemia due to the injected bovine calcitonin.

Mayer and Nibelle (1970) studied sodium fluxes in the freshwater European eel with infusion of saline solutions and found that plasma sodium levels returned to normal within 3 hours after infusion was stopped while sodium efflux continued to increase. They concluded that measurements of plasma concentrations would not be good indications of ion-regulating factors. The stability of the elevated total plasma calcium in the calcium-loaded + bovine calcitonin (Table IV) gave no indication of the effect of the injected hormone. However, a trend towards a decrease in plasma calcium-45 (Table V) and the increased efflux at the gills (Fig. 4) were observed but not reflected in the total plasma calcium determinations. This tends to confirm the conclusion reached by Mayer and Nibelle (1970). Thus, locating and defining the role of the target organ(s) under the control of calcitonin would provide a better understanding of the mechanisms involved in calcium regulation.

The initial increase in plasma total calcium might be part of the mechanism by which calcitonin increases calcium efflux through the gills. The elevation of calcium together with bovine calcitonin's action on the gills might facilitate the initiation of the efflux mechanisms at the gills. Indeed, the presence of calcium-45 in the head chamber of the bovine calcitonin group (Fig. 4b) was higher than the control head chamber values (Fig. 4a) at 30 minutes after the initial injections.

The gills have been identified as one of the target organs for calcitonin with the divided (head/tail) chamber experiment. However, a look at the internal distribution of injected calcium-45 proved inconclusive due to the inaccuracies encountered in the preparation of the various tissue samples for tracer determination. A simpler and better method would be to use a tissue solubilizer such as NCS (NEW) for digesting the tissues. In any event, of the samples in which calcium-45 activity were determined, bovine calcitonin appeared to increase calcium-45 in all the tissues, except gill and muscle (Table VI). The increase in blood calcium-45 (though not statistically significant) with bovine calcitonin might reflect the facilitation of calcium-45 removal by the gills. Also, the increase in tracer in the highly vascularized tissues such as the liver and kidney

could just be a reflection of the increased blood calcium concentrations. The increased skin calcium-45 with bovine calcitonin ($P < 0.05$) would indicate either that the eel skin is a route for calcium clearance or it is a calcium storage site in addition to muscle and bone. As stated earlier, the permeability of the thinner skin of the yellow eel is still not defined. The presence of mucous cells does not preclude its role in the extrusion of calcium from the animal. That the calcium-45 found in the skin samples could be the result of adsorption from the bathing medium is unlikely because the skin sample was taken from the region of the animal that was not immersed in either the head or tail chambers (see Fig. 1). The hypothesis that the skin could be a calcium storage depot is based on the observation by Kirsch (1972) that the silver eel skin is poorly vascularized. Because of this, the skin could act as a passive storage, a 'dump', unlike bone or muscle where active exchanges take place. The foregoing, however, is purely speculative. Experimental evidence has yet to be obtained.

The similar values obtained for muscle and liver calcium-45 (0.004% and 0.006% respectively) supports the observations made on the Japanese eel by Chan (1972) where he proposed that the muscle compartment is a calcium storage site. However, bovine calcitonin injections did not appre-

ciably increase the calcium-45 in this compartment. Thus, muscle is not a target organ for calcitonin. The decrease in gill calcium-45 with bovine calcitonin might be due to the high efflux observed; in other words, calcium does not stay in the tissue and is extruded at a higher rate than the control gills (see Fig. 4), whereas in the control gills, because of the slower efflux, more calcium accumulated.

In the freshwater European eel with direct transfer to seawater, a decrease in urine flow was observed (Kirsch, 1972). In the present study, the amount of urine collected from 4 out of 11 eels used for the experiment was negligible. Conclusions as to the effect of bovine calcitonin on the kidney, therefore, cannot be made. Salmon calcitonin, however, was shown to increase kidney calcium clearance in the North American eel (Fenwick, 1978).

It was possible to pinpoint the gills as a site for increased calcium efflux in the presence of calcitonin, with the calcium-challenge experiment (Table VII; Fig. 4). The results for the control incubated gills (Tables X and XI) gave values approximating those from the calcium-challenged control eels. In the calcium-challenge study, head chamber efflux values were calculated to be $0.33 \mu\text{M/hr}$ per 100 gm

body wt for the control eels; 1.013 $\mu\text{M/hr}$ per 100 gm body wt for the bovine calcitonin-injected ones. Tail chamber values for the control and bovine calcitonin-injected eels were very similar: 0.68 and 0.64 $\mu\text{M/hr}$ per 100 gm body wt, respectively. The control values for the incubated gills in both freshwater and seawater were 0.18 and 0.17 $\mu\text{M/hr}$ per 100 gm body wt respectively (these calculations took the 8 gills into account). The lower values for the incubated gills, approximately a 2-fold decrease, compared to the in vivo gills, could be due to the short time of incubation (6 minutes). If a longer incubation was used, osmotic fluxes of water through the gill epithelium would have to be taken into account. In the European eel, incubation of freshwater- and seawater-eel gills in freshwater for 90 minutes showed an increase in water going into the gills; while an efflux of water was observed when these gills were incubated in seawater (Isaia and Hirano, 1970). However, in the seawater rainbow trout and Japanese eel, a decrease in water loss from incubated gills mediated by prolactin and environmental calcium was observed (Ogawa, 1974, 1975). Also, it is because of this osmotic water flux that Shuttleworth (1972) proposed his continuous gill perfusion system. Thus, the short term incubation in the present study minimized the effects due to osmotic water fluxes. The higher efflux obtained with the in vivo preparation could also be due to the increased circulating calcium from calcium loading and the

action of hexamethonium. Hexamethonium decreases cholinergic vasoconstriction of the gill resulting in an increase in flow to the lamellae whereby more gill surface area is available for ionic exchanges (Wood, 1974, 1975; Wood and Shelton, 1975). In the constant flow preparations (Table IX), epinephrine which has a similar effect in increasing blood flow through the gills by stimulation of the beta-receptors (Wood, op.cit. ; Wood and Shelton, op. cit.), could account for high values obtained in both the control, and salmon calcitonin-perfused gills. Recently, Milhaud et al. (1977) demonstrated that norepinephrine increased calcium influx in the isolated, perfused gill of the Pacific salmon. Norepinephrine has been shown to be a more potent stimulus for the beta-receptors in the gill than epinephrine (Bergman et al., 1974 ; Forster, 1976;; Wood, 1974, 1975;; Wood and Shelton, 1975). Thus, an increase in gill calcium influx with epinephrine appears highly probable. Hence, the data obtained with the continuously perfused gill (Table IX) can not show the effect of salmon calcitonin on calcium efflux per se, due to the use of epinephrine in the perfusate to counteract the increasing vascular gill resistance with time (Table VIII; constant pressure perfusion system). Indeed, the increase in perfused gill resistance with time has been reported in the perfused rainbow trout gill (Richards and Fromm, 1970; Wood, 1974, 1975; Wood and Shelton, 1975), the European eel gill (Rankin and Maetz, 1971) and the Pacific

salmon gill (Milhaud et al., 1977). Thus, hexamethonium would be a preferable drug to use for counteracting the increasing resistance in perfused gills. The stabilizing effect of salmon calcitonin on calcium efflux (Table IX) could mean that the variations observed in the control values were due to the differences in the response of the various gill arches to epinephrine (Wood, 1974). The presence of salmon calcitonin in the perfusate decreased the fluctuations but its effect on calcium efflux was minimal. However, this could be due to the aforementioned effects of epinephrine. In the incubated gill study, epinephrine was not used in the perfusion before the gills were incubated. An increase in calcium efflux was not observed in both the freshwater and seawater gills with salmon calcitonin (Tables X and XI). However, the significant decrease in efflux in the salmon calcitonin + corpuscles of Stannius extract group when compared to the corpuscles of Stannius extract-treated might mean that in freshwater, salmon calcitonin counteracted the effect of corpuscles of Stannius on gill calcium fluxes (Table X). In seawater, the effect of corpuscles of Stannius extract seems to dominate in that the values for the salmon calcitonin + corpuscles of Stannius extract, and corpuscles of Stannius extract-treated groups did not differ but they were significantly higher than the control of salmon calcitonin alone (Table II).

The efflux data on the European eel were 13.86 $\mu\text{Eq/hr/kg}$ (or 0.69 $\mu\text{M/hr}$ per 100 gm body wt) and 20.49 $\mu\text{Eq/hr/kg}$ (or 1.02 $\mu\text{M/hr}$ per 100 gm body wt) for control and salmon calcitonin-perfused gills respectively (Peignoux-Deville et al., 1978). These authors based their calculations on only one gill arch and did not take all eight arches into account in expressing the rate as per kg body weight. Multiplying the above values by 8 gives: 5.54 $\mu\text{M/hr}$ per 100 gm body wt. and 8.16 $\mu\text{M/hr}$ per gm body wt respectively. These values seem relatively high and, with the absence of a proper control preparation, the results reported by these authors could be due to a decrease in the viability of the preparation. This viability is reflected in a drop in perfusion rate through leakage into the bathing medium (Wood, 1974). Also, any changes in rate were not recorded by these authors for the duration of a perfusion period of 100 minutes. Results from the present study (Table IX), the drop in rate in the double-labelled perfused small eel gills would reflect an increased leakage from the preparation. However, the efflux values (control: 0.66 $\mu\text{M/hr}$ per 100 gm body wt; salmon calcitonin-perfused: 0.34 $\mu\text{M/hr}$ per 100 gm body wt - corrected for 8 gills) are much lower than those reported for the European eel. One explanation could be that the mean weight of the eels was 450 gm (Peignoux-Deville et al., 1978) which would mean a larger gill surface area for ionic transport or passive leakage. The large eels

used (Table IX) had a mean body weight of about 900 gm and, with a relatively constant flow rate, showed efflux values ($0.11 \mu\text{M/hr}$ per 100 gm body wt) which were still lower than in the European eel. However, the effect of epinephrine on increasing permeability of the large eel gill (Kirsch, 1972) has to be taken into account when looking at the efflux data for the gills in the present study. Further experimental evidence is needed to quantify the absolute rate of calcium efflux in the perfused gill.

Ma (1976) reported an increased influx of calcium with salmon calcitonin perfusion in the North American eel. The mathematical handling of the values, however, did not lend credence to the effect observed.

Milhaud et al (1977) reported a decrease in influx of calcium with salmon calcitonin perfusion of gills from pre-pawning Pacific salmon. They concluded that in seawater-adapted fish, salmon calcitonin decreases the influx of calcium through the gills. In Table X, the opposing effect of salmon calcitonin on the effect of corpuscles of Stannius extract in the freshwater yellow eel gill together with the foregoing observation might imply that salmon calcitonin causes a decrease in the permeability of the gill to calcium

exchanges. The inability of salmon calcitonin in the seawater gills to effect an increase in calcium efflux (Table XI) does not preclude the possibility of a net calcium influx into the gills. Experiments carried out with calcium-47 and calcium-45 on either side of the gill epithelium would provide much better information on calcium fluxes through the tissue and on the effect of calcitonin and corpuscles of Stannius extracts on this tissue.

In the incubated gills (Tables X and XI), though the same concentrations of isotopes were used in the perfusates, there were differences in gill concentrations of these tracers. Recently, a calcium-binding peptide (MW 3500), has been isolated in gill, gut and kidney tissue of the freshwater European eel (Hearn et al., 1978). This peptide is different from the calcium-binding protein found in the chick intestinal epithelium by Wasserman et al (1968) (Hearn et al., 1978). The presence of such a peptide implies that it could facilitate calcium transport or participate in the storage of ionic calcium in cells. In comparing Tables X and XI, the freshwater control and salmon calcitonin-perfused gills have a higher ($P < 0.01$) gill calcium than the corresponding seawater gills although calcium efflux was not different. This might be due to the fact that the freshwater eel has to conserve calcium in a low calcium environ-

ment. Thus, an increase in the calcium-binding peptide in gill tissues would conserve calcium and maintain intracellular calcium at physiological levels. In the seawater-adapted eel, because of the influx of calcium from the environment - which would bring all the efflux mechanisms into action - the role of the calcium-binding peptide is not as important as in the freshwater eel.

Salmon calcitonin or corpuscles of Stannius extract act to increase calcium stored in the freshwater gills. This is indicated by the fact that gill calcium-45 concentration in the freshwater and seawater eels treated with salmon calcitonin, and corpuscles of Stannius extract (Table XI) were similar. Also, increased calcium-45 efflux is observed in the seawater eels only. The results also pointed to a dominant role played by corpuscles of Stannius extract in increasing calcium efflux from seawater gills. This is drawn from the observation that calcium efflux values in the corpuscles of Stannius and corpuscles of Stannius + salmon calcitonin groups are similar. Further, the salmon calcitonin-treated gills had an increased calcium-45 concentration but efflux was not different from that of the controls. Under extreme conditions, however, as with the calcium-challenge experiment, calcitonin may also participate or enhance the action of corpuscles of Stannius secretions in calcium extrusion from the animal (Table VII; Fig. 4).

The use of tritiated-inulin in the perfusion experiments (Table IX, X, XI) demonstrated that there is an efflux of this substance from the gills regardless of whether the constant flow or the incubation procedures were used. Also, the difference in magnitude of inulin efflux (about 3 times higher in the incubated gill than the continuously perfused gill) would point to the possibility that there are channels or "holes" through which the inulin moves to the external medium. Inulin is lost through the gills at the same rate in the freshwater and seawater eels. This observation of inulin loss through the gills was reported in the European eel by Masoni and Payan (1974). Lam (1969) studied inulin efflux from the gills of the stickleback and proposed that pinocytosis, reverse pinocytosis, diffusion along water channels and passage through "holes" left during cell renewal. In the present study, pinocytosis and reverse pinocytosis was not demonstrated; however, passage through "holes" left during cell renewal or water channels could explain the inulin movement in the freshwater gills. On the other hand, passage through "holes" may be an explanation since it was demonstrated that in the Japanese eel and the rainbow trout, an increase in external calcium concentration decrease water flow through the gill epithelium (Ogawa, 1975). Also, Masoni and García-Romeu (1972, cited in Masoni and Payan, 1974) showed that chloride cells in the gills of seawater eels can accumulate and concentrate inulin. Since chloride

cells have been shown to participate in ionic exchanges (Maetz and Bornancin, 1975), it is not unlikely that these cells would also remove inulin from the circulation and extrude it into the bathing medium (Masoni and Payan, 1974).

The result of higher concentrations of gill calcium-45 and inulin in the freshwater group treated with salmon calcitonin + corpuscles of Stannius extract with no increase in efflux of either substance may indicate that calcitonin caused either an increase of uptake of the tracers by the chloride cells or epithelial cells; or an accumulation of the tracers in the passive channels with the mucosal opening of these channels closed. This effect is also observed in the gills of seawater eels which were treated with salmon calcitonin (increased gill calcium and inulin concentration with no increase in efflux rates of either isotope). Autoradiographs of these perfused gills would provide further insight as to the location of calcium-45 and tritiated-inulin in the present investigation.

There is a high correlation (0.88) between the efflux rates of the two tracers, obtained with the incubated gills in freshwater and seawater. No conclusions can be drawn as to whether these tracers are transported independently or

whether the same mechanism(s) are involved. Because, calcium was moved against a concentration gradient in seawater and the chloride cells do accumulate inulin (Masoni and Garcia-Romeu, 1972, cited in Masoni and Payan, 1974), the processes involved can be assumed to be energy dependent. No demonstration has yet been made of calcium transport by chloride cells. The identification of a gill calcium ATPase in the rainbow trout (Ma et al., 1974) and the North American eel (Penwick, 1976, 1979), makes the idea of separate mechanisms for calcium and inulin exchanges attractive. A demonstration of the presence of the calcium ATPase in chloride cells, however, has not been made. Based on the present results, the conclusion is that calcium and inulin efflux is mediated by corpuscles of Stannius secretions.

Calcium efflux rates obtained from the large freshwater eel gills was 1.33 $\mu\text{M/hr}$ per gm tissue dry wt (Table IX). In a separate study, calcium influx for this size eel was found to be 0.085 $\mu\text{M/hr}$ per gm dry weight (So, pers. comm.); while Ma and Copp (1977) reported a value of 334 pmole/min/100 gm dry weight (or 0.20 $\mu\text{M/hr}$ per gm dry weight). In the European eel, Peignoux-Deville et al. (1978) reported a value of 0.0168 $\mu\text{Eq/hr/kg}$ (or 0.67 $\mu\text{M/hr}$ per gm dry weight). It would seem that the efflux obtained in the present study far exceeds that of the influx of calcium in

the freshwater eel. The influx reported in these studies, however, was based on a perfusion time of a maximum of 100 minutes. In the present study, a time of about 120 minutes (2 hours) was required to load the gills with the isotopes. This was determined by monitoring the "plateauing" of the isotopes appearing in the collected effluent. Therefore, the low influx values could be accounted for by the accumulation of the external calcium-45 in the epithelial cells before it is released into the circulation. The calcium-binding peptide (Hearn *et al.*, 1978) might play an important role. Preloading the gills with *in vivo* injections of calcium-45 prior to extirpation for perfusion studies and as stated earlier, the employment of two different isotopes of calcium (calcium-47 and calcium-45) would be a better approach to the study of calcium fluxes at the gills.

Calcitonin has also been shown to have an effect on other parameters in animal systems. In rats, (Williams *et al.*, 1972), sheep (Barlet, 1972), and man (Bijvoet *et al.*, 1971), calcitonin caused an increased urinary sodium excretion together with an increased urinary volume. The removal of the ultimobranchial body in frogs resulted in similar changes (Robertson, 1974). This means, however, that in frogs, calcitonin has an effect opposite to that found in mammals. In the Japanese eel, a decrease in serum osmotic pressure is observed with calcitonin (Orimo *et al.*, 1972).

In the lungfish, calcitonin caused a significant diuresis (Pang and Sawyer, 1975). . . In the goldfish, a diminished endogenous calcitonin effected by partial ultimobranchialectomy caused a decrease in the hematocrit (Fenwick, 1975); whereas an increase in hematocrit was observed with the European eel with exogenous calcitonin (Chan et al., 1968). Thus, calcitonin is not exclusively concerned with calcium regulation and the possibility of its role in osmoregulation requires further investigation.

In the present study, the effect of calcitonin on the gills under 'normal' physiological conditions is negligible but in times of calcium stress, as was demonstrated in mammals, the gills do respond to calcitonin and an increased calcium efflux is observed. Corpuscles of Stannius secretions appeared to play a more important role on gill calcium fluxes.

In conclusion, the gills of the North American eel have been demonstrated to be a target organ for calcitonin in times of calcium stress. No effect is observed in the freshwater- or seawater-adapted eel gill preparations with either the constant flow system or the perfused, incubated gill system. Corpuscles of Stannius extract was shown to

increase gill calcium and inulin efflux in freshwater- and seawater-adapted yellow eels, with the effect more apparent in the seawater-adapted ones. Plasma total calcium measurements are inadequate indicators of the effect of the injected hormone.

Appendix A

THE EFFECT OF CORPUSCLES OF STANNIUS EXTRACTS IN RATS

The hypocalcemic action of the secretion(s) from the corpuscles of Stannius in fishes is well documented. However, the question of whether this effect of the secretion(s) extends to other animal systems had not been examined, whereas the hypocalcemic effect of salmon calcitonin in mammals was well known. Thus, an investigation of the effect of the hypocalcemic substance(s) from the corpuscles of Stannius was attempted with an application of a modified rat bioassay for calcitonin (Kenny, 1971).

Intravenous injections of eel corpuscular extracts elicited a dose-dependent hypocalcemic response from young male rats. Injections of eel muscle and kidney tissue extracts had no effect on plasma calcium in the assay animals. Also, Stannius corpuscles from the brown bullhead did not induce a hypocalcemia. It appears that, as in the injection of fish calcitonin into mammals, the other hypocalcemic substance(s) found in fish (hypocalcin/ teleocalcin) is also effective in a mammalian system, that of a young rat. Application of the rat bioassay to further examine the nature of the hypocalcemic secretion(s) from the corpuscles of Stannius was proposed.

The above findings were presented as a report in the
Canadian Journal of Zoology (see following pages).

PREVIOUSLY COPYRIGHTED MATERIAL

LEAVES 103, 104, 105

HYPOCALCEMIC ACTION OF EEL STANNIUS CORPUSCLE IN RATS

NOT MICROFILMED

MAY BE OBTAINED FROM:

E. Leung and James C. Fenwick
Department of Biology
University of Ottawa,
Ottawa, Ontario
Canada, K1N 6N5

BIBLIOGRAPHY

- Adachi, I., K. Abe, M. Tanaka, S. Miyakawa, S. Kumaoaka, N. Nagata and M. Sasaki. 1974. Phosphaturic effect of I.V. administered calcitonin in man. *Endocrinol. Jap.* 21:317-322.
- Aldred, J.P., R.K. Stubbs, W.R. Herman, R.A. Zeedyk and J.W. Bastian. 1970. Effects of porcine calcitonin on some urine electrolytes in the rat. *Acta Endocr.* 65:737-750.
- Aliapoulos, M.A., P. Goldhaber and P.L. Munson. 1966. Thyrocalcitonin inhibition of bone resorption induced by parathyroid hormone in tissue culture. *Science* 151:330-331.
- Ardailou, R., J.P. Fillastre, G. Milhaud, F. Rousselet, P. Delaunay and G. Richet. 1968. Renal excretion of phosphate, calcium and sodium during and after a prolonged thyrocalcitonin infusion in man. *Proc. Soc. exp. Biol. Med.* 131:56-60.
- Aurbach, G.D. and D.A. Heath. 1974. Parathyroid hormone and calcitonin regulation of renal function. *Kid. Int.* 6:331-345.
- Avioli, L.V., S.J. Birge, S. Scott and W. Shieber. 1969. Role of the thyroid gland during glucagon-induced hypocalcemia in the dog. *Amer. J. Physiol.* 216:939-945.
- Avramides, A., R.K. Baker and S. Wallach. 1974. Metabolic effects of synthetic salmon calcitonin in Paget's disease of bone. *Metabolism* 23:1037-1046.
- Barlet, J.P. 1972. Effect of porcine, salmon and human calcitonin on urinary excretion of some electrolytes in sheep. *J. Endocrinol.* 55:153-161.
- Barlet, J.P. and J.M. Garel. 1974. Physiological role of calcitonin in pregnant goats and ewes. In: Calcium Regulating Hormones. Proc. V. Parathyroid Conf. pp.119-121. R.V. Talmage, M. Owen and J.A. Parsons (eds.). Excerpta Medica, Amsterdam.
- Bélanger, L.F. 1971. The ultimobranchial gland of birds and the effects of nutritional variations. *J. Exptl. Zool.* 178:125-138.

- Bélanger, L.F. and D.H. Copp. 1972. The skeletal effects of prolonged calcitonin administration in birds, under various conditions. In: Calcium, Parathyroid Hormone and the Calcitonins. Proc. IV. Parathyroid Conf. R.V. Talmage and P.L. Munson (eds.). Excerpta Medica, Amsterdam.
- Bélanger, L.F., M.T. Dimond and D.H. Copp. 1973. Histological observations on bone and cartilage of growing turtles treated with calcitonin. Gen. Comp. Endocrinol. 20:297-304.
- Bell, N.H. 1975. Further studies on the regulation of calcitonin release in vitro. Horm. Metab. Res. 7:77-83.
- Bell, N.H. and P.H. Stern. 1970. Effects of changes in serum calcium on hypocalcemic response to thyrocalcitonin in the rat. Amer. J. Physiol. 218:64-68.
- Bellamy, D. 1961. Movements of potassium, sodium and chloride in incubated gills from the silver eel. Comp. Biochem. Physiol. 3:125-135.
- Bellamy, D. and I. Chester Jones. 1961. An apparatus for studying the gain and loss of electrolytes by the eel. Comp. Biochem. Physiol. 3:223-226.
- Bergman, H.L., K.R. Olson and P.O. Fromm. 1974. The effects of vasoactive agents on the functional surface area of isolated-perfused gills of rainbow trout. J. Comp. Physiol. 94:267-286.
- Biddulph, D.M. and P.F. Hirsch. 1973. Role of thyrocalcitonin in restricting hypercalcemia in the golden hamster. Endocrinology 92:1328-1333.
- Bijvoet, O.L.M., J. van der Sluys Veer, H.R. de Vries and A.T.J. van Koppen. 1971. Natriuretic effect of calcitonin in man. New Eng. J. Med. 284:681-688.
- Borle, A.B. 1969. Effects of thyrocalcitonin on calcium transport in kidney cells. Endocrinology 85:194-199.
- Bradshaw, W.N. and W.W. Sutton. 1970. The influence of the environment, reproductive activity and calcitonin on serum calcium and phosphate in the brown bullhead (Ictaluridae). Proc. W. Virg. Acad. Sci. 42:119-120.
- Butler, D.G. 1969. Corpuscles of Stannius and renal physiology in the eel (Anguilla rostrata). J. Fish. Res. Bd. Can. 26(3):639-654.

- Butler, D.G. 1972. Failure to observe changes in selected metabolites following removal of the Stannius corpuscles from the freshwater North American eel, Anquilla rostrata. J. Fish. Res. Bd. Can. 29:1362-1364.
- Care, A.D., R.F.L. Bates, R. Swaminathan, C.G. Scanes, M. Peacock, E.B. Mawer, C.M. Taylor, H.F. DeLuca, S. Tomlinson and J.L.H. O'riordan. 1975. The control of parathyroid hormone and calcitonin secretion and their interaction with other endocrine systems. In: Calcium Regulating Hormones. Proc. V. Parathyroid Conf. pp. 100-110. R.V. Talmage, M. Owen and J.A. Parsons (eds.). Excerpta Medica, Amsterdam.
- Care, A.D., T. Duncan and D. Webster. 1967. Thyrocalcitonin and its role in calcium homeostasis. J. Endocrinol. 37:155-167.
- Carpenter, S.J. and H.L. Heyl. 1974. Fine structure of the corpuscles of Stannius of Atlantic salmon during the freshwater spawning journey. Gen. Comp. Endo. 23:212-223.
- Chan, D.K.O. 1968. Endocrine regulation of calcium and inorganic phosphate balance in the freshwater-adapted teleost fish, Anquilla anquilla and Anquilla japonica. In: Progress in Endocrinology. Proc. III Int. Cong. Endo., Mexico. pp. 709-716. C. Gual and F. J.G. Ebling (eds.) Excerpta Medica, Amsterdam.
- Chan, D.K.O. 1972. Hormonal regulation of calcium balance in teleost fish. Gen. Comp. Endo. Supp. 3:411-420.
- Chan, D.K.O. and Chester Jones, I. 1968. Regulation and distribution of plasma calcium and inorganic phosphate in the European eel, Anquilla anquilla L.) Endocrinology 42:109-117.
- Chan, D.K.O., I. Chester Jones, I.W. Henderson and J.C. Rankin. 1967. Studies on the experimental alteration of water and electrolyte composition of the eel (Anquilla anquilla L.). J. Endocrinol. 37:297-317.
- Chan, D.K.O., I. Chester Jones and W. Mosley. 1968a. Pituitary and adrenocortical factors in the control of the water and electrolyte composition of the freshwater eel (Anquilla anquilla L.). J. Endocrinol. 42:91-98.
- Chan, D.K.O., I. Chester Jones and R.W. Smith. 1968b. The effect of mammalian calcitonin on the plasma levels of calcium and inorganic phosphate in the European eel (Anquilla anquilla L.). Gen. Comp. Endo. 11:243-254.

- Chan, D.K.O., J.C. Rankin and I. Chester Jones. 1969. Influences of the adrenal cortex and the corpuscles of Stannius on osmoregulation in the European eel (Anquilla anguilla L.) adapted to freshwater. Gen. Comp. Endo. Suppl. 2:342-353.
- Chartier, M.-M., C. Milet, E. Lopez, F. Lallier, E. Martelly and S. Warrot. 1977. Modifications morphologiques, cytologiques et biochimiques de la branchie d' Anquilla anguilla L. apres ablation des corpuscles de Stannius. J. Physiol. Paris. 73:23-36.
- Chester Jones, I., D.K.O. Chan and J.C. Rankin. 1969. Renal function in the European eel (Anquilla anguilla): Effects of the caudal neurosecretory system, corpuscles of Stannius, neurohypophyseal peptides and vasoactive substances. J. Endocrinol. 43:21-31.
- Chester Jones, I. and I.W. Henderson. 1965. Electrolyte changes in the European eel (Anquilla anguilla L.). J. Endocrinol. 32:iii-iv.
- Chester Jones, I., I.W. Henderson, D.K.O. Chan, J.C. Rankin, W. Moseley, J.J. Brown, A.F. Léver, J.I.S. Robertson and M Tree. 1966. Pressor activity in extracts of the corpuscles of Stannius from the European eel (Anquilla anguilla L.). J. Endocrinol. 34:393-408.
- Clark, J.D. and A.D. Kenny. 1969. Hog thyrocalcitonin in the dog: urinary calcium, phosphorous, magnesium and sodium responses. Endocrinology 86:1199-1205.
- Clark, N.B. 1971. The ultimobranchial bodies of reptiles. J. Exptl. Zool. 178:115-124.
- Clark, N.B. and W.R. Fleming. 1963. The effect of mammalian parathyroid hormone on bone histology and serum calcium levels in Fundulus kansae. Gen. Comp. Endo. 3:461-467.
- Cohn, D.V., J.J. Morrissey, R.R. MacGregor and J.W. Hamilton. 1978. The role of calcium in the biosynthesis and secretion of parathormone. In: Comparative Endocrinology. Proc. VIII Int. symp. Comp. Endocrinol. pp. 273-278. P.J. Gaillard and H.H. Boer (eds.). Elsevier/North-Holland Biomed. Press, Amsterdam.
- Copp, D.H. 1969. The ultimobranchial glands and calcium regulation. In: Fish Physiology. Vol. 2 : 377-398. Hoar & Randall.
- Copp, D.H. 1970. Endocrine regulation of calcium metabolism. Ann. Rev. Physiol. 32:61-86.

- Copp, D.H., C.E. Brooks, B.S. Low, F. Newsome, R.K. O'Dor, C.O. Parkes, V. Walker and E.G. Watts. 1970. Calcitonin and ultimobranchial function in lower vertebrates. In: Calcitonin 1969. Proc. II Int. Symp. pp. 281-294. S.F. Taylor (ed.). Heinemann, London.
- Copp, D.H., P.G.H. Byfield, C.R. Kerr, F. Newsome, V.E. Walker and E.G. Watts. 1972. Calcitonin and ultimobranchial function in fishes and birds. In: Calcium, Parathyroid Hormone and the Calcitonins. Proc. IV Parathyroid Conf. pp. 12-20. R.V. Talmage and P.L. Munson (eds.). Excerpta Medica, Amsterdam.
- Copp, D.H., E.C. Cameron, B.A. Cheney, A. George, F. Davidson and K.G. Henze. 1962. Evidence for calcitonin - A new hormone from the parathyroid that lowers blood calcium. Endocrinology 70:638-649.
- Copp, D.H., D.W. Cockcroft and Y. Keuh. 1967. Calcitonin from ultimobranchial glands of dogfish and chickens. Science 158:924-925.
- Copp, D.H. and A.V. Kuczerpa. 1968. A new bioassay for calcitonin and effect of age and dietary calcium on the response. In: Calcitonin. Symposium on Thyrocalcitonin and the C cells. pp. 18-24. S.F. Taylor (ed.). Heinemann, London.
- Copp, D.H. and S.W.Y. Ma. 1978. Endocrine control of calcium metabolism in vertebrates. In: Comparative Endocrinology. Proc. VIII Int. Symp. Comp. Endocrinol. pp. 243-253. P.J. Gaillard and H.H. Boer (ed. s.). Elsevier/North-Holland Biomed. Press. Amsterdam.
- Copp, D.H. and C.O. Parkes. 1968a. Extraction of calcitonin from ultimobranchial tissue. In: Parathyroid Hormone and Thyrocalcitonin (calcitonin), Montreal, 1967. pp. 74-82. Excerpta Medica, Amsterdam.
- Copp, D.H. and C.O. Parkes. 1968b. Nature, function and control of calcitonin. In: Progress in Endocrinology, Proc. III Int. Cong. Endocrinol, Mexico, 1968. pp. 704-708. C. Gual and F.J.G. Ebling (eds.). Excerpta Medica, Amsterdam.
- Dacke, C.G. 1975. Effect of salmon calcitonin on calcium and sodium efflux in the European eel (Anquilla anquilla L.). J. Physiol. Lond. 246:75P-76P.
- Dacke, C.G., W.R. Fleming and A.D. Kenny. 1971. Plasma calcitonin levels in fish. The Physiologist 14:127.

- Deftos, L.J., A.E. Bury, G.P. Mayer, J.F. Habener, P.R. Singer, D. Powell, L. Krook, E.G. Watts and J.T. Potts, Jr. 1972. Radioimmunoassays for calcitonins: clinical and experimental studies. In: Endocrinology 1971. Proc. III Int. Symp. pp.89-97. S.F. Taylor (ed.) Heinemann, London.
- Deftos, L.F., J., E.G. Watts, D.H. Copp and J.T. Potts, Jr. 1974. A radioimmunoassay for salmon calcitonin. Endocrinology 94:155-160.
- DeLuca, H.F. 1975. Calcium metabolism. Acta Orthop. Scand. 46:286-314.
- DeLuca, H.F. 1976. Recent advances in our understanding of the metabolism of vitamin D and its regulation. Clin. Endocrinol. 5 suppl: 97s-108s.
- Deville, J. and E. Lopez. 1970. Le corps ultimobranchial du Saumon, Salmo salar L. Etude histophysiologique a diverses etapes de son cycle vital en eau douce. Arch. Anat. Microsc. Morphol. Exp. 59:393-402.
- Dousa, T.P. 1974. Effects of hormones on cyclic AMP formation in kidneys of nonmammalian vertebrates. Amer. J. Physiol. 226:1193-1197.
- Fenwick, J.C. 1974. The corpuscles of Stannius and calcium regulation in the North American eel (Anguilla anguilla LeSueur). Gen. Comp. Endo. 23:127-135.
- Fenwick, J.C. 1975. Effect of partial ultimobranchialectomy on plasma calcium concentration and on some related parameters in goldfish (Carassius auratus L.) during acute transfer from freshwater to 30% seawater. Gen. Comp. Endo. 25:60-63.
- Fenwick, J.C. 1976. Effect of stanniectomy on calcium activated adenosinetriphosphatase activity in the gills of freshwater adapted North American eel, Anguilla rostrata LeSueur. Gen. Comp. Endo. 29:383-387.
- Fenwick, J.C. 1978. Role of the ultimobranchial body and the corpuscles of Stannius in the regulation of calcium in the eel. In: Comparative Endocrinology. Proc. VIII Int. Symp. Comp. Endocrinol. pp.255-258. P.J. Gaillard and H.H. Boer (eds.). Elsevier/North-Holland Biomed. Press. Amsterdam.
- Fenwick, J.C. 1979. Calcium-activated adenosinetriphosphatase activity in the gills of freshwater and seawater-adapted eels (Anguilla rostrata). Comp. Biochem. Physiol. 62B:67-70.

- Penwick, J.C. and M.E. Forster. 1972. Effects of stanniectomy and hypophysectomy on total plasma cortisol levels in the eel (Anquilla anquilla L.). Gen. Comp. Endocrinol. 19:184-191.
- Penwick, J.C. and Y.P. So. 1974. A perfusion study of the effect of stanniectomy on the net influx of calcium-45 across an isolated eel gill. J. Exptl. Zool. 188:125-131.
- Fleming, W.R., J. Brehe and R. Hanson. 1973. Some complicating factors in the study of the calcium metabolism of teleosts. Amer. Zool. 13:793-797.
- Fontaine, M. 1964. Corpuscles de Stannius et regulation ionique (Ca, Na, K) du milieu interieur de l'anguille, Anquilla anquilla. Compt. Rend. Acad. Sci. 259:875-878.
- Fontaine, M., N. Delerue, E. Martelly, J. Marchelidon and C. Milet. 1972. Role des corpuscles de Stannius dans les echanges de calcium d'un poisson teleosteen, l'anguille (Anquilla anquilla L.) avec le milieu ambiant. C.R. Acad. Sci. Paris Serie D, 275:1523-1528.
- Forster, M.E. 1976a. Effects of catecholamines on the heart and on branchial and peripheral resistance of the eel, Anquilla anquilla (L.). Comp. Biochem. Physiol. 55C:27-32.
- Forster, M.E. 1976b. Effects of adrenergic blocking drugs on the cardiovascular system of the eel, Anquilla anquilla (L.). Comp. Biochem. Physiol. 55C:33-36.
- Foster, G.V., F.H. Doyle, P. Bordier and H. Matrajt. 1966. Effect of thyrocalcitonin on bone. Lancet 2:1428.
- Friedman, P. and L.G. Raisz. 1965. Thyrocalcitonin: inhibition of bone resorption in tissue culture. Science 150:1465.
- Fromm, P.O. 1968. Some quantitative aspects of ion regulation in teleosts. Comp. Biochem. Physiol. 27:865-869.
- Garel, J.M., A.D. Care and J.P. Barlet. 1974. A radioimmunoassay for ovine calcitonin: an evaluation of calcitonin secretion during gestation, lactation and fetal life. J. Endocrinol. 62: 497-509.
- Gray, T.K., F. Bieberdorf and J.J. Fordtran. 1973. Thyrocalcitonin and the jejunal absorption of calcium, water, and electrolytes in normal subjects. J. Clin. Invest. 52/12:3084-3088.

- Gray, T.K., and P.L. Munson. 1969. Thyrocalcitonin: evidence for physiological function. *Science* 166:512-513.
- Haas, H., M.A. Dambacher, J. Guncaga and T. Lauffenbruger. 1971. Renal effects of calcitonin and parathyroid extract in man. *J. Clin. Invest.* 50:2689-2702.
- Hanke, W., K. Bergerhoff and D.K.O. Chan. 1967. Histological observations on pituitary ACTH-cells, adrenal cortex and the corpuscles of Stannius of the European eel Anquilla anquilla L.). *Gen. Comp. Endo.* 9:64-75.
- Harell, Arie, Binderman, Itzhak and G.A. Rodan. 1973. The effect of calcium concentration on calcium uptake by bone cells treated with thyrocalcitonin (TCT) hormone. *Endocrinology* 92:550-555.
- Hayslett, J.P., M. Epstein, D. Spector, J.D. Myers and V.H. Murdaugh. 1972. Lack of effect of calcitonin on renal function in the elasmobranch, Squalus acanthias. *Comp. Biochem. Physiol.* 43A:223-226.
- Hayslett, J.P., M. Epstein, D. Spector, J.D. Myers, H.V. Murdaugh and F.H. Epstein. 1971. Effect of calcitonin on sodium metabolism in Squalus acanthias and Anquilla rostrata. *Bull. Mt. Desert Is. Biol. Lab.* 11:33-35.
- Hearn, P.R., S. Tomlinson, H. Mellersh, C.J. Kenyon, C.J. Preston and P.G. Russell. 1978. High affinity calcium binding peptides from the gills, gut and kidneys of the freshwater eel (Anquilla anquilla). *Biochim. Biophys. Acta.* 536:429-432.
- Heersche, J.N.M., R. Marcus and G.D. Aurbach. 1974. Calcitonin and the formation of 3'5'-AMP in bone and kidney. *Endocrinology* 94:241-247.
- Hendler, R.W. 1964. Procedure for simultaneous assay of two beta-emitting isotopes with the liquid scintillation counting technique. *Anal. Chem.* 7:110-120.
- Heynen, G. and P. Franchimont. 1974. Human calcitonin radioimmunoassay in normal and pathological conditions. *Europ. J. Clin. Invest.* 4:213-222.
- Hirano, T., S. Mashiko and S. Utida. 1972. Central nervous system control of osmoregulation in the eel (Anquilla japonica). *Comp. Biochem. Physiol.* 43A:537-544.

Hirsch, P.F., C.W. Cooper, T.C. Peng, H.J. Burford, S.U. Toverud, T.K. Gray, D. Juan, D.A. Ontjes, J.F. Hennessy and P.L. Munson. 1975. Effects, functions, and endocrine interrelations of thyrocalcitonin. In: Calcium-regulating Hormones. Proc. V Parathyroid Conf. pp.89-99. R.V. Talmage, M. Owen and J.A. Parson (eds.). Excerpta Medica, Amsterdam.

Hsu, W.H. and C.W. Cooper. 1975. Hypercalcemic effect of catecholamines and its prevention by thyrocalcitonin. Calcif. Tiss. Res. 19:125-137.

Isaia, J and T. Hirano. 1975. Effect of environmental salinity change on osmotic permeability of the isolated gill of the eel, Anquilla anquilla L. J. Physiol. Paris. 70:737-747.

Kallio, D.M., P.R. Grant and C. Minkin. 1972. Ultrastructural effects of calcitonin on osteoclasts in tissue culture. J. Ultrastruct. Res. 39:205-216.

Kamiya, M. 1967. Changes in ion and water transport in isolated gills of the cultured eel during the course of salt adaptation. Annot. Zool. Japon. 40(3):123-129.

Kenny, A.D. 1972. Introductory remarks. In: Calcium, Parathyroid Hormone and the Calcitonins. Proc. IV Parathyroid Conf. (1971). pp.9-11. R.V. Talmage and P.L. Munson (eds.) Excerpta Medica, Amsterdam.

Kenny, A.D. and C.A. Heiskell. 1965. Effect of crude thyrocalcitonin on calcium and phosphate metabolism in rats. Proc. Soc. Exp. Biol. Med. (N.Y.) 120:269-271.

Kirsch, R. 1972. The kinetics of peripheral exchanges of water and electrolytes in the silver eel (Anquilla anquilla L.) in fresh water and in sea water. J. Exptl. Biol. 57:489-512.

Klein, D.C. and R.V. Talmage. 1968. Thyrocalcitonin suppression of hydroxyproline release from bone. Proc. Soc. Exp. Biol. Med. 127:95-99.

Krishnamurthy, V.G. 1976. Cytophysiology of corpuscles of Stannius. Int. Rev. Cyto. 46:177-249.

Lam, T.J. 1969. Evidence of loss of C -inulin via the head region of the threespine stickleback, Gasterosteus aculeatus, form trachurus. Comp. Biochem. Physiol. 28:459-463.

Liskova-Kiar, M. 1978. Effect of calcitonin and estradiol on parathyroid extract-induced bone resorption in vitro. Can. Fed. Biol. Soc. 21:162. (abstr. 8648).

- Lopez, E., M.-M. Chartier-Baraduc and J. Deville. 1971. Mise en evidence de l'action de la calcitonine porcine sur l'os de la truite Salmo gairdneri soumise a un traitement demineralisant. C.R. hebd. Seanc. Acad. Sci. Paris. 272:2600-2603.
- Lopez, E., J. Deville and E. Bagot. 1968. Etude histophysiologique du corps ultimobranchial d'un teleosteen Anguilla anguilla L. au cours d'hypercalcemies experimentales. C.R. Acad. Sci. Paris, Serie D. 267:1531-1534.
- Lopez, E., J. Peignoux-Deville, F. Lallier, E. Martelly and C. Effects of calcitonin and ultimobranchialectomy (UBX) on calcium and bone metabolism in the eel, Anguilla anguilla L. Calcif. Tiss. Res. 20:173-186.
- Louw, G.N., P.D. Stahl and A.D. Kenny. 1969. Thyrocalcitonin studies in elasmobranch fish. Zool. Afri. 4(1):95-98.
- Louw, G.N., W.W. Sutton and A.D. Kenny. 1967. Action of thyrocalcitonin in the teleost fish, Ictalurus melas. Nature 215:888-889, 1967.
- Lwowski, E.S. 1978. The corpuscles of Stannius and calcified tissues in the eel, Anguilla anguilla(L.). Comp. Biochem Physiol. 59A:183-188.
- Ma, S.W.Y. 1976. Studies on the mechanisms of branchial calcium transport in teleost fish, with special reference to a calcium-stimulated ATPase in the gill plasma membranes. Ph.D. Thesis. University of British Columbia, Vancouver, B.C.
- Ma, S.W.Y. and D.H. Copp. 1978. Purifications, properties and action of a glycopeptide from the corpuscles of Stannius which affect calcium metabolism in the teleost. In: Comparative Endocrinology. Proc. VIII Int. Symp. Comp. Endocrinol. pp. 283-286. P.J. Gaillard and H.H. Boer (eds.). Elsevier/North-Holland Biomed. Press. Amsterdam.
- Ma, S.W.Y., Y. Shami, H.H. Messer and D.H. Copp. 1974. Properties of calcium-ATPase from the gill of rainbow trout (Salmo Gairdneri). Biochim. Biophys. Acta. 345:243-251.
- MacIntyre, I., P.G.H. Byfield, L. Galante, E.W. Matthews, J.M. Mosely and N.J.Y. Woodhouse. 1972. Studies of the effects of human synthetic calcitonin in experimental animals and man. In: Endocrinology 1971. Proc. III Int. Symp. pp. 21-26. S.F. Taylor (ed.) Heinemann, London.

- MacIntyre, I., K.W. Colston, I.M.A. Evans, E. Lopez, S.J. MacAuley, J. Peignoux-Deville, E. Spanos and M. Szelke. 1976. Regulation of vitamin D: An evolutionary view. Clin. Endocrinol. 5 suppl:85s-95s.
- MacIntyre, I., I.M.A. Evans and R.G. Larkins. 1977. Vitamin D. Clin. Endo. 6:65-79.
- Maetz, J. 1971. Fish gills: mechanisms of salt transfer in fresh water and sea water. Phil. Trans. Roy. Soc. Lond. B. 262:209-249.
- Maetz, J. 1976. Transport of ions and water across the epithelium of fish gills. In: Lung Liquids. Ciba Found. Symp. 38 (new series). pp.133-159. Elsevier.Excerpta Medica.North-Holland, Amsterdam.
- Maetz, J. and M. Bornancin. 1975. Biochemical and biophysical aspects of salt excretion by chloride cells in teleosts. Fortschr. Zool. 23:322-362.
- Maetz, J., P. Payan and G. de Renzis. 1976. Controversial aspects of ionic uptake in freshwater animals. In: Perspectives in Experimental Biology, volume 1 Zoology, pp. 77-92. P.S. Davis (ed.). Pergamon Press, Oxford.
- Marx, S.J. and G.D. Aurbach. 1975. Renal receptors for calcitonin: coordinate occurrence with calcitonin-activated adenylate cyclase. Endocrinology 97:448-453.
- Marx, S.J., S.A. Pedak and G.D. Aurbach. 1972. Preparation and characterization of a hormone-responsive renal plasma membrane fraction. J. Biol. Chem. 247:6913-6918.
- Mashiko, K. and K. Jozuka. 1962. Studies on the calcium uptake by teleost fishes I. Calcium-45 uptake by the crucian carp. Sci. Rep. University Kanazawa 8:107-126.
- Mashiko, K. and K. Jozuka. 1964. Absorption and excretion of calcium by teleost fishes with special reference to routes followed. Annot. Zool. Japon. 37:41-50.
- Masoni, A. and P. Payan. 1974. Urea, inulin and para-amino-hippuric acid (PAH) excretion by the gills of the eel, Anguilla anguilla L. Comp. Biochem. Physiol. 47A:1241-1244.
- Massry, S.G., R.M. Friedler and J.W. Coburn. 1973. Excretion of phosphate and calcium. Physiology of their renal handling and relation to clinical practice. Arch. Intern. Med. 131:828-859.

- Matthews, J.L., J.H. Martin, E.J. Collins, J.W. Kennedy III and E.L. Powell, Jr. 1972. Immediate changes in the ultrastructure of bone cells following thyrocalcitonin administration. In: Calcium, Parathyroid Hormone and the Calcitonins. Proc. IV Parathyroid Conf. pp. 375-382. R.V. Talmage and P.L. Munson (eds.) Excerpta Medica, Amsterdam.
- Mayer, N. and J. Maetz. 1967. Axe hypophyso-interrenalien et osmoregulation chez l'anguille en eau de mer. Etude de l'animal intact avec notes sur les perturbations de l'équilibre mineral produites par l'effet de "Choc". C.R. Acad. Sci. Paris Serie D. 264:1632-1635.
- Mayer, N. and J. Nibelle. 1970. Kinetics of the mineral balance in the eel, Anguilla anguilla, in relation to external salinity changes and intravascular saline infusions. Comp. Biochem. Physiol. 35:553-566.
- McWhinnie, D. J. and T.G. Scopelliti. 1978. A comparative study of the influence of calcitonin on plasma mineral and volume dynamics in the amphibians, Rana pipiens and Xenopus laevis, and the rat. Comp. Biochem. Physiol. 61A:487-495.
- Milet, C., M.M. Chartier and E. Lopez. 1978. Effect of Stannius corpuscles (SC) on morphology, cytology calcium-- binding proteins, in relation to calcium transfer in the gills of the European eel, Anguilla anguilla L. In: Comparative Endocrinology. Proc. VIII Int. Symp. Comp. Endo. pp. 263-267. P.J. Gaillard and H.H. Boer. (eds.) Elsevier/North-Holland Biomed. Press. Amsterdam.
- Milhaud, G., J.C. Rankin, L. Bolis and A.A. Benson. 1977. Calcitonin: Its hormonal action on the gill. Proc. Natl. Acad. Sci. 74(10):4693-4696.
- Minkin, C., J.J. Reynolds and D.H. Copp. 1971. Inhibitory effect of salmon and other calcitonins on calcium release from mouse bone in vitro. Can. J. Physiol. Pharma. 49:263-267.
- Morii, H. and H.P. DeLuca. 1967. Relationships between vitamin D deficiency, thyrocalcitonin, and parathyroid hormone. Amer. J. Physiol. 213:358-362.
- Moss, M.L. 1963. The biology of acellular teleost bone. Ann. N.Y. Acad. Sci. 109:337-350.
- Moss, M.L. 1965. Studies of the acellular bone of teleost fish. Acta Anat. 60:262-276.

- Munson, P.L. and T.K. Gray. 1970. Function of thyrocalcitonin in normal physiology. Fed. Proc. 29/3:1206-1208.
- Nagata, N., M.Saski, N. Kimura and K. Nakane. 1975. Effects of porcine calcitonin on the metabolism of calcium and cyclic AMP in rat skeletal tissue in vivo.
- Newsome, F.E., R.K. O'Dor, C.O. Parkes and D.H. Copp. 1973. A study of the stability of calcitonin biological activity. Endocrinology 92:1102-1106.
- Noda, T. and K. Narita. 1976. Amino acid sequence of eel calcitonin. J. Biochem. (Jap. Biochem. Soc.) 79:353-359.
- Nordin, B.E.C., A. Hodgkinson and M. Peacock. 1967. The measurement and meaning of urinary calcium. Clin. Orthop. 52:293-322.
- Norris, W.P., W.Chavin and L.S. Lombard. 1963. Studies of calcification in a marine teleost. Ann. N.Y. Acad. Sci. 109:312-336.
- O'Dor, R.K., C.O. Parkes and D.H. Copp. 1969. Biological activities and molecular weights of ultimobranchial and thyroid calcitonins. Comp. Biochem. Physiol. 29:295-300.
- Ogawa, M. 1963. On the corpuscles of Stannius of goldfish treated with sea water. Sci. Rep. Sait. Univer. Series B. 4(3):181-191.
- Ogawa, M. 1967. Fine structure of the corpuscles of Stannius and the interrenal tissue in goldfish, Carassius auratus. Z. Zellforsch. Mikrosk. Anat. 81:174-189.
- Ogawa, M. 1968. Osmotic and ionic regulation in goldfish following removal of the corpuscles of Stannius or the pituitary gland. Can. J. Zool. 46:669-676.
- Ogawa, M. 1974. The effects of bovine prolactin, sea water and environmental calcium on water influx in isolated gills of the euryhaline teleosts, Anquilla japonica and Salmo gairdneri. Comp. Biochem. Physiol. 49A:545-553.
- Ogawa, M. 1975. The effects of prolactin, cortisol and calcium-free environment on water influx in isolated gills of Japanese eel, Anquilla japonica. Comp. Biochem. Physiol. 52A:539-543.

- Ogawa, M., R.K. Pang, P.K.T. Pang and H. Sokabe. 1978. Identification of the renin-like substance in the corpuscles of Stannius with hypocalcin in the Japanese eel and killifish. In: Comparative Endocrinology. Proc. VIII Int. Symp. Comp. Endo. pp. 289 (abstr). P.J. Gaillard and H.H. Boer (eds.). Elsevier/North-Holland Biomed. Press, Amsterdam.
- Oguri, M. 1973. Seasonal histological changes in the ultimobranchial gland of goldfish. Bull. Jap. Soc. Sci. Fish. 39:851-858.
- Okita, G., J.K. Kabara, F. Richardson and G.V. LeRoy. 1957. Assaying compounds containing H-3 and C-14. Nucleonics 15:111-114.
- Olson, B.E., H.F. DeLuca, and J.T. Potts, Jr. 1972. The effect of calcitonin and parathyroid hormone on calcium transport of isolated intestine. In: Calcium, Parathyroid Hormone and the Calcitonins. Proc. IV. Parathyroid Conf. pp. 240-246. R.v. Talmage and P.L. Munson (eds.). Excerpta Medica, Amsterdam.
- Orimo, H., M. Ohata, T. Fujita, M. Yoshikawa, T. Higashi, J. Abe, S. Watanabe and K. Otani. 1972a. Ultimobranchial calcitonin of Anguilla japonica. In: Endocrinology 1971. Proc. III Int. Symp. pp.48-54. S.f.Taylor (ed.) Heinemann, London.
- Orimo, H., T. Fujita, M. Yoshikawa, S. Watanabe, M. Otani and J. Abe. 1972b. Ultimobranchial calcitonin of the eel, Anguilla japonica. Endocrinol. Japon. 19(3):299-302.
- Otani, M., H. Yamauchi, T. Meguro, S. Kitazawa, S. Watanabe and H. Orimo. 1976. Isolation and characterization of calcitonin from pericardium and esophagus of eel. J. Biochem. (Jap. Biochem. Soc.) 79:345-352.
- Pang, P.K.T. 1969. The hormonal control of serum calcium, phosphate and magnesium levels in the killifish, Fundulus heteroclitus: with special reference to calcitonin. Amer. Zool. 9:1081. abstr.
- Pang, P.K.T. 1971a. The relationship between corpuscles of Stannius and serum electrolyte regulation in killifish, Fundulus heteroclitus. J. Exp. Zool. 178:1-8.
- Pang, P.K.T. 1971b. Calcitonin and ultimobranchial glands in fishes. J. Exp. Zool. 178:89-100.

- Pang, P.K.T., N.B. Clark and K.S. Thomson. 1974c. Hypocalcemic activities in the ultimobranchial bodies of lungfishes, Neoseratodus forsteri and Lepidosiren paradoxa and teleosts, Fundulus heteroclitus and Gadus morhua. Gen. Comp. Endo. 17:582-585.
- Pang, P.K.T. 1973. Endocrine control of calcium-metabolism in teleosts. Amer. Zool. 13:775-792.
- Pang, P.K.T. and R.K. Pang. 1974. Environmental calcium and hypocalcin activity in the Stannius corpuscles of the channel catfish, Ictalurus punctatus (Rafinesque). Gen. Comp. Endo. 23:239-241.
- Pang, P.K.T., R.K. Pang and W.H. Sawyer. 1973. Effects of environmental calcium and replacement therapy on the killifish, Fundulus heteroclitus. after the surgical removal of the corpuscles of Stannius. Endocrinology 93:705-710.
- Pang, P.K.T., R.K. Pang and W.H. Sawyer. 1974. Environmental calcium and the sensitivity of killifish (Fundulus heteroclitus) in bioassays for the hypocalcemic response to Stannius corpuscles from killifish and cod (Gadus morhua). Endocrinology 94:548-555.
- Pang, P.K.T. and G.E. Pickford. 9 1967. Failure of hog thyrocalcitonin to elicit hypocalcemia in the teleost fish, Fundulus heteroclitus. Comp. Biochem. Physiol. 21:573-578.
- Pang, P.K.T. and W. Sawyer. 1975. Parathyroid hormone preparations, salmon calcitonin, and urine flow in the South American lungfish, Lepidosiren paradoxa. J. Exp. Zool. 193:407-412.
- Pang, P.K.T., M.P. Schreiber, F. Balbontin and R.K. Pang. 1978. Prolactin and pituitary control of calcium regulation in the killifish, Fundulus heteroclitus. Gen. Comp. Endo. 36:306-316.
- Peignoux-Deville, J. 1977. Role du corps ultimobranchial (C.U.B.) dans la regulation du metabolisme calcique chez les poissons teleosteens. Ph.D. Thesis, L'Universite Pierre et Marie Curie, Paris, France.
- Peignoux-Deville, J., E. Lopez, F. Lallier, E. Martelly-Bagot and C. Milet. 1975. Responses of the ultimobranchial body in eels (Anguilla anguilla L.) maintained in sea water and experimentally matured, to injections of synthetic calcitonin. Cell. Tiss. Res. 164:73-83.

- Peignoux-Deville, J., C. Milet and E. Martelly. 1978. Effets de l'ablation du corps ultimobranchial et de la perfusion de calcitonine sur les flux de calcium au niveau des branchies de l'anguille (Anquilla anguilla L.). Ann. Biol. Anim. Bioch. Biophys. 18:119-126.
- Potts, Jr. J.T. 1976. Parathyroid hormone and calcitonin. In: Peptide Hormones. pp.191-142. J.A. Parsons (ed.) Univ. Park Press, London.
- Prosser, C.L. 1973. Comparative Animal Physiology. Third edition. W.B. Saunders Company, Philadelphia. 966 pp.
- Puschett, J.B., W.S. Beck, Jr., A. Jelonek and P.C. Fernandez. 1974. Study of the renal tubular interactions of thyrocalcitonin, cyclic adenosine 3',5'-monophosphate, 25-hydroxycholecalciferol, and calcium ion. J. Clin. Invest. 53:756-767.
- Raisz, L.G. 1970. Physiological and pharmacologic regulation of bone resorption. New Eng. J. Med. 282:909-916.
- Raisz, L.G. and P.J. Bingham. 1972. Effect of hormones on bone development. Ann. Rev. Pharmacol. 12:337-352.
- Rankin, J.C., D.K.O. Chan and I. Chester Jones. 1967. Kidney function in the European eel, Anquilla anguilla. Gen. Comp. Endo. 9:484-499.
- Rankin, J.C. and J. Maetz. 1971. A perfused teleostean gill preparation: vascular actions of neurohypophysial hormones and catecholamines. J. Endocrinol. 51:621-635.
- Rasmussen, H. 1971. Ionic and hormonal control of calcium homeostasis. Am. J. Med. 50:567-588.
- Rasmussen, H. and D.B. Goodman. 1977. Relationships between calcium and cyclic nucleotides in cell activation. Physiological Reviews 57:421-509.
- Rasmussen, H., M. Wong, D. Bikle and D.P.B. Goodman. 1972. Hormonal control of the renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol. J. Clin. Invest. 51:2502-2504.
- Reynolds, J.J. and J.T. Dingle. 1970. A sensitive in vitro method for studying the induction and inhibition of bone resorption. Calcif. Tiss. Res. 4:339-349.

- Richards, B.D. and P.O. Fromm. 1969. Patterns of blood flow through filament and lamellae of isolated perfused rainbow trout (Salmo gairdneri) gills. Comp. Biochem. Physiol. 29:1063-1070.
- Richards, B.D. and P.O. Fromm. 1970. Sodium uptake by isolated-perfused gills rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. 33:303-310.
- Robertson, D.R. 1969. The ultimobranchial body of Rana pipiens VIII. Effects of extirpation upon calcium distribution and bone cell types. Gen. Comp. Endocrinol. 12:479-490.
- Robertson, D.R. 1972. Calcitonin in amphibians and the relationship of the paravertebral lime sacs with carbonic anhydrase. In: Ca: Calcium, Parathyroid Hormone, and the Calcitonins. Proc. IV Parathyroid Conf. pp.21-28. R.V. Talmage and P.L. Munson (eds.) Excerpta Medica, Amsterdam.
- Robertson, D.R. 1974. Effects of the ultimobranchial and parathyroid glands on sodium and water excretion in the frog. Endocrinology 94:940-946.
- Robertson, D.R. 1975. In vitro transport of calcium by the frog intestine and the effect of vitamin D-3. Comp. Biochem. Physiol. 51a:705-710.
- Roos, B.A., L.L. Bundy, R. Bailey and L.J. Deftos. 1974. Calcitonin secretion in vitro part 1: preparation of monolayer C cell cultures. Endocrinology 95(4):1142-1149.
- Roos, B.A., L.L. Bundy, E.A. Miller and J.J. Deftos. 1975. Calcitonin secretion by monolayer cultures of human C-cells derived from medullary thyroid carcinoma. Endocrinology 97:39-45.
- Roos, B.A. and L.J. Deftos. 1976. Regulation of calcitonin secretion in vitro. Clin. Endocrinol. 5 (suppl):217s-222s.
- Russell, R.G.G. 1976. Regulation of calcium metabolism. Ann. Clin. Biochem. 13:518-539.
- Salako, L.A., A.J. Smith and R.N. Smith. 1971. The effects of porcine calcitonin on renal function in the rabbit. J. endocrinol. 50:485-491.
- Samson, P.J., R.E. Stacey and F. Bronner. 1969. Further studies on the role of thyrocalcitonin in calcium homeostasis and metabolism. Biochem. Med. 3:252-270.

- Shuttleworth, T.J. 1972. A new isolated perfused gill preparation for the study of the mechanisms of ionic regulation in teleosts. *Comp. Biochem. Physiol.* 43(1A):59-65.
- Shuttleworth, T.J. and R.F.H. Freeman. 1974. Net fluxes of water in the isolated gills of Anquilla dieffenbachii. *J. Exp. Biol.* 60:769-781.
- Simmons, D.J. 1971. Calcium and skeletal tissue physiology in teleost fishes. *Clin. Orthop.* 76:244-280.
- So, Y.P. and J.C. Fenwick. 1977. Relationship between net calcium-45 influx across a perfused isolated eel gill and the development of post-stanniectomy hypercalcemia. *J. Exp. Zool.* 200(2):259-264.
- Sokabe, H. 1974. Phylogeny of the renal effects of angiotensin. *Kidney Intern.* 6:263-271.
- Steel, R.G.D. and J.H. Torrie. 1960. Principles and Procedures of Statistics, with special reference to the Biological Sciences. MacGraw-Hill, New York. 481 pp.
- Sturtridge, W.C. and M.A. Kumar. 1967. Calcium homeostasis in chronic thyrocalcitonin deficiency. *Endocrinology* 81:1297-1300.
- Suryawanshi, S.A. and S.M. Mahajan. 1976. Role of ultimobranchial body and corpuscle of Stannius in regulation of the plasma calcium and phosphorus levels in the teleost Heteropneustes fossilis. *Acta biol. Acad. Sci. Hung.* 27(4):269-274.
- Swaminathan, R., R.F.L. Bates and A.D. Care. 1972. Fresh evidence for a physiological role of calcitonin in calcium homeostasis. *J. Endocrinol.* 54(3):525-526.
- Talmage, R.V., J.J.B. Anderson and C.W. Cooper. 1972. The influence of calcitonins on the disappearance of radiocalcium and radiophosphorus from plasma. *Endocrinology (Philadelphia)* 90(5):1185-1191.
- Talmage, R.V., L.A. Whitehurst and J.J.B. Anderson. 1973. Effect of calcitonin and calcium infusion on plasma phosphate. *Endocrinology* 92:792-798.
- Tanzer, F.S. and J.M. Navia. 1973. Calcitonin inhibition of intestinal phosphate absorption. *Nat. New Biology* 242:221-222.
- Tauber, S.D. 1967. The ultimobranchial origin of thyrocalcitonin. *Proc. Nat. Acad. Sci. USA* 58:1684-1687.

- Tesch, F.-W. 1977. The Eel, Biology and Management of Anguillid Eels. P.H. Greenwood (ed. English edition). Chapman and Hall Ltd. London. 4434 pp.
- Timm, N.H. 1975. Multivariate Analysis with applications in education and psychology. Brooks/Cole Pub. Comp., Calif. 689 pp.
- Tisserand-Jochem E-M, A. Eyquem, J. Peignoux, and C. Calmettes. 1976. Detection in the serum of patients treated by salmon calcitonin of anti-calcitonin antibodies by indirect immuno-fluorescence on sections of eel ultimobranchial membrane. *Ann. D'Immunologie (Paris)* 127 (2): 121.
- Urist, M.R. 1967. Avian parathyroid physiology: including a special comment on calcitonin. *Amer. Zool.* 7:883-895.
- Urist, M.R., S. Uyeno, E. King, M. Okada and S. Applegate. 1972. Calcium and phosphorus in the skeleton and blood of the lungfish, Lepidosiren paradoxa, with comment on humoral factors in calcium homeostasis in the osteichthyes. *Comp. Biochem. Physiol.* 42A:393-408.
- Van Noorden, S. and A.G.E. Pearse. 1972. The ultimobranchial gland of the dogfish Scyliorhinus canicula L. I. immunofluorescence cross-reactivities with anti-sera to different calcitonins. *Histochemie* 39:97-101.
- Wang, C.G. and D.L. Willis. 1965. Radiotracer Methodology in Biological Science. Prentice-Hall Inc., N.J. 382 pp.
- Watts, E.G. 1973. Calcium and calcitonin studies in Pacific salmon, Genus Oncorhynchus, and rainbow trout, Salmo gairdneri. Ph.D. Thesis. University of British Columbia, Vancouver, B.C.
- Watts, E.G., D.H. Copp, K. Dawson, F. Hui and E. McGowan. 1970. Calcitonin in the rainbow trout, Salmo gairdneri. *Proc. Can. Fed. Biol. Soc.* 13:78, abstr. #295.
- Watts, E.G., D.H. Copp and L.J. Deftos. 1975. Changes in plasma calcitonin and calcium during the migration of salmon. *Endocrinology* 96:214-218.
- Wendelaar Bonga, S.E. 1978. The role of environmental calcium and magnesium ions in the control of prolactin secretion in the teleost Gasterosteus aculeatus. pp. 259-262. P.J. Gaillard and R.H. Boer (eds.), Elsevier/North-holland Biomed. Press, Amsterdam.

- Wendelaar Bonga, S.E. and J.A.A. Greven. 1978. The relationship between prolactin cell activity, environmental calcium and plasma calcium in the teleost Gasterosteus aculeatus. Observations on stanniectomized fish. Gen. Comp. Endo. 36:90-101.
- West, T.E.T., J.L.H. O'Riordan, D.H. Copp, R.F.L. Bates and A.D. Care. 1973. The effect of hypocalcemia on the secretion of calcitonin. J. Endocrinol. 56:463-470.
- Wood, C.M. 1974 A critical examination of the physical and adrenergic factors affecting blood flow through the gills of the rainbow trout. J. Exptl. Biol. 60:241-265.
- Wood, C.M. 1975. A pharmacological analysis of the adrenergic and cholinergic mechanisms regulating branchial vascular resistance in the rainbow trout (Salmo gairdneri). Can. J. Zool. 53:1569-1577.
- Wood, C.M. and G. Shelton. 1975. Physical and adrenergic factors affecting systemic vascular resistance in the rainbow trout: a comparison with branchial vascular resistance. J. Exptl. Biol. 63:505-523.
- Williams, C.C., E.W. Matthews, J.M. Moseley and I. MacIntyre. 1972. The effects of synthetic human and salmon calcitonin on electrolyte excretion in the rat. Clin. Sci. 42:129-137.
- Yamauchi, H., M. Matsuo, A. Yoshida and H. Orimo. 1978. Effect of eel calcitonin on serum electrolytes in the eel, Anguilla japonica. Gen. Comp. Endo. 34:343-346.