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The Role of the Gills in the Regulation of Calcium Homeostasis in the American Eel, *Anguilla rostrata* (LeSueur)

by

Ying Peng So

A Thesis
presented to the School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in the
Department of Biology

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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Publications resulting from the work embodied in this thesis:

Fenwick, 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. Exp. Zool. 199: 125-131.

So, Y.P. and J.C. Fenwick. 1977. Relationship between net ^{45}Ca influx across a perfused isolated eel gill and the development of post-stanniectomy hypercalcemia. J. Exp. Zool. 200:259-264.

So, Y,P. and J.C. Fenwick. 1979. *In vivo* and *in vitro* effect of Stannius corpuscle extract on the branchial uptake of ^{45}Ca in stanniectomized North American eels (*Anguilla rostrata*). Gen. Comp. Endocrinol. 37:143-149.

Fenwick, J.C. and Y.P. So. 1980. Effect of an angiotensin on the net influx of calcium across an isolated perfused eel gill. Can. J. Zool. (in press).

ABSTRACT

The role of the gills in calcium homeostasis was examined in the American eel, Anguilla rostrata (LeSueur).

The present data suggest a cause-effect relationship exists between the surgical removal of the corpuscles of Stannius and an increase in branchial calcium influx. Stanniectomy was consistently followed by an increase in plasma total calcium and an increase in the branchial calcium influx as measured by the isolated perfused gill preparation. Both parameters could be corrected by in vivo treatment with Stannius corpuscle extract. Additionally, the Stannius corpuscle extract inhibited ^{45}calcium influx in vitro. Calcitonin produced an anti-hypercalcemic role, possibly by increasing branchial calcium efflux, but did not lower blood calcium levels. The post-stanniectomy hypercalcemia did not appear to affect the rate of branchial calcium influx whereas a high concentration of calcium ions or magnesium ions in the external environment prevented the branchial calcium influx rate from rising to its predicted levels.

The present study on the eel indicates that the gills are involved in calcium homeostasis and that they are a target organ for the Stannius corpuscle hypocalcemic

hormone(s).

RÉSUMÉ

Le rôle des branchies dans l'homéostasie du calcium fut étudié chez l'anguille américaine, Anguilla rostrata (LeSueur).

Les données suggerent qu'il existe une relation de cause à effet entre l'ablation chirurgicale des corpuscules de Stannius et un accroissement du flux d'éntree au niveau des branchies. L'ablation des corpuscules de Stannius était toujours suivie par un accroissement du calcium total dans le plasma et d'un accroissement du flux d'entrée du calcium au niveau des branchies, mesuré à l'aide d'une préparation de branchies isolées et perfusées. Ces deux parametres pouvaient etre corrigés par un traitement in vivo d'un extrait de corpuscules de Stannius. En outre, l'extrait de corpuscules de Stannius inhibe le flux d'entrée de calcium in vitro. La calcitonine joua un role anti-hypercalcémique, en accroissant possiblement le flux de sortie du calcium des branchies, mais ne diminua pas les niveau de calcium sanguin. L'hypercalcémie post-opératoire ne sembla pas affecter le taux du flux d'entrée du calcium au niveau des branchies tandis qu'une concentration élevée d'ions de calcium et de magnesium dans l'environnement externe empecha l'accroissement jusqu'au

niveau prévu du taux du flux d'entrée du calcium.

Cette étude sur l'anguille indique que les branchies sont liées à l'homéostasie du calcium et qu'elles sont un organe cible pour les hormones hypocalcémiques des corpuscules de Stannius.

ACKNOWLEDGEMENTS

I gratefully acknowledge my supervisor, Dr. James C. Fenwick, for his encouragement and assistance both during the research period and the preparation of this thesis. I would like to thank Mrs. Julie Fenwick for her effort in correcting grammar and spelling in this thesis.

I would like to thank Dr. Thomas W. Moon for his helpful comments.

The opportunity for invaluable conversation with both Dr. Mizuho Ogawa of Saitama University, Japan and Dr. S.E. Wendelaar Bonga of Nijmegen University, the Netherlands during their visit to Ottawa is highly appreciated.

I also express my appreciation to Miss E. Sullivan and Mrs. A. Hurd at the Department of Applications for their advice in statistical analysis of the data.

Special thanks are due to Mr. Nino Cavacciuti in the construction of the experimental apparati, Mr. J. Helie for final preparation of figures for this thesis, and Mr. Alain Baril and Mr. Eric Leung for their assistance and support.

Finally, I would like to express my gratitude to my wife, Karen, for her understanding and forbearance during the critical period.

The 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. Fenwick.

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INTRODUCTION

The divalent cation calcium is a key regulator of many biological processes ranging from the maintenance of structural integrity of membranes to, inter alia, coenzymic functions, excitation-secretion coupling at nerve terminals, coupling of excitation to contraction, secretory processes and the coagulation of blood. It can play such a role because cells have devices that maintain a low concentration of calcium ions in the cytosol (Racker, 1980). This maintenance of low and relatively constant cytosolic calcium ions is considerably facilitated in the vertebrates by effective homeostatic mechanisms which hold the level of extracellular calcium ions within acceptable levels (Bygrave, 1978a). Additionally, calcium is a major structural component of tissues such as bone, teeth and scales wherein it acts as a tissue 'hardener' and substantially contributes to the biomechanical role of these tissues. In other words, there are two main lines of evolutionary pressures operating upon calcium homeostasis in animals. Firstly, there is the role of calcium ions in the various dynamic physiological and biochemical processes. Secondly, there is the role of calcium in the development and maintenance

of mineralized skeletons.

In tetrapods, these two functions of calcium are closely linked by the fact that the bone is also used extensively as a repository for calcium during high intake and as a reservoir when calcium intake is low. The necessity for the use of bone in this fashion seems to result from the fact that most tetrapods are essentially land-based and have but intermittent access to environmental calcium by way of their food and drinking water. In these animals the exchange of calcium between the bone and the body fluids, and vice versa, is facilitated by the presence of bone cells (osteocytes and osteoclasts), which under the stimulus of various hormonal factors can direct the movement of calcium ions from the body fluids to bone or from the bone to the body fluids.

The situation in fish, however, appears to be quite different. Unlike their tetrapod descendants, the fish do not have only intermittent access to environmental calcium. Instead, they have continuous access to the virtually limitless supply of calcium dissolved in their aqueous habitat so it is possible for the fish to divorce the calcium involved in the regulation of body fluid calcium from that involved in the mineralization of their osseous tissue. This difference is evidenced by the following observations. The majority of bony fish have no enclosed osteocytes and as such are said to have acellular bone (Moss, 1963, 1966). Fish, especially those which

possess cellular bone, show an extremely low rate of bone calcium turnover (Moss, 1963; Fleming, 1974). Fish even in freshwater live in a medium with ample supplies of calcium; they have large surface areas used for exchange, especially the gills, through which exchange of calcium might take place, and fish have been shown to have a marked capacity to withdraw calcium from the environment (Phillips et al., 1960; Moss, 1963; Fleming et al., 1964; Podoliak and Holden, 1965a, 1965b, 1966). Finally, neither the presence of cellular nor acellular bone, nor differences in external environmental concentration of calcium seem to be correlated with the attainment and maintenance of plasma calcium homeostasis in fish (Moss, 1965). Therefore, whereas it appears that the calcium involved in body fluid calcium homeostasis in tetrapods exist in a bone - body fluid continuum, in fish the calcium exists in a body fluid - environment continuum. In other words, the calcium reservoir for calcium homeostasis in fish is different from that employed by the tetrapods. Regardless of this difference, however, and for the reasons cited earlier, fish must maintain calcium homeostasis and in order to do this must have some means of controlling the exchange of calcium between the body fluids and their aqueous surroundings. Unfortunately, very little is known concerning this regulation in fish and it will be the purpose of this thesis to shed some light on the endocrine factors involved in calcium homeostasis in the American eel, Anguilla rostrata (LeSueur).

Fish can and do regulate plasma calcium levels (Table I) even when faced with the considerable difference in calcium concentration which exists between freshwater and seawater. For example, the euryhaline American eel can maintain a relatively constant plasma calcium level (around 2.3 mM) regardless of the variation of external calcium concentration they face, ranging from 10 mM in seawater to 0.45 mM in freshwater.

Being co-existent with the external environment, fish that live in high calcium environments face potential excess calcium uptake and therefore must possess some means of preventing hypercalcemia. Fish in low calcium environments must have some means of gaining access to the calcium to prevent hypocalcemia. In both instances fish must have some methods of regulating their exchange. The most reasonable method for this regulation would be by way of the endocrine system, since this system is known to exert important regulatory influence on the exchange of monovalent ions. Unfortunately, the endocrine involvement in calcium homeostasis in fish is not so well understood as in mammals.

In mammals the primary hormonal control of calcium homeostasis involves a close integration between the activities of three hormones, namely, parathormone (PTH) from the parathyroid glands, calcitonin (CT) from the ultimobranchial or 'C' cells and the active metabolite(s) of cholecalciferol (vitamin D_3) especially the 1,25-dihydroxy

Table I

*Calcium Levels in Environment and Plasma of Different Animals

Environment			mM calcium
Standard seawater			10.48
Brackish water			2.00 - 5.00
Freshwater (hard)			3.98
Freshwater (soft)			0.07
Lake Huron			0.90
Ottawa River			0.45
Invertebrate			
Marine coelenterate, Aurelia			9.70
Lobster, Homarus			15.82
Crab, Maia			13.61
Vertebrate			
Fish			
Cyclostome	(seawater)	Myxine	6.25
	(freshwater	Lampetra	1.96
Chimaerid			4.30
Elasmobranch		Raja	6.00
		Marine shark, C. Leucas	4.50
		Freshwater shark, C. Leucas nicaraguensis	3.00
Teleost	Eel	A. anguilla	2.86
		A. japonica	3.50
		A. rostrata	2.22 - 2.48
	Rainbow trout, S. gairdneri		2.48
	Killifish, H. fundulus		2.28 - 2.68
	Stickleback, G. aculeatus		2.15
Amphibian			
Green frog, R. pipiens			1.70
Reptile			
Snake, R. tigrinus tigrinus			3.30
Bird			
Chicken, G. domesticus			2.53
Mammal			
Rat			3.10
Human, H. sapiens			2.35

Adapted from Prosser and Brown, 1961; Fontaine, 1964; Chan, 1972; Wilson, 1972; Pang, 1973; Fenwick, 1974; Copp and Ma, 1978; Wendelaar Bonga, 1978; Dacke, 1979.

derivative produced in the kidney. Fish, however, lack a parathyroid gland so that presumably no PTH as such exists in them (Fleischman, 1947; Hoar, 1951; Pickford, 1953). But prolactin may perform a similar hypercalcemic role (Pang, 1973; Pang et al., 1978; Wendelaar Bonga, 1978; Wendelaar Bonga and Greven, 1978). Further, in fish, the role of vitamin D_3 metabolites in calcium metabolism is very poorly understood. Calcitonin, a potential hypocalcemic agent in mammals under certain circumstances, has produced very inconsistent effects in fish (Pang and Pickford, 1967; Louw et al., 1967, 1969; Chan et al., 1968; Bradshaw and Shutton, 1970; Copp et al., 1970, 1972a, 1972b; Lopez et al., 1971; Pang, 1971a; Hayslett et al., 1971, 1972; Dacke, 1972; Watt, 1973; Hirano et al., 1981). This leaves the corpuscle of Stannius as the only well established fish endocrine gland which is involved in calcium homeostasis.

The corpuscles of Stannius are glands unique to the ray-finned fish (Ogawa, 1969). They are oval, ductless glands found on or in the mesonephric kidney of holostean and teleostean fish (Ogawa, 1969; Krishnamurthy, 1976). Their number varies from one pair in many teleosts up to 25 pairs in the holostean, Amia calvia. Bauchot (1953), De Smet (1962) and Krishnamurthy and Bern (1969) have suggested that the large number of corpuscles in holosteans and their apparent decline in teleosts reflect an evolutionary trend for the corpuscles of Stannius in fish; that is, they

have less functional importance in the higher teleosts.

Embryonically the corpuscles of Stannius may develop as outgrowths of the pronephric uriniferous tubules and this differentiates them from the adrenocortical tissue (Huot, Giacomini, cited in Ogawa, 1969; De Smet, 1962). In some fish (Colisa lilia), they may originate from the mesonephric tubules (Krishnamurthy, 1967). Anatomically the corpuscles have a rich vascular network supplied with blood from the renal portal system (Chester Jones et al., 1969; Wendelaar Bonga et al., 1977). Thus substances released from the gland will pass through the kidney before entering the systemic circulation. In addition, as recently reported in the stickleback, Gasterosteus aculeatus, the corpuscles are penetrated by sympathetic nerves coming from a small subvertebral ganglion (Unsicker, 1977; Wendelaar Bonga et al., 1977).

Several functional roles have been attributed to the corpuscles of Stannius (Fontaine, 1964; Leloup and Leloup-Hatey, 1964; Leloup-Hetay, 1964; Chester Jones et al., 1966; Fontaine, 1967; Ogawa, 1969, 1977; Kenyon et al., 1980). The most consistently observed effect following the surgical removal of the Stannius corpuscles (stanniectomy) has been the appearance of a marked but transient hypercalcemia. Characteristically the plasma calcium level begins to increase within a few days after the glands are removed, peaks after two or three weeks and returns towards normal values after six weeks (Fontaine, 1967; Chan, 1972; Fenwick

and Forster, 1972). This post-stanniectomy hypercalcemia can be prevented by administration of Stannius corpuscle extract (Rankin et al., 1967; Chan et al., 1969; Pang, 1971b; Pang et al., 1973) or by auto- or homo-transplantation of Stannius corpuscles (Chester Jones et al., 1967; Fenwick and Forster, 1972). Accordingly, it is now generally accepted that the corpuscles of Stannius produce and release an active hormone which plays a hypocalcemic role in teleosts.

Attempts have been made to isolate this putative hormone and to identify its chemical nature. From the various reports in the literature it is clear that two main lines of thought have evolved. One suggests a homology between the cells of Stannius corpuscles and those of mammalian adrenal cortex, and implies that the secretory product is steroidal in nature (Krishnamurthy, 1968, 1976; Subhadar and Rao, 1974). The other suggests that the secretory product is proteinaceous. In the last two decades, however, various embryological, biochemical and histochemical studies have provided strong reasons for favouring the latter hypothesis (Chieffi and Botte, 1963; Hanke and Chester Jones, 1966; Idler and Freeman, 1966; Oguri, 1966; Fujita and Honma, 1967; Ogawa, 1968; Bana, 1968; Tomasulo et al., 1970; Cohen et al., 1972, 1975; Carpenter and Heyl, 1974). Recently, Pang et al. (1974), based on the bioassay of crude Stannius corpuscle extract on killifish, Fundulus heteroclitus have named a heat labile proteinaceous mater-

ial obtainable from the corpuscles of Stannius 'Hypercalcin' in reference to its hypocalcemic role in calcium metabolism in fish. This material has now been equated with a renin-like or angiotensin-like substance (Nishimura, 1978; Ogawa et al., 1978). Very recently, a second active principle has been isolated from the Stannius corpuscles of salmon. This material has been identified as a glycoprotein with a molecular weight of about 3,000 daltons and an isoelectric point of approximately 5.0 (Ma and Copp, 1978). Preliminary amino acid analysis indicates that the glycopeptide contains aspartic acid, threonine, serine, glutamic acid, glycine, alanine and valine. No known basic or aromatic amino acid was found. The authors (Ma and Copp) have suggested the name 'Teleocalcin' for this active principle in order to differentiate it from Pang's 'Hypocalcin'.

In spite of these advances made towards elucidating the chemical nature of the Stannius corpuscle hormone(s), there remains a paucity of information concerning its target organ and mechanism of action. For example, neither how post-stanniectomy hypercalcemia occurs nor the source of the calcium that is involved in its development is known. Several hypothesis have, however, been postulated.

The kidney is an important calcium regulatory organ in mammals and it has been suggested that changes in the renal handling of calcium following stanniectomy result in the observed hypercalcemia. Indeed post-stanniectomy

hypercalcemia in European eel Anguilla anguilla and Japanese eel Anguilla japonica has been reported to result from a decrease in renal calcium excretion (Chester Jones et al., 1967, 1969; Rankin et al., 1967; Chan et al., 1969; Chan, 1972). This is supported by the observation that extracts of corpuscles of Stannius promoted calciuresis (Rankin et al., 1967; Chan et al., 1969). Conversely, however, Butler (1969) and Fenwick (1974a, 1978) showed that in the American eel, Anguilla rostrata, urinary calcium excretion did not decrease following stanniectomy. Rather, two weeks after the operation urinary calcium excretion was markedly elevated due to a combination of increased tubular load and increased relative calcium clearance (C_{Ca}/C_{in}). Further, Pang (1971b) has reported the presence of calculi in the kidney ducts of hypercalcemic stanniectomized killifish and this is more consistant with increased renal calcium excretion than with increased tubular calcium absorption.

Butler's observation (1969) led him to suggest that the hypercalcemia resulted from a reduction in the net rate of calcium deposition in bone, but this suggestion has not been corroborated by subsequent studies. For example it was shown that stanniectomy was followed by a highly significant increase in the deposition of ^{45}calcium in the bone of yellow eel, Anguilla anguilla (Fontaine et al., 1970 cited in Krishnamurthy, 1976). Further, Lopez (1970) reported that the rate of osteoclastic resorption was decreased at the peak of hypercalcemia and this coincided

with a decrease in the number of osteoclasts and an increase in the degree of bone mineralization. However, whether the decrease of osteoclastic resorption occurred before or after the peaking of hypercalcemia remains obscure. Recent indirect evidence also indicates that post-stanniectomy hypercalcemia is not caused by an increased rate of bone resorption (Lwowski, 1978). Finally, in vitro studies on the incubation of dried defatted eel bone in eel plasma indicated that under these conditions eel bone alone could not support the characteristic level of plasma calcium seen in hypercalcemia (Fenwick, 1974b). This latter study was, however, done with dead bone so that the results must be viewed with some skepticism.

The scales are another possible source of calcium from within the fish. This tissue, in some if not many teleosts, is known to be an important repository for calcium (White et al., 1964; Podoliak and Holden, 1965a) and this calcium is available for resorption (Simmons, 1971). But in the eel the scales are acellular and rudimentary when compared with other species of fish (Tesch, 1977) and this must reduce their calcium storage potential.

Muscle also may be an important source of exchangeable calcium and in fish is able to accumulate five times as much calcium as tetrapod muscles (Chan, 1972). However, in the Japanese eel, the cellular calcium content in muscle rises markedly (120%) after stanniectomy (Chan,1972) and this may preclude the possibility of muscle being the

source of the calcium responsible for the hypercalcemia. All these conclusions point to the suggestion that the calcium responsible for post-stanniectomy hypercalcemia is not mobilized from internal stores. Instead, and in view of the apparent continuum which exists between the calcium in fish plasma and the calcium in the environment, the most likely source of the extra plasma calcium is external calcium. This is supported by the observation that fish have the ability to absorb calcium from their environment (Mashiko and Jozuka, 1961, 1962, 1964; Moss, 1963; Rosenthal, 1963; Fleming et al., 1964, 1967), that the appearance of post-stanniectomy hypercalcemia is dependent upon the availability of exogenous calcium (Fontaine, 1967; Fenwick and Forster, 1972; Pang et al., 1973; Fenwick, 1974) and by the net increase of ^{45}calcium influx into stanniectomized eels (Fontaine et al., 1972).

In fish, the gut, skin and gills are the sites of most intimate contact between internal and external environment and represent the most likely sites of ionic change. Food can be a source of calcium but dietary calcium in the intestine cannot alone induce hypercalcemia in fed killifish (Pang et al., 1973). Further, post-stanniectomy hypercalcemia has been reported in unfed freshwater eels (Fontaine, 1964; Chester Jones and Henderson, 1965; Chan et al., 1967; Fenwick and Forster, 1972; Fenwick, 1974a) and fish in freshwater drink very little (Maetz and Skadhauge, 1968). In other words, the participation of the gut is not obliga-

tory in the development of post-stanniectomy hypercalcemia. In fish such as the carp or silver eel, the integument is poorly vascularized and has a thick epidermis and considerable mucous, all of which contribute to the relative impermeability of the skin to water and electrolytes (Mashiko and Jozuka, 1964; Fromm, 1968; Kirsch, 1972; Tesch, 1977). However yellow eels have relatively thin skin so that minor exchange may occur (Chester Jones et al., 1962).

Simmons (1971), Fontaine et al. (1972) and Fenwick (1974a) have all implied that the gills, at least in unfed freshwater fish, are the most important sites for calcium uptake from the environment. This is reasonable when one considers that the gill filaments have a total surface area up to 50 times the body surface (Gray, 1954) and are the site where blood comes into the most intimate contact with the external environment. Indeed, within the gill filaments the blood is separated from the external environment by a very thin layer of cells only one to 3 micrometers in thickness (Hughes and Morgan, 1973). Moreover, the relatively high cardiac output allows frequent circulation of the total blood volume through the gill lamellae in a short period of time (Satchel, 1971). Certainly the gill is a multipurpose organ and is specialized for the clearance of waste products of nitrogenous metabolism, maintenance of acid-base balance and respiratory gas exchanges (Maetz, 1971). More importantly to this thesis, it is well documented that active transport of sodium and potassium

ions occurs across the gills, or more specifically across the chloride cells which are situated at the base of the secondary lamellae of the gill filaments (Maetz and Bornancin, 1975). With all these things considered, it did not seem unreasonable to look to the gills as a possible target organ for the Stannius corpuscle hormone and to postulate that the calcium involved in post-stanniectomy hypercalcemia was entering through the gills. Consequently the main thrust of this thesis was oriented towards elucidating the relationship, if any, between the Stannius corpuscles and the gills at least so far as calcium homeostasis is concerned.

Earlier studies on calcium exchange between fish and their environment were conducted in vivo. The common experimental approach was to study calcium influx by placing the whole intact fish in ^{45}calcium prelabelled medium for certain periods of time and then measuring the appearance and distribution of the isotope within the animal (Asano et al., 1956; Borough et al., 1957; Rosanthal, 1956, 1960; Mashiko and Jozuka, 1962; Flèming et al., 1973) or by monitoring the disappearance of ^{45}calcium from the medium (Fontaine et al., 1972). Calcium efflux was measured by loading the fish with ^{45}calcium by intraperitoneal injection and then observing the appearance of the isotope in the external medium (Fontaine et al., 1972; Dacke, 1975). While these techniques do provide some data on overall cal-

cium exchange rates they do not separate the function of the gills from other possible exchange sites. An improvement in this regard has been achieved by enclosing the posterior half of the fish body with a rubber sac (Mashiko and Jozuka, 1961, 1964) and evaluating the ^{45}calcium fluxes across the anterior or posterior regions. However these *in vivo* preparations tend to suffer from handling and cannulation effects (Wood and Randall, 1971), lack of flexibility in carrying out the experiments, possible intervention of various feedback mechanisms, the effects of stress and the presence of uncontrolled endogenous hormonal levels. This can be observed from the post-anaesthetized alteration of various hormonal levels and levels of different electrolytes, particularly calcium ions in the trout (Houston *et al.*, 1969, 1971a, 1971b; Fromm, 1971). Furthermore, experimental observations by the above methods still are unable to specify the role of the branchial epithelium. Indeed even the perfusion of whole isolated head (Schiffman, 1961) suffers from the possibility that some exchange is occurring across the skin and/or the epithelium of the buccal and branchial cavities.

For these reasons I decided to adopt a recently developed technique which involves the perfusion of isolated, individual hemibranchs. This preparation, which was originally designed to study the vascular action of neurohypophyseal hormones (Rankin and Maetz, 1971), has in the hands of Shuttleworth (1972) proven to be very useful in

the study of ionic regulation. Indeed, Shuttleworth (1972) obtained results with this preparation which were in accord with the accepted role of the gill as established by experiments on intact fish. Therefore, this technique was deemed to be the most appropriate method for studying branchial calcium regulation. By this method, the gill per se can be studied free from endogenous neural and hormonal influence.

The general approach of these experiments was first to measure the effect on stanniectomy on ^{45}calcium influx across isolated perfused gills and to relate the observed effects to the state of calcium homeostasis in whole eels. The second series of experiments involved the investigation of the action of two hypocalcemic principles, namely calcitonin and the factor(s) from the extract of Stannius corpuscles, on the control of calcium movement across the isolated perfused gill. Finally studies were carried out to test the influence of calcium ions and magnesium ions on the calcium influx.

MATERIALS AND METHODS

1. Experimental Animals

The eels used throughout this study were immature yellow female American eels, Anguilla rostrata (LeSueur), procured from a commercial fish dealer in Quebec City, Quebec, Canada. The eels, 500 grams to one kilogram in weight, were caught in the St. Lawrence River near Quebec City. After being brought to Ottawa, they were held at 10 ± 1 ^{o}C in a recirculating living stream[1] filled with dechlorinated Ottawa tap water (0.45 mM Ca^{2+}). They were not fed. The photoperiod was not strictly controlled but approximated 12 hours of light alternating with 12 hours of darkness. Occasionally a few drops of a 1% aqueous solution of malachite green were added to the water to inhibit fungal growth. The circulated dechlorinated water was changed partially every week to reduce the incidence of bacterial and fungal infections, to remove the mucous secreted by the fish and to maintain constant calcium levels in water. The calcium level in the water was also checked periodically and did not vary.

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[1]Frigid Units Inc., Toledo, Ohio.

2. Surgical Techniques

A. Anaesthetization

Fish were anaesthetized in an aqueous solution of Tricaine methane sulfonate[2] at a concentration of .2 grams per liter until the eels were observed to have lost their righting response. The induction of a suitable state of anaesthetization was checked by pinching the ventral fin with tissue forceps. If the fish did not respond it was judged to be ready for surgery.

B. Tagging

All experimental animals were tagged by the split-ring and plate method of Vladykov (1970). The tag consisted of a numbered plastic tag on a metal ring which was split diagonally. The mouth of the eel was opened and the open ring was pushed through the skin of the lower jaw so that it encircled the jaw bone. The tagged fish had no difficulty in irrigating their gills and the tags remained firmly attached for the whole experimental period.

The disadvantage of this method was that the wound caused by the ring did not always heal. In several cases, marked necrosis and/or minor bleeding of the surrounding tissue were noted at the site of the ring. Nevertheless,

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[2] formerly MS-222, Sandoz or Ethyl-m-Aminobenzoate methane sulfonic acid salt, Sigma.

this method of tagging proved satisfactory for the present study.

C. Stanniectomy (Surgical removal of corpuscles of Stannius)

The technique used for stanniectomy and sham-operation was similar to that described by Butler (1969), Fenwick and Forster (1972) and Fenwick (1974).

After anaesthetization, the eels were placed ventral side up on a one-meter fiberglass tray lined with a dry paper towel. One centimeter lateral to the vent, a 4 to 5 cm long incision was made through the body wall parallel to the left lateral line. Bleeding from the capillaries of the body wall was stopped by hot-wire cautery. The edges of the body wall were retracted and the urinary bladder was deflected laterally to expose the paired corpuscles situated on the midventral surface of the posterior kidney (Figure 1). Sometimes the corpuscles of Stannius were found embedded in the kidney near the subcardinal (postcardinal) vein. The corpuscles were removed with a pair of fine forceps. To facilitate hemostasis a small square of absorbable gelatin sponge[3] was placed at the removal site of the corpuscles. The urinary bladder and the surrounding tissue were restored to their original position and the incision was sutured with No. 10 cotton thread. Sham-operations were performed in a

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[3]Gelfoam: The Upjohn Co., Michigan.

Figure 1

Position of the corpuscles of Stannius.

The paired corpuscles of Stannius (arrows) were situated on the ventral aspect of the posterior kidney in the American eel Anguilla rostrata (LeSueur). (a, the anterior end of the fish; p, the posterior end of the fish.)


a

similar way without the removal of corpuscles of Stannius.

At the end of the experimental period autopsies were performed to assure that the complete removal of the Stannius corpuscles had been achieved.

D. Blood Sampling

a. Caudal Vessels Approach

This method was employed in various experimental time periods before the sacrifice of the fish. Blood samples of about 0.5 ml were taken from the caudal vessels of MS-222 anaesthetized or unanaesthetized fish into heparinized 1-ml disposable syringes fitted with one inch long 23 gauge hypodermic needles. The sampling of blood was achieved by inserting the needle at an angle of 60° (posterior to anterior) mid-ventrally through the surface of the fish body at a site about 10 cm posterior to the vent.

b. Ventral Aorta Approach

This method was usually employed at the end of various experimental time periods when the fish were killed for gill isolation and cannulation. The eels were killed by decapitation at the level of ventral aorta, immediately cranial to the pectoral fins. The blood samples were collected directly from the heart into heparinized 100-ml beakers.

c. Preparation of Plasma

Fresh blood was immediately centrifuged at 500 x g for 10 minutes, the plasma was decanted into a 2-ml disposable plastic beaker or clean polyetnylene centrifuge tube and was stored frozen at -40 ^{o}C until calcium analysis. The blood cells were discarded.

E. Gill Cannulation and Perfusion Technique

The cannulation of individual isolated hemibranchs and the constant-flow branchial perfusion technique followed closely the techniques described by Shuttleworth (1972).

a. Cannulation Technique

i. Cannula

The afferent arterial cannula to connect the afferent artery of the isolated hemibranch to the perfusion pump consisted a 40-cm long piece of polyethylene tubing[4] connected to a 23 gauge hypodermic needle. The tip of the needle was protected by a short length (0.4 cm) of polyethylene tubing (Figure 2). This piece of tubing also acted as an 'anchor' once inserted into the vessel. The efferent arterial cannula for connecting the efferent artery to the collection pipette was of the same design as the afferent arterial cannula except that the cannula was only 15 cm

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[4]PE 50: Clay Adam; o.d.:0.1 cm, i.d.:0.06 cm.

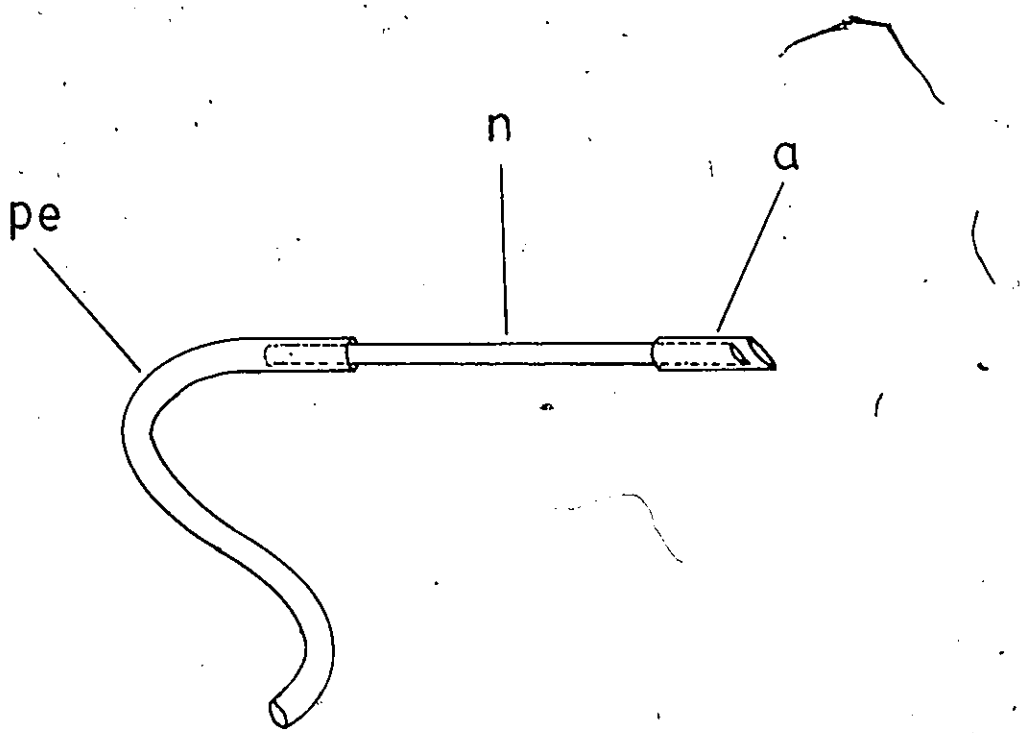
Figure 2

Diagram showing the type of cannula used for cannulating the afferent and efferent arteries.

n = shaft of a hypodermic needle, No. 23 gauge.

pe = polyethylene tubing (PE 50).

a = polyethylene tubing acting as an 'anchor' and protecting the pointed tip of the needle.


n
a
pe

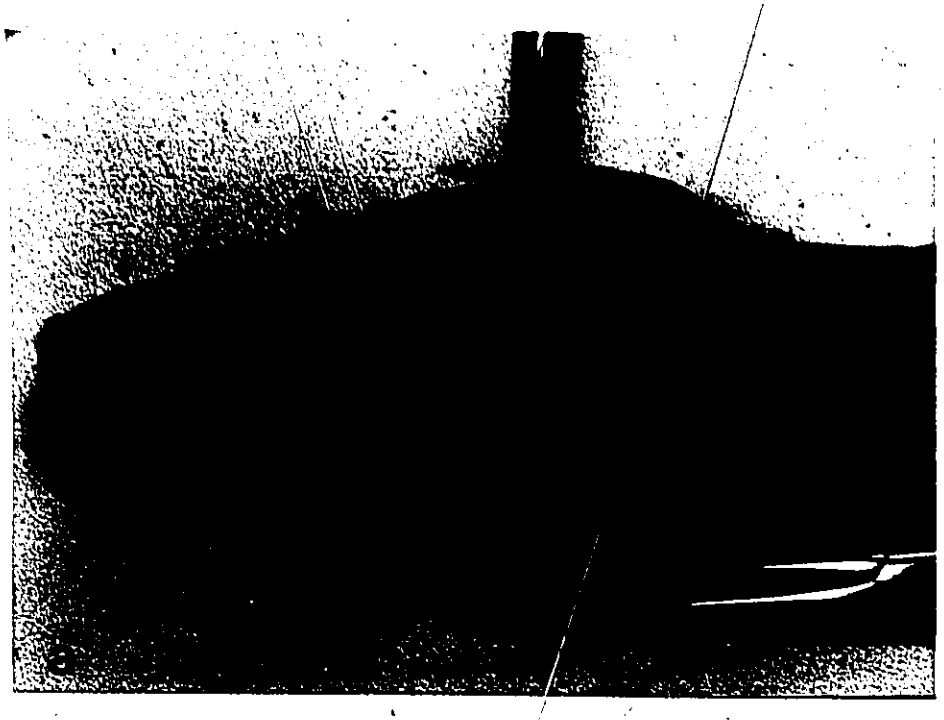
long.

ii. Gill Cannulation

At the end of the experimental period, eels were killed and plasma collected as described earlier. The head was pithed with a piece of wire to reduce the movement of the eel which otherwise occurred at the time of gill cannulation. For better artery isolation two balls of cotton about 2.5 cm in diameter were pushed into the buccal cavity to raise the ventral aorta and afferent branchial arteries. The head was placed ventral side up on a piece of moulded styrofoam and the first pair of gills, the afferent arteries and the ventral aorta were exposed by cutting open the opercular cavity along a line running from the opercular opening to the tip of the lower jaw. The primary filaments of the adjacent more posterior second arch were trimmed off in order to expose the first afferent artery (Figure 3a). The artery was opened by making a small V-shaped nick and the afferent arterial cannula, with flowing heparinized perfusion fluid (Table II), was inserted through the nick and secured with double ligatures. The hemibranch was flushed continuously with perfusion fluid at a rate of 3 ml hr^{-1}. The cannulated end of the gill arch was then freed from the branchial apparatus and a second ligature was tied around the arch and the cannula holding them together. The cannula was then withdrawn until the 'anchor' caugnt on the ligature. The first efferent artery on the upper pharynx (Figure 3b) was

Figure 3

Position of the afferent and efferent arteries. Head of the eel dissected to show the position of first right afferent artery (a, arrow) and first right efferent artery (b, arrow).




b

Table II

Composition of the Perfusion Fluid and Bathing Medium in mM

	NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$NaHCO_3$	NaH_2PO_3
Perfusion* Fluid	119.68	2.68	1.63	15.00	3.66
Bathing Medium	1.19	0.027	0.45	0.15	0.037

*0.1% w/v of glucose was added to the perfusion fluid and it was then filtered through a 0.45 μ Millipore disposable filter. 100 I.U. ml^{-1} of ammonium heparin was added just prior to use.

traced and exposed. It was cut at the point where it joined the artery to the anterior head region and was cannulated in the same manner as the afferent artery. At this time the perfusion fluid could be seen flowing out from the open end of the efferent cannula. The whole perfused hemibranch was then dissected free and was placed in a 100-ml beaker filled with 10 ^{o}C bathing medium (Table II). It was flushed continuously at 3 ml hr^{-1} with perfusion fluid for 10 to 15 more minutes or until the gills were cleared of blood as indicated by a marked blanching.

b. Perfusion Technique

The constant flow of perfusion fluid through the isolated hemibranch was achieved by using a variable speed motor-driven syringe pump[5] fitted with a 20-ml BD disposable syringe filled with appropriate perfusion fluid (Figure 4). Immediately prior to use, 100 I.U. of ammonium heparin were added to each ml of perfusion fluid. The bathing medium was maintained at 10 ± 0.5 ^{o}C by a Lauda refrigerated circulator[6] and was vigorously mixed by bubbling oxygen through it. The gill was then equilibrated for 15 minutes at a perfusion rate of 0.8 ml hr^{-1}. The perfusion fluid was collected from the efferent arterial cannula by allowing it to flow into a 0.1 ml pipette graduated in 0.01 ml divisions. Perfusion

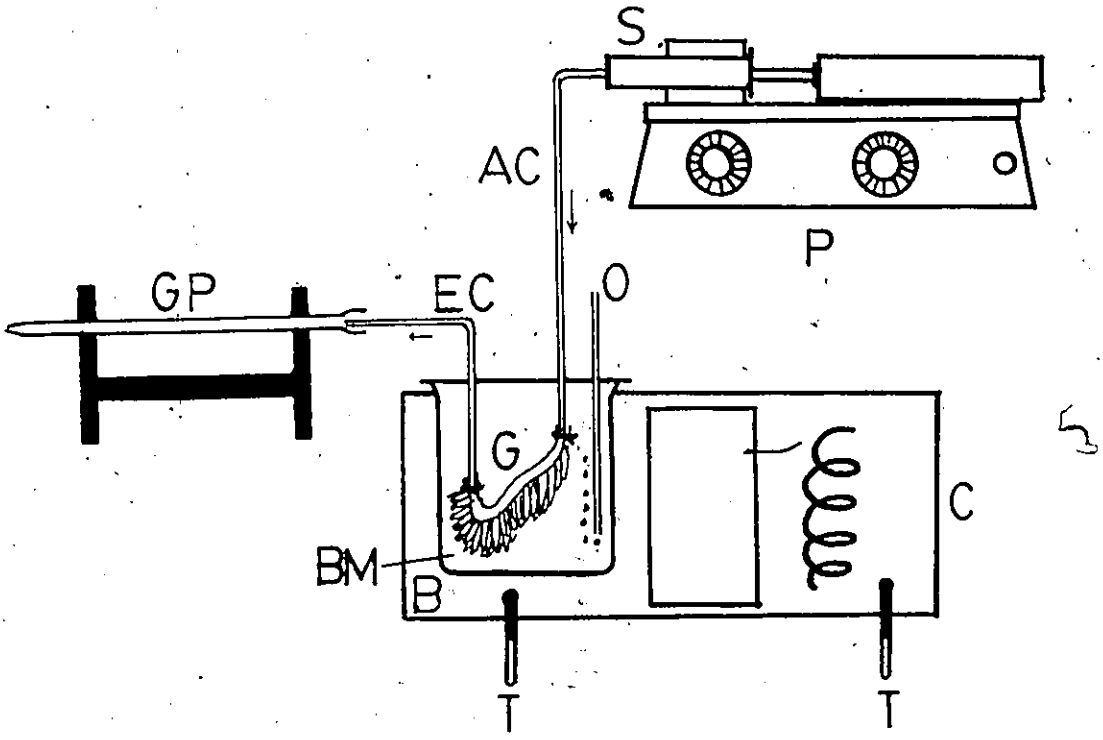
[5]Sage Model 352, Orion Research Inc.

[6]Model K-2/R: Messgerate-Werk Lauda, Germany.

Figure 4

Schematic Diagram of Gill Perfusion Preparation.

B, constant temperature water bath;
C, temperature control circulator;
G, isolated hemibranch;
O, oxygen line;
P, Perfusion pump;
S, syringe fill with perfusion fluid;
T, thermometer;
AC, afferent arterial cannula;
EC, efferent arterial cannula;
BM, bathing medium;
GP, graduated pipette.


S
AC
P
GP
EC
O
G
C
BM
B
T
T

and collection rates were recorded as a check for leaks. Preparations with effluent flow rates less than 90% of the pumping rate were discarded. After equilibration a 0.1 ml sample of perfusion fluid was taken as blank. For influx studies the gill preparation was then transfered to a jar filled with 50 ml of fresh bathing medium prelabelled with two microcuries of ^{45}calcium[7]. This gave a specific activity of 2.2 microcuries per mg of calcium in the bathing medium or about 5,000 CPM per 0.1 ml of bathing medium (approximately 60% efficiency). The jar was covered with parafilm in order to prevent splashing and to hold the cannula in place. The effluent perfusion fluid was collected by removing and replacing the collection pipettes every 15 minutes for the periods indicated in the Results section. The effluent perfusion rate during every 15-minute interval was monitored also to determine CPM uptake (see Table III). A 0.1 ml aliquot was withdrawn from each 15-minute perfusion fluid sample for counting; the excess effluent perfusion fluid was discarded. Sampling was done with an adjustable Gilson pipette calibrated for 100 µl. Samples of the bathing medium were collected also at the end of every alternate 15-minute interval. Each sample was placed in an individual scintillation vial along with 6 ml of scintil-

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[7] aqueous ^{45}calcium chloride with a specifity of 22.73 microcuries per microgram of calcium, Amersham-Searle Co., Oakville, Ontario.

lant[8] and was counted to constant error in a Beckman LS-233 Liquid Scintillation Counter or a Wallac LKB 1215 Rackbeta Liquid Scintillation Counter. For efflux studies 5 microcuries of ^{45}calcium were added to each ml of the perfusion fluid. This gave an activity of about 10^7 CPM per ml of perfusion fluid. A 0.1 ml sample of bathing medium was taken every 15 minutes for the periods indicated in the results section.

2. Analytical Techniques

A. Plasma Total Calcium Measurement

Plasma total calcium was routinely measured by an atomic absorption flame photometer[9]. A 0.1 ml aliquot of plasma sample (unknown) and calcium standard solution (ranging from 0.5 mM to 10 mM) were diluted with 1 ml of an aqueous solution of lanthanum chloride (1.13×10^{-2} mM). The lanthanum served to suppress ionization interference by providing an excess of free electrons, thereby preventing the ionization of calcium. One ml of lanthanum chloride solution was used as the blank and the unknown values were read from the standard curve.

Plasma total calcium levels in small samples were

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[8]Aquasol-2, New England Nuclear.

[9]Atomsob-Model 82-720, Jarrel-Ash Waltham, Mass.

measured fluorometrically[10]. A 20 µl sample of unknown plasma or calcium standard solution was diluted with 5 ml of working reagent which was prepared by diluting 7 ml of calcein stock[11] to one liter with 0.8 N KOH. The calcium concentration of plasma was evaluated from the standard curve.

B. ^{45}Calcium Measurement

Initially ^{45}calcium samples were counted using a Beckman LS-233 Liquid Scintillation Counter. Later, a Wallac LKB 1215 Rackbeta Liquid Scintillation Counter was obtained and used. In the Beckman LS-233 the samples were counted for 2 minutes in channel A with a window widtn of zero to 650 (the full window for ^{45}calcium spectrum). Channel B, filled with a tritium (^{3}H) spectrum isoset module was used for the purpose of enumerating the channel ratios required for adjusting the quenched rate.

When the Wallac LKB 1215 Rackbeta Liquid Scintillation Counter was used, the samples were counted twice for 5 minutes using its external standard for evaluating their efficiency. The ^{45}calcium spectrum in Channel 1 was set from 50 to 175.

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[10]Model 111 Turner Fluorometer, G.K. Turner Associates.

[11]Calcein: 2', 7'-{[bis(Carboxymethyl)-Amino]-Methyl}-Fluorescein or Fluorescein-methylene-iminodiacetic acid, Sigma. The stock was prepared in a concentration of 1 mg calcein per ml of 0.8 N KOH.

a. Quenching Corrections

Quenching is any reduction of efficiency in the energy transfer process in the scintillation solution (Wang and Willis, 1965). It reduces the detection efficiency of the samples and lowers the count obtained on the scintillation counter during sample counting. The extent of quenching varies considerably with different sample materials and as different types of samples were studied a quenching curve was established to determine the counting efficiency of the different unknown samples.

i. Quenching Curve for Beckman Liquid Scintillation Counter

To accomplish the preparation of a quenching curve for each possible type of sample, a series of standard samples (10 to 20) were made. Aliquots (0.1 ml) of ^{45}calcium chloride with known activity (around 10^4 DPM) and as little quenching as possible were used when preparing these standards. The first sample in the quenched series was unaltered. Each additional sample standard had a quenching agent added which was similar to the quenching agent in the samples being measured in the experiments. The amount of the quenching agent was progressively increased for each sample. The general quenching agents used were Sudan IV (colour quencher), distilled water and ethanol (dilution quencher), hydrochloric acid and sodium hydroxide (acid and base quencher), and chloroform (or acetone)

(chemical quencher) (Wang and Willis, 1965). All these samples were counted to constant error with the same window settings as described earlier. From the known count, the efficiency for each standard sample was calculated by dividing the observed count (quenched value) by the calculated count (theoretical value)[12]. The quenching curve with the counting efficiency versus the known channel ratio was used to evaluate the actual rate of unknown experimental samples.

ii. Quenching Curve for Wallac LKB Liquid Scintillation Counter

The quenching calibration for the Wallac LKB 1215 Rackbeta Liquid Scintillation Counter was simpler than that for the Beckman Liquid Scintillation Counter. The first of the three methods mentioned in its manual was used for ^{45}calcium quenching. Only one sample of any activity (with the activity about 10^4 DPM) was used. After each measurement, 0.1 ml of quenching agent (carbon tetrachloride) was

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[12]The theoretical value was adjusted for decay by the following formula (Wang and Willis, 1965):

$$\text{Log}_{10}N_{10} = \text{Log}_{10}N_0 - 0.4343\lambda t$$

where N = the number of atoms of a radioisotope present at a given time,
N_0 = the number of radioactive atoms present in the sample at the time zero,
λ = the decay constant, and
t = the time for disintegration of the isotope.

added progressively. The curve for efficiencies versus external standard ratios for the sample was automatically recorded and stored in the machine programme.

At the time when the experimental samples were measured, its quenched rate was automatically adjusted to 100% efficiency and the corrected count was given subsequently on the print out.

C. Gill Dry Weight Measurement

At the end of the experiments the isolated perfused gills were rinsed for 5 minutes in fresh unlabelled bathing medium with perfusion fluid perfused continuously at 6 ml hr^{-1} to flush out any isotope that was present in blood capillaries of the gills. They were then injected carefully via the afferent arterial cannula with a pulse of malachite green to mark the perfused area of the gill filaments. After they were removed and blotted lightly, the filaments marked with malachite green were trimmed off from the gill arches and were placed in separate preweighed glass scintillation vials. The dry weight of the gill filaments was determined after drying in an oven at 100 ^{o}C for approximately twenty-four hours.

D. Preparation of Gill Tissue for Liquid Scintillation Counting

The residual ^{45}calcium present in the gill filaments was evaluated after the gill had been perfused in the

^{45}calcium-labelled bathing medium. To measure the radioactivity, the tissue had to be transformed into liquid state. The method used for preparation was modified from that by Lindsay and Kurnick (1969) and Adams et al. (1970).

After the dry weight of the gill filaments were obtained, the dry samples were allowed to imbibe with a few drops of distilled water in a glass scintillation vial; this enhanced tissue digestion. One ml of NCS solubilizer[13] was then added. The mixture was swirled gently and digested overnight at 50 ^{o}C until the sample appeared homogenous when shaken. To achieve a low background, high counting efficiency, less quenching and improved sample clarity, the completely dissolved sample was neutralized at room temperature with glacial acetic acid before adding 6 ml of scintillant, Aquasol-2. Usually the digested samples were clear pale yellow in color so that decolorization was unnecessary. The tissue sample was then counted with the same procedure as used for the fluid samples described earlier.

E. Preparation of Tissue Extracts for Isolated Gill Perfusion Study

Extracts of Stannius corpuscles or other tissues such as kidney and liver were prepared by homogenizing 20 mg of fresh tissue in one ml of ice cold perfusion fluid (Table I)by means of a Potter Elvehjem tissue grinder fitted

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[13]Amersham Searle Co.

with a teflon pestle. Extracts to be used in gill perfusion studies and most of those to be injected into the animals were then acidified to pH 2 with 1 N HCl, centrifuged at 40,000 x g for 20 minutes at 0 °C, adjusted back to pH 7.4 with 0.8 N KOH and re-centrifuged as above. The supernatant was collected and stored as stock solution at -40 °C until use. The residue was discarded. The acidification step was necessitated by the presence of an acid labile pressor material present in Stannius corpuscles (Chester Jones et al., 1966) which otherwise caused a marked reduction in effluent perfusion rate.

Prior to gill perfusion, the stock extract solution was diluted to a concentration of 2.5 mg (extract from 2.5 mg of tissue) per ml with heparinized perfusion fluid and filtered through a 0.45 μ Millipore disposable filter. The final concentration of extracts used for gill perfusion was equivalent to approximately one half (in weight) of a corpuscle[14] per milliliter of perfusion fluid.

F. Terminology

For the purpose of this thesis the term '^{45}calcium influx' refers to the measured amount of ^{45}calcium which crosses from the bathing medium to the perfusion fluid and stays there; while the term '^{45}calcium efflux' refers to the converse situation. The terms 'calcium influx' and 'calcium

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[14]The average weight of one corpuscle of Stannius is 5 mg.

efflux' refer to the movement of cold calcium as calculated from ^{45}calcium influx and ^{45}calcium efflux values in concert with the specific activities of ^{45}calcium in the outside bathing medium and perfusion fluid respectively (see Table III and IV). Net ^{45}calcium influx and efflux and net calcium influx and efflux were calculated according to the following formulae:

Net ^{45}calcium influx = ^{45}calcium influx - ^{45}calcium efflux

Net ^{45}calcium efflux = ^{45}calcium efflux - ^{45}calcium influx

Net calcium influx = calcium influx - calcium efflux

Net calcium efflux = calcium efflux - calcium influx

4. Experimental Protocol

A. Effect of Stanniectomy on Plasma Calcium Level and Branchial ^{45}Calcium Flux

a. Effect of Stanniectomy on the ^{45}Calcium Influx across an Isolated Perfused Eel Gill

This experiment was to measure the rate of ^{45}calcium influx from the ^{45}calcium labelled bathing medium into individual perfused isolated gills in two week stanniectomized, sham-operated and control eels.

Three groups of 7 eels (control, sham-operated

and stanniectomized) were used. Two weeks after the various surgical procedures the fish were decapitated and the gills were perfused as described earlier. Blood samples were taken at the time of surgery and the end of the two weeks post-surgical period in order to measure the effect of various manipulations on plasma calcium levels. Samples of effluent perfusion fluid were collected for five consecutive 15-minute intervals for a total of 75 minutes. The dry weight of perfused gill filaments were also determined. Branchial ^{45}calcium influx and total calcium influx were calculated using the equations in Table III.

b. Effect of Stanniectomy on the ^{45}Calcium Efflux across an Isolated Perfused Eel Gill

This experiment was designed to study the effect of stanniectomy on ^{45}calcium efflux across isolated perfused eel gills.

The experiment was carried out by isolating and cannulating the first pair of gills from the operated animals. A modified system (Figure 5) was used (Leung, 1979). Instead of driving the perfusion fluid directly out of syringes and into the gills, two oxygen filled 20 ml syringes were used to force the appropriate perfusion fluid out of closed vacutainer tubes and into the afferent cannulae. The vacutainer tube functioned as a bubble trap and attenuated the rate of pressure changes. One of the

Table III

Calculation for
Branchial ^{45}Calcium Influx and
Total Calcium Influx

I. Branchial ^{45}calcium influx corrected from various CPM in bathing medium (5×10^4 CPM ml^{-1}):

$$\frac{P\ R}{W} \times \frac{5 \times 10^4}{B} \text{ CPM } g^{-1} \text{ dry filament wt } min^{-1}$$

where P = the activity of ^{45}calcium in effluent perfusion fluid (CPM 0.1 ml^{-1})

B = the activity of ^{45}calcium in bathing medium (CPM 0.1 ml^{-1})

R = the effluent perfusion rate ($ml \cdot min^{-1}$)

W = the dry weight of the perfused gill filament (g)

II. Total calcium influx from bathing medium of 0.45 mM Ca^{2+} or 0.45 µmol ml^{-1} Ca^{2+}:

$$\frac{P\ R}{W} \times \frac{5 \times 10^4}{B} \times \frac{0.45}{5 \times 10^4} \text{ µmol } Ca^{2+}\ g^{-1} \text{ dry filament wt } min^{-1}$$

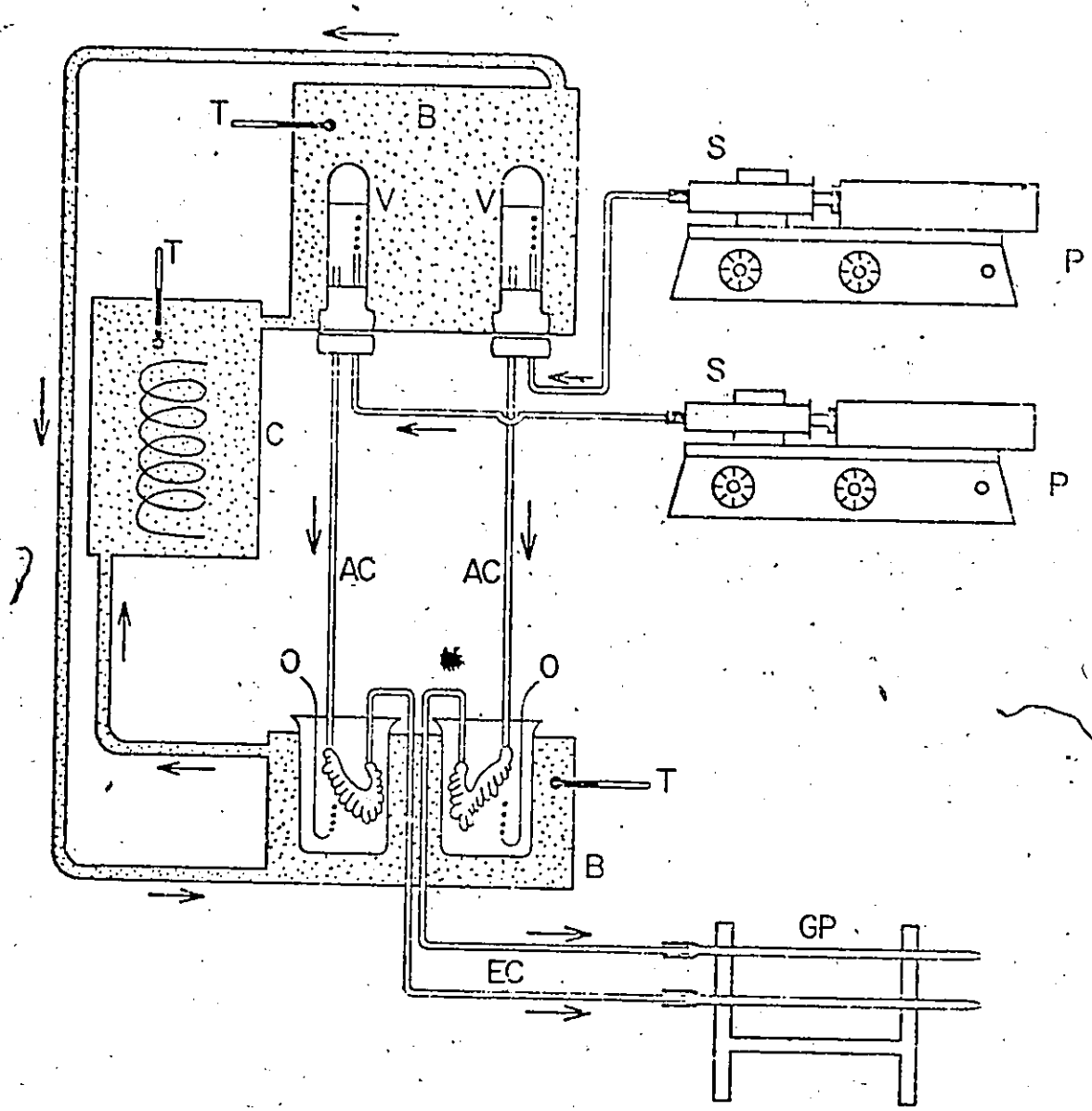
or $\frac{P\ R}{W\ B} \times 0.45$ µmol Ca^{2+} g^{-1} dry filament wt min^{-1}

or $\frac{P\ R}{W\ B} \times 27$ µmol Ca^{2+} g^{-1} dry filament wt hr^{-1}

Figure 5

Alternate Set Up for Simultaneous Measurement of Branchial ^{45}Calcium Influx and Efflux.

B, constant temperature water bath;
C, temperature control circulator;
O, oxygen line;
P, perfusion pump;
S, syringe filled with oxygen;
T, thermometer;
AC, afferent arterial cannula;
EC, efferent arterial cannula;
GP, graduated pipette;
V, vacutainer filled with appropriate perfusion fluid.


T
B
V
V
S
P
T
S
P
C
AC
AC
O
O
T
B
GP
EC

isolated hemibranchs was used for studying ^{45}calcium influx rate while the other hemibranch was used to measure efflux. Branchial ^{45}calcium efflux and total calcium efflux were calculated from the equation in Table IV.

c. Relationship between ^{45}Calcium Influx across the Perfused Isolated Eel Gill and the Development of Post-stanniectomy Hypercalcemia

This experiment was desinged to observe the changes in and relationship between branchial ^{45}calcium uptake and plasma calcium levels in eels at various time intervals following the surgical removal of corpuscles of Stannius.

Five groups of 7 stanniectomized fish each were used and the experimental procedure was the same as described in Experiment a. The 7 fish in each group were decapitated at two days, four days, one week, two weeks, three weeks and four weeks following stanniectomy. Blood samples were taken before stanniectomy and at the time of sacrifice in order to measure plasma total calcium.

Table IV

Calculation for
Branchial ^{45}Calcium Efflux and
Total Calcium Efflux

I. Branchial ^{45}calcium efflux corrected from various CPM in perfusion fluid (10^7 CPM ml^{-1}):

$$\frac{B\ V}{T\ W} \times \frac{10^7}{P} \text{ CPM } g^{-1} \text{ dry filament wt } min^{-1}$$

where B = the activity of ^{45}calcium in bathing medium (CPM 0.1 ml^{-1})
P = the activity of perfusion fluid (CPM 0.1 ml^{-1})
V = volume of bathing medium (ml)
T = duration of perfusion period (min)
W = the dry weight of perfused gill filament (g)

II. Total Calcium Efflux from perfusion fluid of 1.63 mM Ca^{2+} or 1.63 µmol ml^{-1} Ca^{2+}:

$$\frac{B\ V}{T\ W} \times \frac{10^7}{P} \times \frac{1.63}{10^7} \text{ µmol } Ca^{2+}\ g^{-1} \text{ dry filament wt } min^{-1}$$

or $$\frac{B\ V}{T\ W} \times \frac{1.63}{P} \text{ µmol } Ca^{2+}\ g^{-1} \text{ dry filament wt } min^{-1}$$

or $$\frac{B\ V}{T\ W} \times \frac{97.80}{P} \text{ µmol } Ca^{2+}\ g^{-1} \text{ dry filament wt } hr^{-1}$$

B. Effect of Stannius Corpuscle Extract and Salmon Calcitonin on Branchial ^{45}Calcium Influx and Plasma Calcium Levels

a. In vivo and in vitro Effects of Stannius Corpuscle Extract on the Branchial ^{45}Calcium Influx in Stanniectomized Eels

Two experiments were performed. One experiment was designed to test and compare *in vivo* the effect of previously acidified and unacidified extracts of Stannius corpuscles on the plasma total calcium level in two week stanniectomized eels. The same eels were used to measure the effects of these extracts on ^{45}calcium influx in the gills. This was also a trial experiment to confirm the activity of previously acidified extract. Three groups of six eels each were stanniectomized. On the morning of the eighth day following stanniectomy, the groups were injected intraperitoneally with either perfusion fluid, previously acidified corpuscular extract or unacidified extracts and the injections were repeated daily until the end of the two week post-operative period. The stock extract solution was diluted with perfusion fluid so that the final injection fluid contained the equivalence of 10 mg of extracted tissue per ml; 0.5 ml was injected per kg of fish. Intraperitoneal injections were made at the level of the liver to minimize potential leakage through the incision used to

remove the Stannius corpuscles. Starting on the day of the operations, and continuing throughout the experiment, blood samples for calcium measurement were taken every 2 days during the afternoon. Two weeks after the operations were performed the final plasma samples were collected and the branchial ^{45}calcium uptake was measured on an isolated perfused gill from each fish.

The second experiment was designed to study the in vitro effect of Stannius corpuscle extract on branchial ^{45}calcium uptake during gill perfusion. In this experiment, 2 syringe-pumps were arranged so that one drove a 20 ml syringe full of normal perfusion fluid and the other drove a syringe filled with perfusion fluid containing previously acidified corpuscle extract (an equivalence of 2.5 mg of homogenized corpuscle tissue extract per ml of fluid). The two syringes were connected by a three-way stop-cock to the afferent perfusion cannula (Figure 6).

Three groups of 7 fish were used. Groups 1 and 2 were stanniectomized and group 3 was sham-operated. Two weeks after the operation the fish were killed by decapitation and the gill perfusion performed. The gills from group 1 were perfused with perfusion fluid only. The gills from groups 2 and 3 were perfused with perfusion fluid for the first 45 minutes and then perfusion fluid containing previously acidified corpuscle extract for the next 60 minutes. As a control the effect of previously acidified extracts from eel liver and kidney (an equivalence of

Figure 6

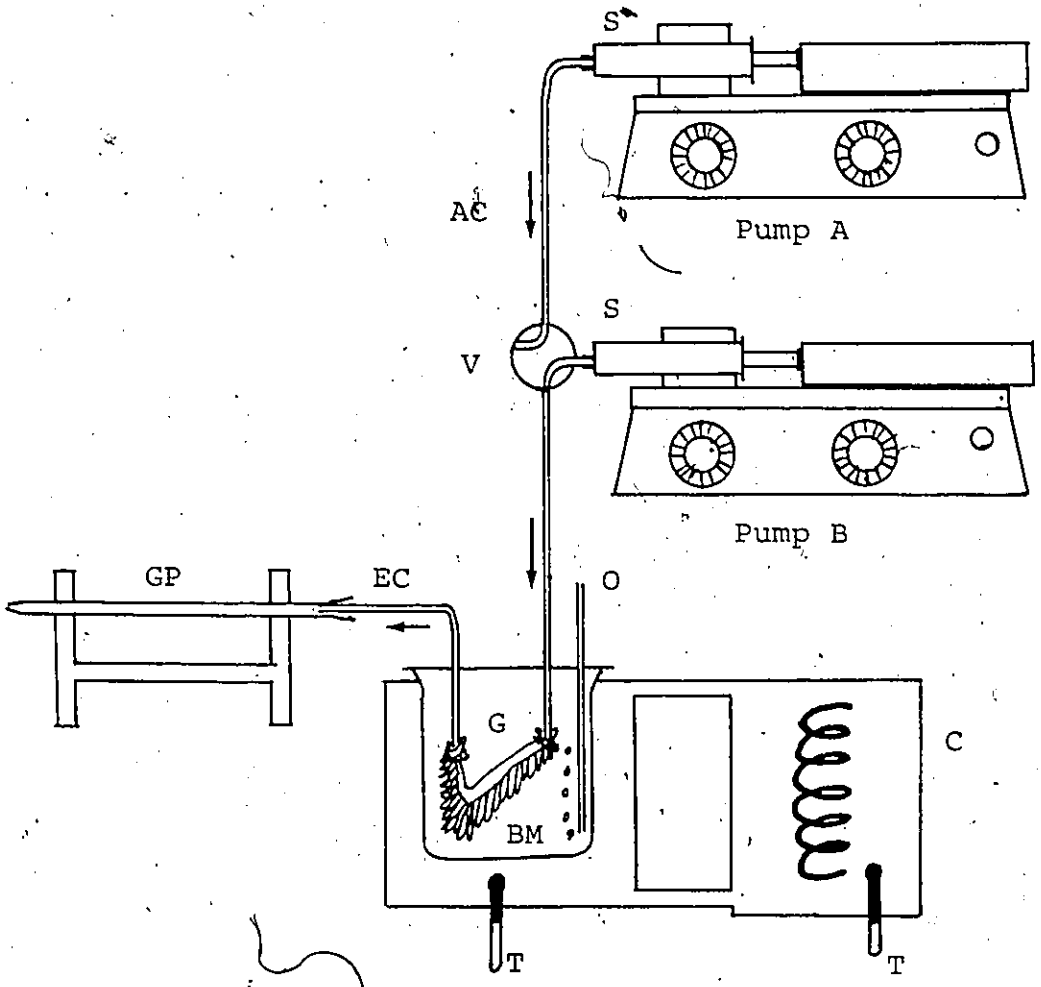
Schematic Diagram of Preparation for Extracts Perfusion.

Pump A: Perfusion pump with syringe filled with perfusion fluid;

Pump B: Perfusion pump with syringe filled with appropriate extract;

V, the three-way stop-cock.

(for details of labels, see legend for Figure 4)


S
AC
Pump A
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2.5 mg of homogenized tissue per ml of fluid) was tested using similar procedures as described for groups 2 and 3.

b. In vivo and in vitro Effect of Salmon Calcitonin on the Branchial ^{45}Calcium Flux in Eels

The purpose of these experiments was to study the in vivo and in vitro effect of salmon calcitonin (SCT) on plasma total calcium and branchial calcium influx in eels.

The experimental design was the same as in section 'a' with the exception that salmon calcitonin[15] was substituted for the Stannius corpuscle extracts. The dosage of salmon calcitonin for intraperitoneal injection was 2 MRC units per kg of fish per day. The SCT was dissolved in perfusion fluid containing 0.1% albumin to reduce loss of SCT due to binding to the walls of the syringe (Smith et al., 1978). The salmon calcitonin used for perfusion was prepared at a concentration of 400 mMRC units per ml in perfusion fluid containing 0.1% albumin. It was then filtered through a 0.45 μ millipore filter prior to gill perfusion.

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[15] Armour Pharmaceutical Co., AL 0977.

C. Effect of External and Internal Concentration of Ca^{2+} and Mg^{2+} on Branchial ^{45}Calcium Transport

a. Effect of High Internal Calcium Concentration on Branchial ^{45}Calcium Influx

The purpose of this experiment was to study the effect of high internal calcium, such as occurred during post-stanniectomized hypercalcemia, on calcium uptake by the gills. In this experiment, three two week stanniectomized freshwater eels were used. The first pair of gills was isolated and perfused simultaneously for 105 minutes using the same set up as shown in Figure 5. One of the gills was perfused with high calcium perfusion fluid (5 mM Ca^{2+}) while the other was perfused with standard perfusion fluid (1.63 mM Ca^{2+}).

b. Effect of Seawater Bathing Medium on Branchial ^{45}Calcium Influx

This experiment was designed to observe the effect of acute exposure to seawater on gill calcium transport.

Four two week stanniectomized freshwater eels were used. After the first pair of isolated gills had equilibrated, one of them was transferred to 50 ml of ^{45}calcium labelled reconstituted 100% seawater bathing

medium (10.4 mM calcium), while the other was placed in the ^{45}calcium labelled standard freshwater bathing medium (Table II). They were then perfused simultaneously for 120 minutes.

c. Effect of Seawater Concentrations of Ca^{2+} and Mg^{2+} in the Bathing Medium on Branchial ^{45}Calcium Influx

These experiments were performed to study the effect of seawater concentrations of calcium and magnesium ions in the otherwise freshwater bathing medium.

In the first experiment three fish were used. The first pair of gills was isolated and cannulated. After equilibration, one of the gills was transferred to freshwater bathing medium with 10 mM calcium chloride, while the other was placed in the freshwater bathing medium to which sufficient mannitol was added to compensate for the osmotic effect of the added calcium chloride (38 mOsm) in the first medium.

The second experiment was similar to the previous one with the exception that the freshwater bathing medium contained 52 mM magnesium chloride instead of 10 mM calcium chloride. The osmotic concentration was again adjusted by adding mannitol to the second medium.

5. Statistical Procedure

Statistical analysis of the results were carried out using the student's 't' test. For multiple comparisons among means one way- or two way-anova were carried out on the data and significance between pairs of means were made using Tukey's ω procedure. For the purpose of this study, a probability of $P < 0.05$ was taken as significant and $P < 0.01$ as highly significant.

RESULTS

1. Evaluation of Perfusion Technique

The isolated perfused gill preparation used proved to be a successful technique for the present study. Leaks were relatively uncommon and when the effluent flow rate was less than 90% of the pumping rate the preparation was discarded. As evidenced by dye marking, a large part of the gills was perfused and the preparation appeared to remain fully functional for at least two hours.

The time required for the perfusion fluid to pass through the gills was checked by perfusing blue food dye through the isolated gill preparation. At a perfusion rate of 0.8 ml per hour, the perfusion fluid required an average of 8.5 minutes to travel from the tip of the perfusion syringe to the afferent branchial artery. Another 5.5 minutes were required before the dye appeared in the efferent branchial artery. This, however, depended partially upon the size of the gills. A further three minutes were needed for the fluid to pass from the efferent artery to the collection pipette. In all, therefore, the fluid took approximately 17 minutes to pass from the syringe to the collection pipette. Usually the first part of the arch to

be perfused was the longest gill filaments at the bend of the gill arch with the other parts of the arch being perfused later. Areas of the gill arch which were tied off during the cannulation were not perfused. For dry gill filament weight determinations only those parts of the gill filaments which were successfully perfused were used.

2. Effect of Stanniectomy on the Plasma Total Calcium Concentration, ^{45}Calcium Influx and Calcium Influx across Perfused Isolated Eel Gills During the Summer

A. Plasma Total Calcium Level

The effect of stanniectomy on the plasma total calcium level in tap water adapted eels is shown in Table V. These data clearly indicate that the plasma total calcium concentration was significantly ($P < 0.05$) higher in stanniectomized fish when compared to sham-operated or control fish two weeks after the operations were performed.

B. ^{45}Calcium Influx

The data in Figure 7 indicate a clear effect of stanniectomy on the rate of ^{45}calcium influx across the isolated gills as compared to the influx rate seen in gills from sham-operated and control eels. Stanniectomy resulted in a significant increase ($P < 0.05$) which was

Table V

Plasma Total Calcium Levels in Stanniectomized, Sham-operated and Control Tap Water Adapted Eels at the time of the Operations and two weeks after the Operations[a]

Groups	Total Plasma Calcium (mM)	
	Before Operations (0 day)	After Operations (14 days)
Stanniectomized	2.58 ± 0.02	5.32 ± 0.13 [b]
Sham-operated	2.62 ± 0.11	2.50 ± 0.10
Control	2.49 ± 0.04	2.54 ± 0.04

[a] All values are means ± S.E.M. (N = 7)

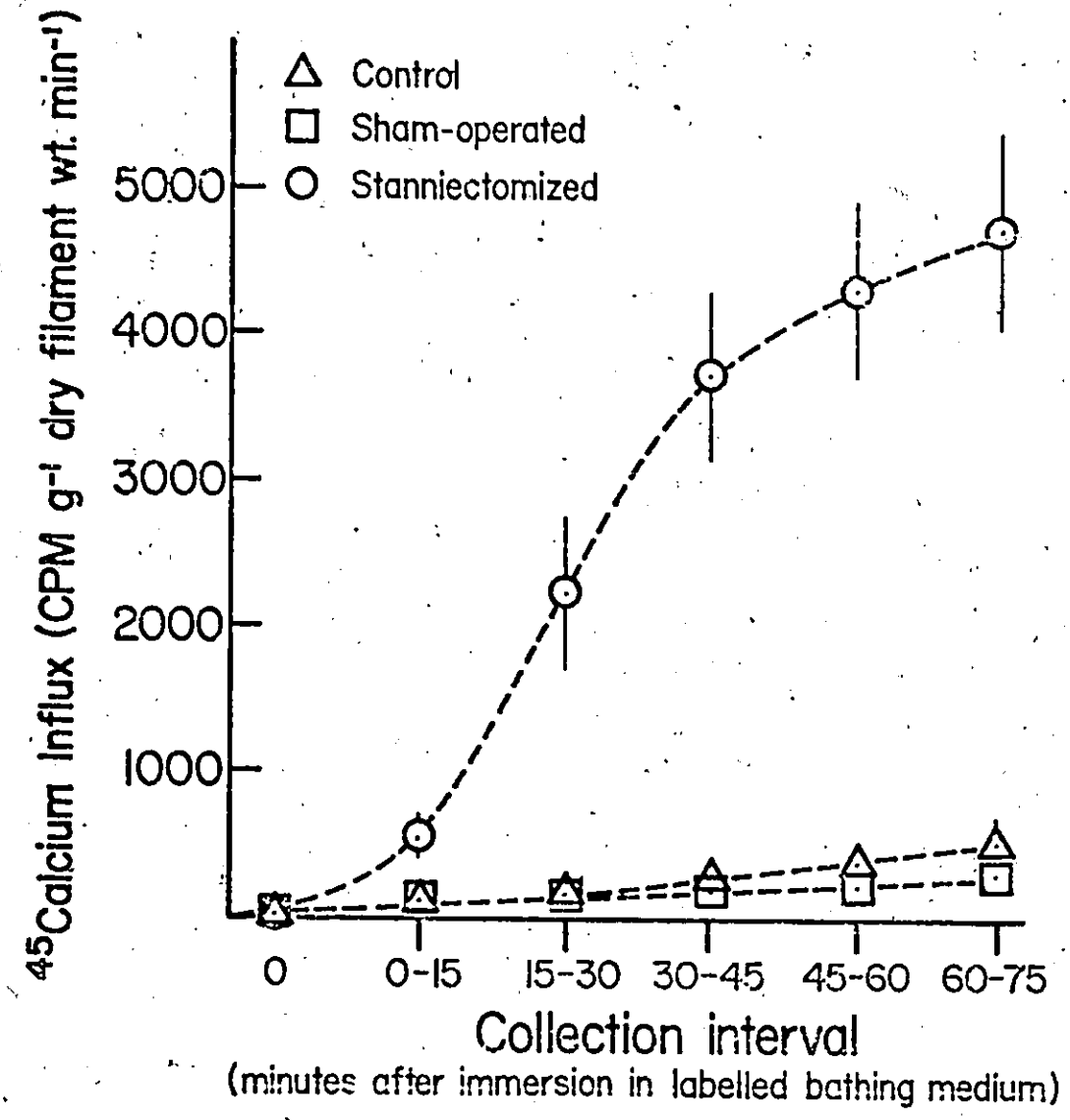
[b] Significantly different ($P < 0.05$) from all other tabulated values.

Figure 7

^{45}Calcium influx into individual perfused isolated gills from control, sham-operated and two week post-stanniectomized eels during the summer, measured as CPM g^{-1} dry filament wt min^{-1}.

Samples of perfusion fluid were collected at every 15 minute interval after immersion in bathing medium prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}).

Center of symbols is at the mean, vertical lines represent the S.E.M. N = 7 in all cases. (S.E.M.'s are not shown for sham-operated and control groups).


Control
Sham-operated
Stanniectomized
5000
4000
3000
2000
1000
^{45}Calcium influx (CPM g^{-1} dry filament wt. min^{-1})
0
0-15
15-30
30-45
45-60
60-75
Collection interval
(minutes after immersion in labelled bathing medium)

already evident during the 0 to 15 minute interval. By the 45 to 60 minute interval, the gills from stanniectomized eels showed a ^{45}calcium influx rate which was 17 fold greater than that of either the sham-operated or control fish. The calculated calcium influx rate for this group during the 60 to 75 minute interval was 2.572 µmol Ca^{2+} g^{-1} dry gill filament wt hr^{-1}. A plot of branchial ^{45}calcium influx against time of perfusion from two week stanniectomized fish described a sigmoid shaped curve. Further, in three of the seven preparations from stanniectomized eels the effluent perfusion fluid collected during the 60 to 75 minute interval had a level of radioactivity which exceeded the radioactivity of the bathing medium. The activity of ^{45}calcium in the bathing medium did not change significantly over these 75 minutes of perfusion. The branchial ^{45}calcium influx into the isolated gills of sham-operated and control fish was very low and did not exceed 500 CPM g^{-1} dry filament wt min^{-1} (equivalent to 0.270 µmol Ca^{2+} dry filament wt hr^{-1}). Values from sham-operated eels were not significantly different from control eel values ($P > 0.05$).

3. Effect of Stanniectomy on ^{45}Calcium Influx and Calcium Influx across Perfused Eel Gills During the Winter

As in the previous experiment (Figure 7), the ^{45}calcium influx rate across the perfused gills from stan-

niectomized eels was significantly ($P < 0.05$) higher than that in sham-operated animals (Figure 8). Accordingly, the calculated calcium influx rate for the interval between the 105th and 120th minute of gill perfusion for the stanniectomized eels (0.187 ± 0.018 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}) was significantly greater ($P < 0.05$) than the calcium influx rates in the sham-operated eels (0.014 ± 0.003 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}). These values were both significantly ($P < 0.01$) lower than the maximal influx rates calculated for the summer fish (see Figure 7). This indicates a seasonal effect on branchial calcium influx.

4. Effect of Stanniectomy on ^{45}Calcium Efflux and Calcium Efflux across Perfused Isolated Eel Gills During the Winter

The data shown in Figure 9 indicate that at no time during the two hours of perfusion was there a significant ($P < 0.05$) difference in the rate of ^{45}calcium efflux between the gills from sham-operated eels and the gills from stanniectomized eels. Correspondingly, calcium efflux rates from the stanniectomized (0.002 ± 0.0006 μmol Ca^{2+} g^{-1} dry gill filament wt hr^{-1}) and sham-operated eels (0.003 ± 0.0004 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}) were not significantly different ($P > 0.05$). These calcium efflux values were, however, significantly less ($P < 0.01$) than the calcium influx values shown in the preceeding

Figure 8

^{45}Calcium influx into individual perfused isolated gills from sham-operated and two week post-stannniectomized eels during the winter, measured as CPM g^{-1} dry filament wt min^{-1}.

Samples of perfusion fluid were collected at every 15 minute interval after immersion in bathing medium prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}).

Center of symbols is at the mean, vertical lines represent the S.E.M. N = 4 in all cases. (S.E.M.'s are not shown for the sham-operated group).

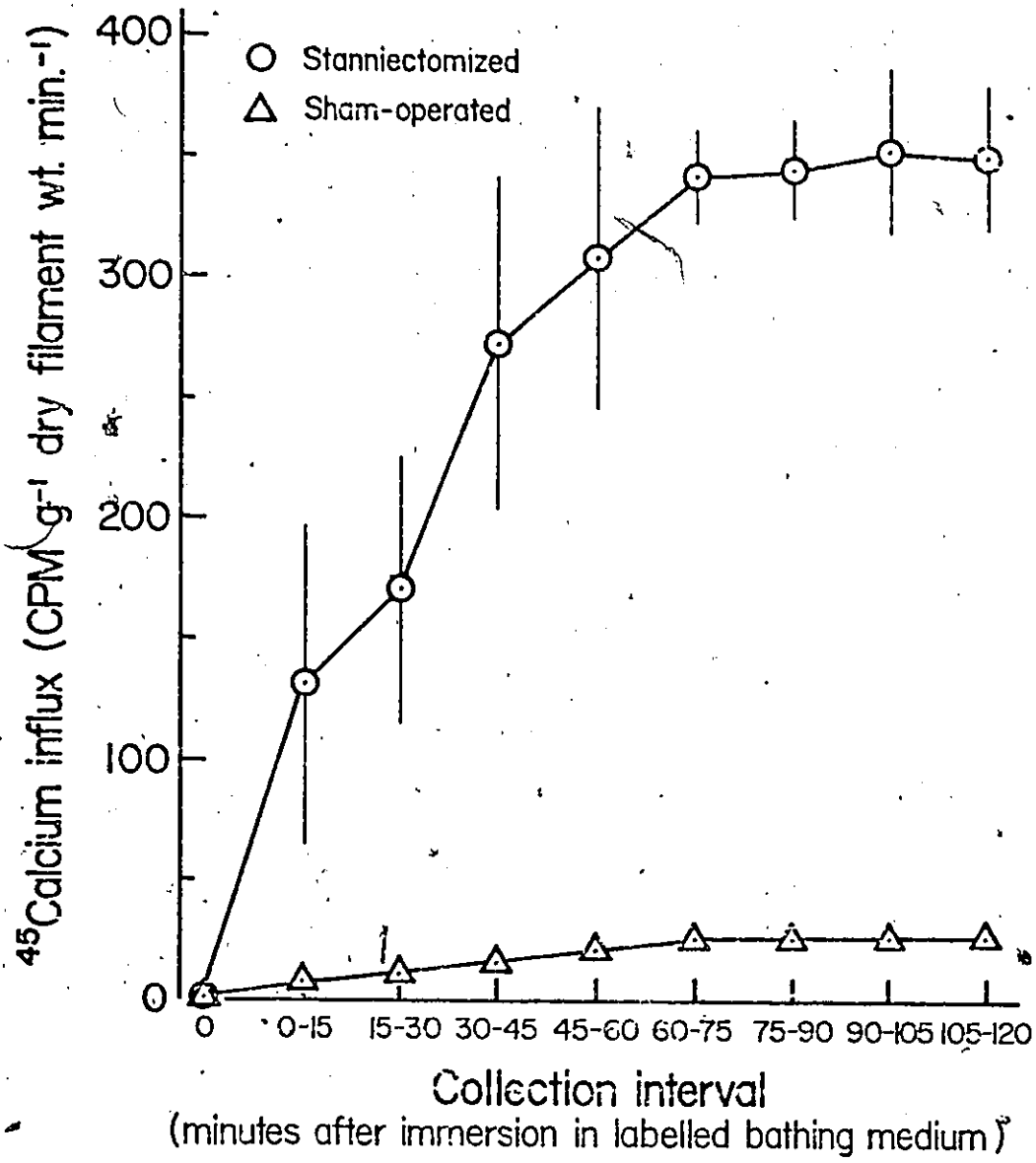
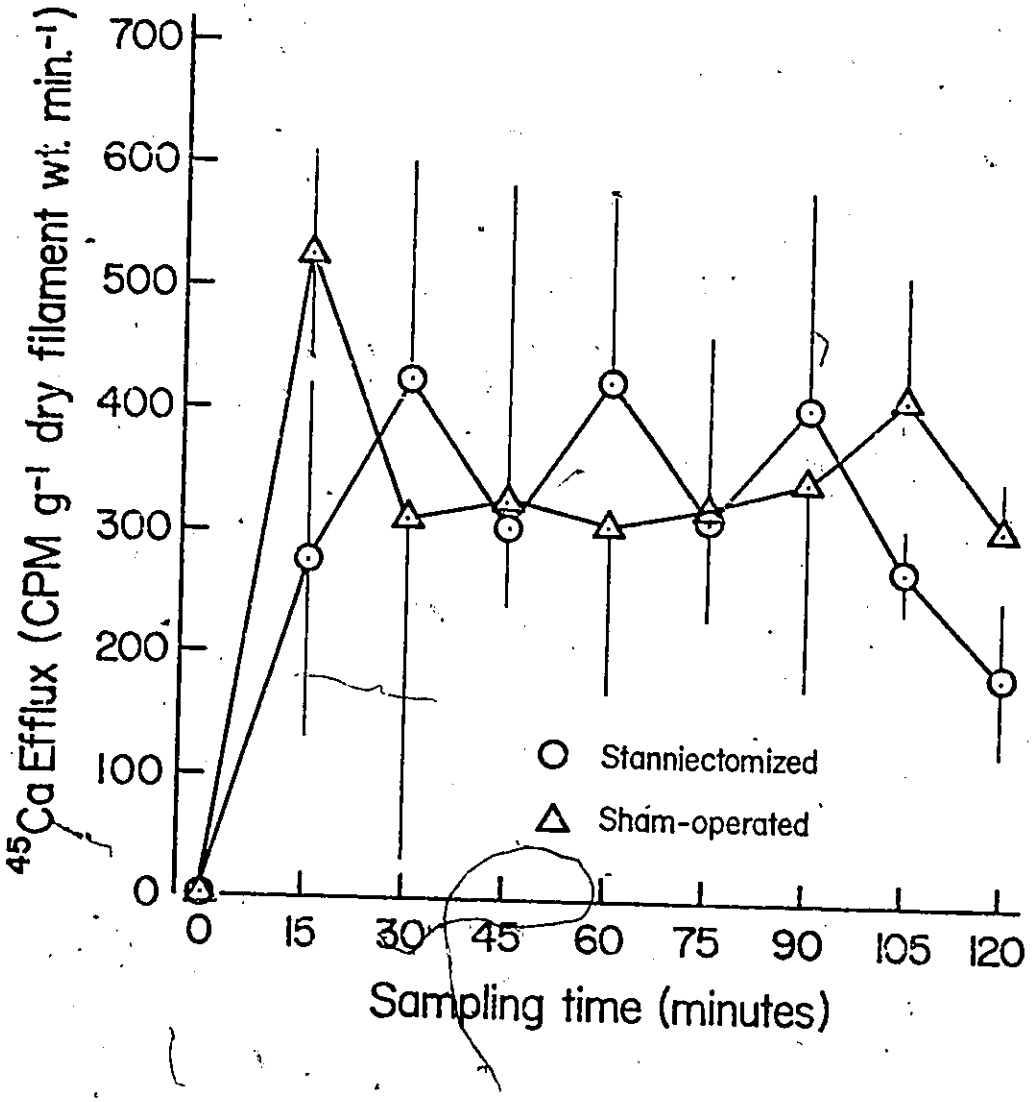

Stanniectomized
Sham-operated
^{45}Calcium influx (CPM g^{-1} dry filament wt. min.$^{-1}$)
400
300
200
100
0
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
105-120
Collection interval
(minutes after immersion in labelled bathing medium)

Figure 9

^{45}Calcium Efflux from individual perfused isolated gills from sham-operated and two week post-stanniectomized eels during the winter, measured as CPM g^{-1} dry filament wt min^{-1}.

Samples of bathing medium were collected at 15 minute intervals after starting to perfuse the gills with perfusion fluid prelabelled with ^{45}calcium (10^6 CPM 0.1 ml^{-1}).

Center of the symbols is at the mean, vertical lines represent the S.E.M. N = 4 in all cases.


700
600
500
400
300
200
100
0
45Ca Efflux (CPM g-1 dry filament wt. min.-1)
0
15
30
45
60
75
90
105
120
Sampling time (minutes)
Stanniectomized
Sham-operated

section. In other words, the gill from the stanniectomized and sham-operated groups both showed net calcium influx amounting to 0.185 ± 0.018 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1} and 0.012 ± 0.003 μmol Ca^{2+} g^{-1} dry gill filament wt hr^{-1} respectively.

5. Relationship between ^{45}Calcium Influx across the Perfused Isolated Eel Gill and the Development of Post-stanniectomy Hypercalcemia

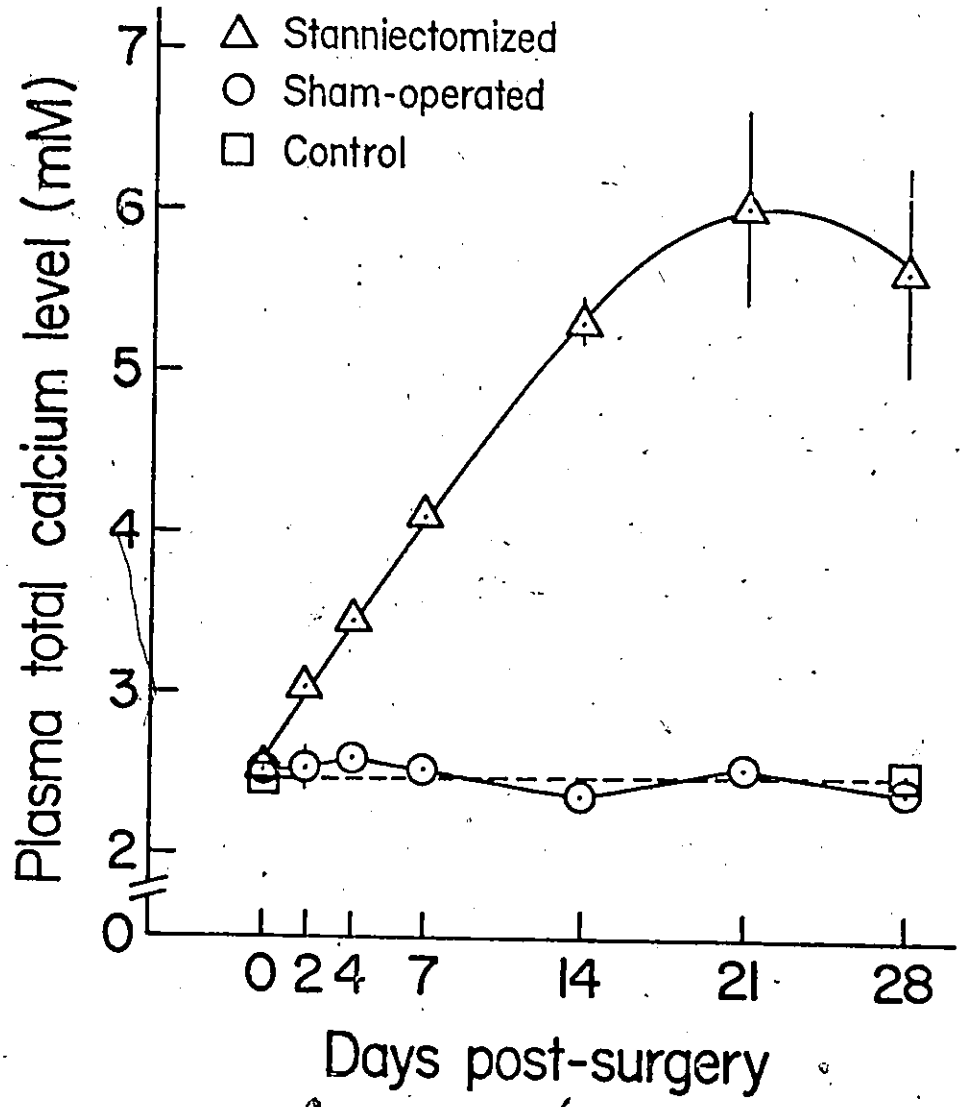
A. Development of Post-stanniectomy Hypercalcemia

The changes in plasma total calcium levels in tap water adapted eels during the first four weeks following the surgical removal of the corpuscles of Stannius are shown in Figure 10. These data show that the total plasma calcium level rose slightly during the first two days but was not significantly ($P > 0.05$) elevated until after 4 days had passed. The values started to peak after two weeks and showed a slight, though non-significant, decline during the fourth week. Plasma calcium levels did not change significantly ($P > 0.05$) in either the sham-operated or control groups.

Figure 10

The plasma total calcium levels in eels after various post-stanniectomy time periods.

Center of the symbols is at the mean, vertical lines represent S.E.M. and N = 7 in all cases.


Stanniectomized
Sham-operated
Control
Plasma total calcium level (mM)
7
6
5
4
3
2
0
0 2 4 7 14 21 28
Days post-surgery

B. ^{45}Calcium Influx across the Isolated Perfused Eel Gill at Different Post-stanniectomy Periods

Branchial ^{45}calcium influx into the gills from the stanniectomized eels after the different post-operation periods (Figure 10) is shown in Figure 11. During the first 15 minutes of perfusion there were no significant differences between ^{45}calcium influx rates into gills taken from the various groups. After 15 minutes, the ^{45}calcium influx rates from one week and two week stanniectomized eel gills were significantly higher than the control and sham-operated group. By the third and fourth weeks, the ^{45}calcium influx into the stanniectomized eel gills had returned to control levels.

In order to compare the ^{45}calcium influx rates by the gills from control, sham-operated and stanniectomized fish at various post-operative periods, the influx rates (expressed as μmol Ca^{2+} g^{-1} dry gill filament wt hr^{-1}) during the 15 minute interval between the 60th and 75th minutes of perfusion were taken and are summarized in Figure 12. Stabilized maximum influx rates occurred at this interval. The data indicate that after the operation, the rate of calcium influx increased slightly after two days and on the seventh post-operative day was significantly increased to approximately 3,000 CPM g^{-1} dry gill filament wt min^{-1} or 1.62 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}. Three weeks after stanniectomy, the branchial calcium uptake had re-

Figure 11

^{45}Calcium influx into individual perfused isolated gills from eels after various post-stanniectomy time periods, measured as CPM g^{-1} dry filament wt min^{-1}.

Influx rates were measured on the gills from a) control eels, b) sham-operated eels, c) 2 day post-stanniectomized eels, d) 4 day post-stanniectomized eels, e) one week post-stanniectomized eels, f) 2 week post-stanniectomized eels, g) 2 week post-stanniectomized eels, and h) 4 week post-stanniectomized eels.

Samples of perfusion fluid were collected for each 15 minute interval after immersion of the perfused gills in the bathing medium prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}).

Center of the symbols is at the mean, vertical lines represent the S.E.M. N = 7 in all cases.

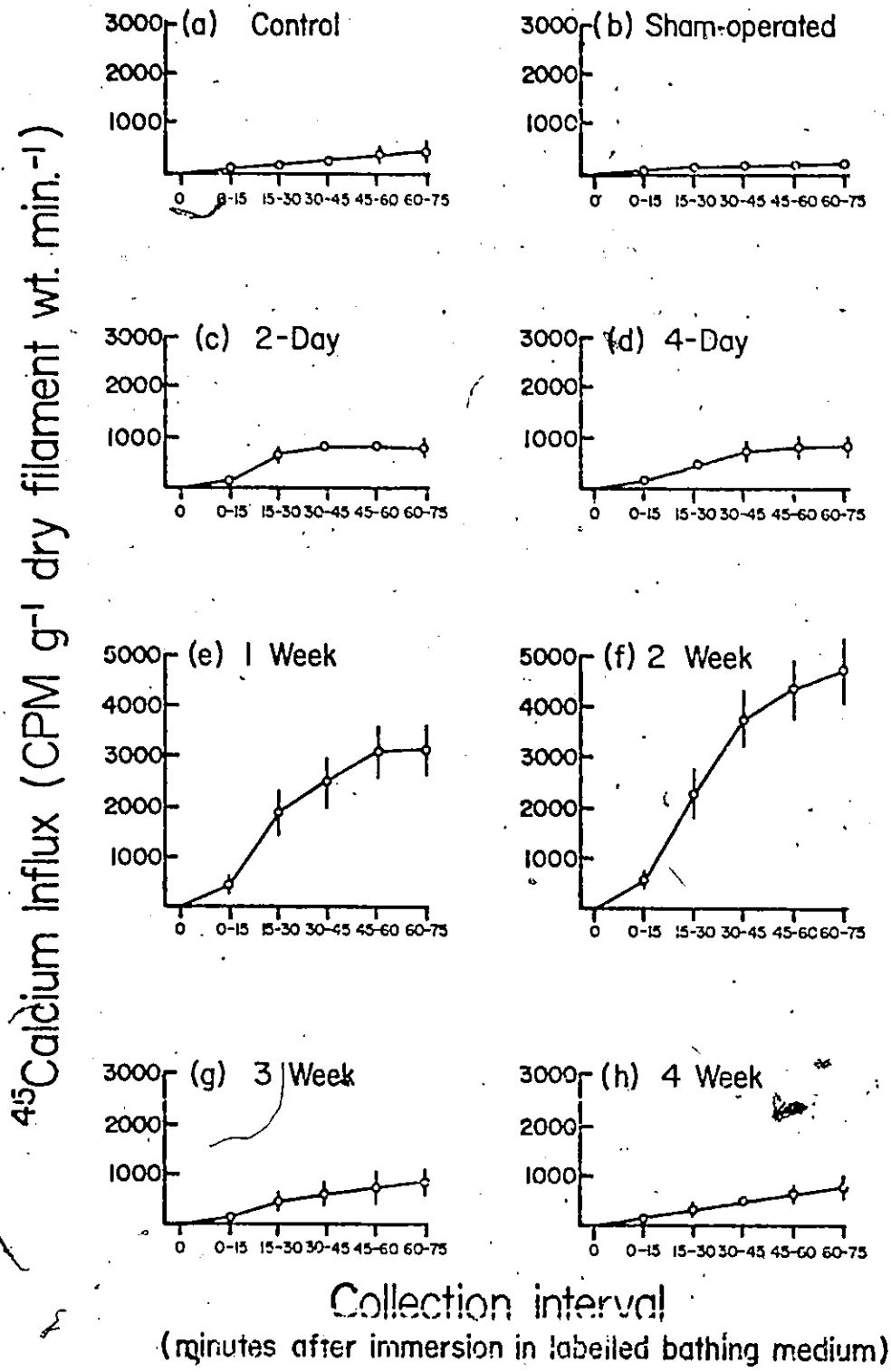
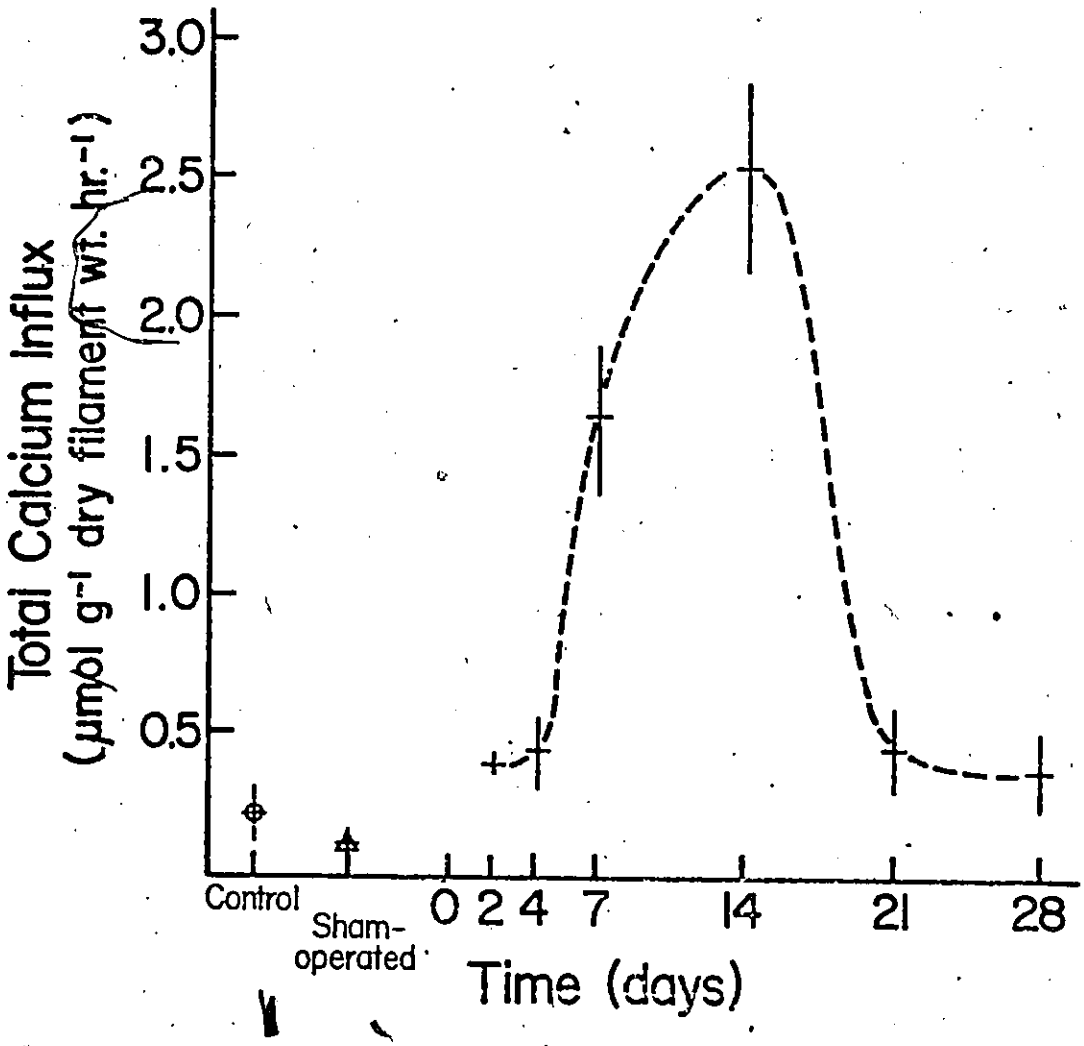

(a) Control
(b) Sham-operated
(c) 2-Day
(d) 4-Day
(e) 1 Week
(f) 2 Week
(g) 3 Week
(h) 4 Week
3000
2000
1000
5000
4000
0
0-15
15-30
30-45
45-60
60-75
45Calcium Influx (CPM g-1 dry filament wt. min.-1)
Collection interval
(minutes after immersion in labelled bathing medium)

Figure 12

Summary of the total calcium influx rates into individual perfused isolated gills from control and sham-operated eels and from eels killed at various time periods after stanniectomy.

The rates were expressed as μmol Ca^{2+} g^{-1} dry filament wt hr^{-1} and represent the rates at 60 to 75 minute interval of perfusion.

Horizontal lines are at the mean, vertical lines represent the S.E.M. N = 7 in all cases.


Total Calcium Influx
(μmol g⁻¹ dry filament wt. hr.⁻¹)
3.0
2.5
2.0
1.5
1.0
0.5
Control
Sham-operated
0 2 4 7 14 21 28
Time (days)

turned to a level similar to the rates in two day or four day post-stanniectomized fish, and remained near the control level after four weeks.

C. Residual ^{45}Calcium in Gill Filaments

The residual ^{45}calcium which remained in the gill filaments after perfusion of the gills from fish of various post-stanniectomized periods was measured (Table VI). This table shows that there were no significant difference in ^{45}calcium activity in the perfused gills at different intervals after stanniectomy. The fish which were stanniectomized for two weeks, however, appeared to have slightly greater amounts of ^{45}calcium in the gill filaments after the perfusion studies.

6. In Vivo Effects of Stannius Corpuscle Extracts on Plasma Total Calcium, Branchial ^{45}Calcium Influx into Gills from Stanniectomized Eels, and Residual ^{45}Calcium in Gill Filaments following Perfusion

A. Effect of Untreated and Previously Acidified Stannius Corpuscle Extract on Plasma Total Calcium

The changes in plasma calcium levels in two week post-stanniectomized eels treated on the final six days

Table VI

Residual ^{45}calcium in individual isolated perfused gill filaments from eels killed at various times following stanniectomy (Figure 12).[a]

GROUPS	Residual Calcium[b] ($\times 10^4$ CPM g^{-1} dry filament wt)
2 day post-stanniectomy	19.11 ± 2.26 (5)
4 day post-stanniectomy	17.43 ± 2.83 (6)
1 week post-stanniectomy	18.92 ± 1.89 (7)
2 week post-stanniectomy	22.20 ± 2.24 (7)
3 week post-stanniectomy	15.54 ± 2.08 (5)
4 week post-stanniectomy	14.82 ± 1.23 (7)

[a]The gills had been immersed in ^{45}calcium labelled bathing medium (5,000 CPM 0.1 ml^{-1}) and perfused for 75 minutes.

[b]Values are means ± S.E.M. (Number of animals)

with daily intraperitoneal injections of either perfusion fluid (Placebo), untreated corpuscular extract or previously acidified corpuscle extracts are shown in Figure 13. All these groups demonstrated post-stanniectomy hypercalcemia six days after the operation. In control injected fish the plasma calcium levels continued to increase and had about doubled by the end of the two week experimental period ($P < 0.05$). In the two extract injected groups the plasma calcium levels did not differ from their pre-operative levels nor from each other ($P > 0.10$) by the end of the extract injection period. This calcium lowering effect was seen on the first day of injection (Figure 13) just 3 to 5 hours after the first injection. It was also noticed that three consecutive daily injections of approximately one corpuscle per kg of fish per day were potent enough to bring the plasma calcium levels to their original normal values.

B. Branchial ^{45}Calcium Influx after Prolonged Intraperitoneal Injection of Corpuscle Extracts

The branchial ^{45}calcium influx from the preceeding groups of stanniectomized eels at the end of the experiment (i.e. 14 days post-operatively and after 6 daily intraperitoneal injections) are shown in Figure 14. The average ^{45}calcium influx (413 ± 71 CPM g^{-1} dry filament wt min^{-1}; equivalent to 0.223 ± 0.038 µmol Ca^{2+} g^{-1} dry fila-

Figure 13

The effect of intraperitoneal injection of Stannius corpuscle extracts on the plasma calcium levels.

Six daily injections of previously acidified Stannius corpuscle extract (⊙—⊙), unacidified Stannius corpuscle extract (△—△), and perfusion fluid alone (□—□) were started respectively in the morning of the 8th day after stanniectomy and the first plasma sample was taken in the afternoon of the 8th day. Extracts were given at the rate of 5 mg of extracted corpuscles kg^{-1} of eel day^{-1} contained in 0.5 ml of the perfusion fluid.

Centers of the symbols represent the means and the vertical lines indicate the S.E.M. N = 6 in all experiments.

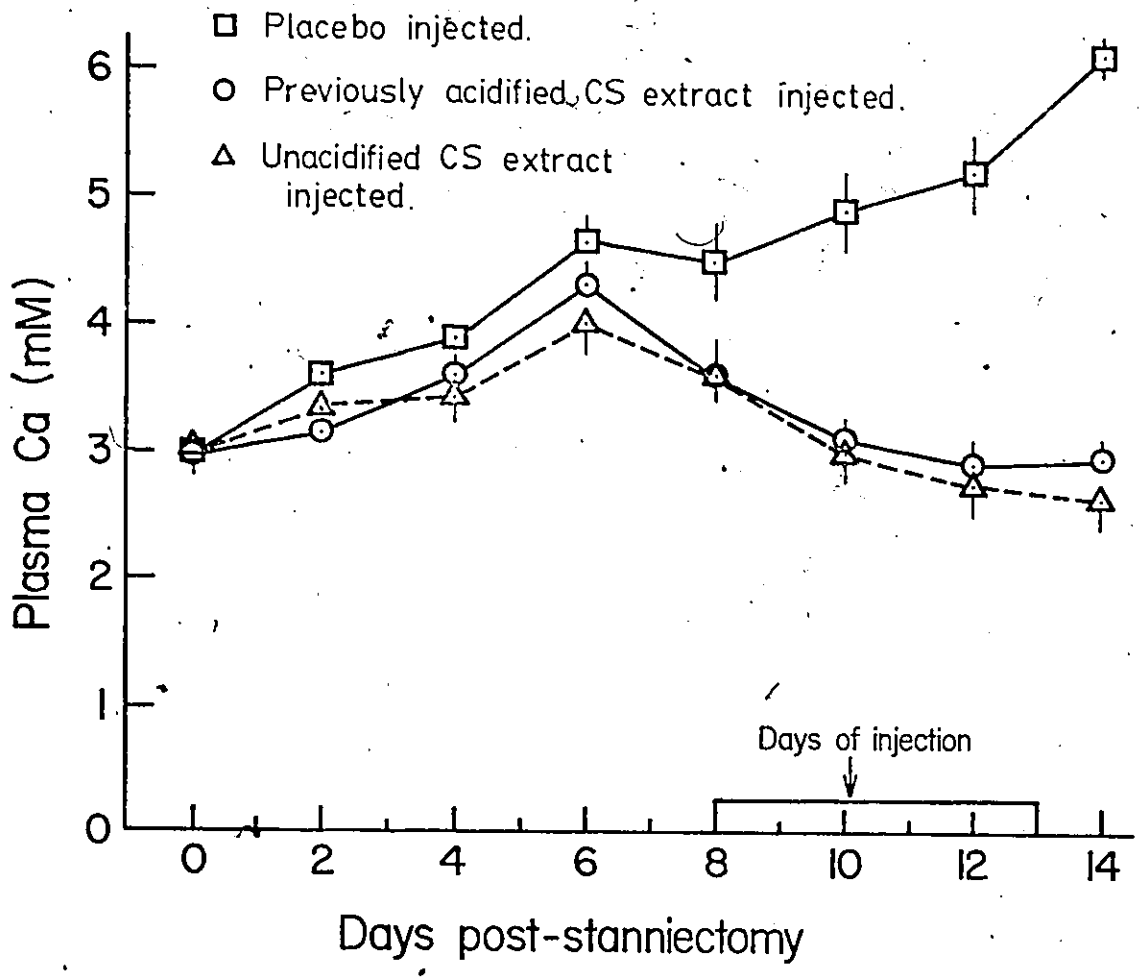

Placebo injected.
Previously acidified CS extract injected.
Unacidified CS extract injected.
Plasma Ca (mM)
0
1
2
3
4
5
6
Days of injection
0
2
4
6
8
10
12
14
Days post-stanniectomy

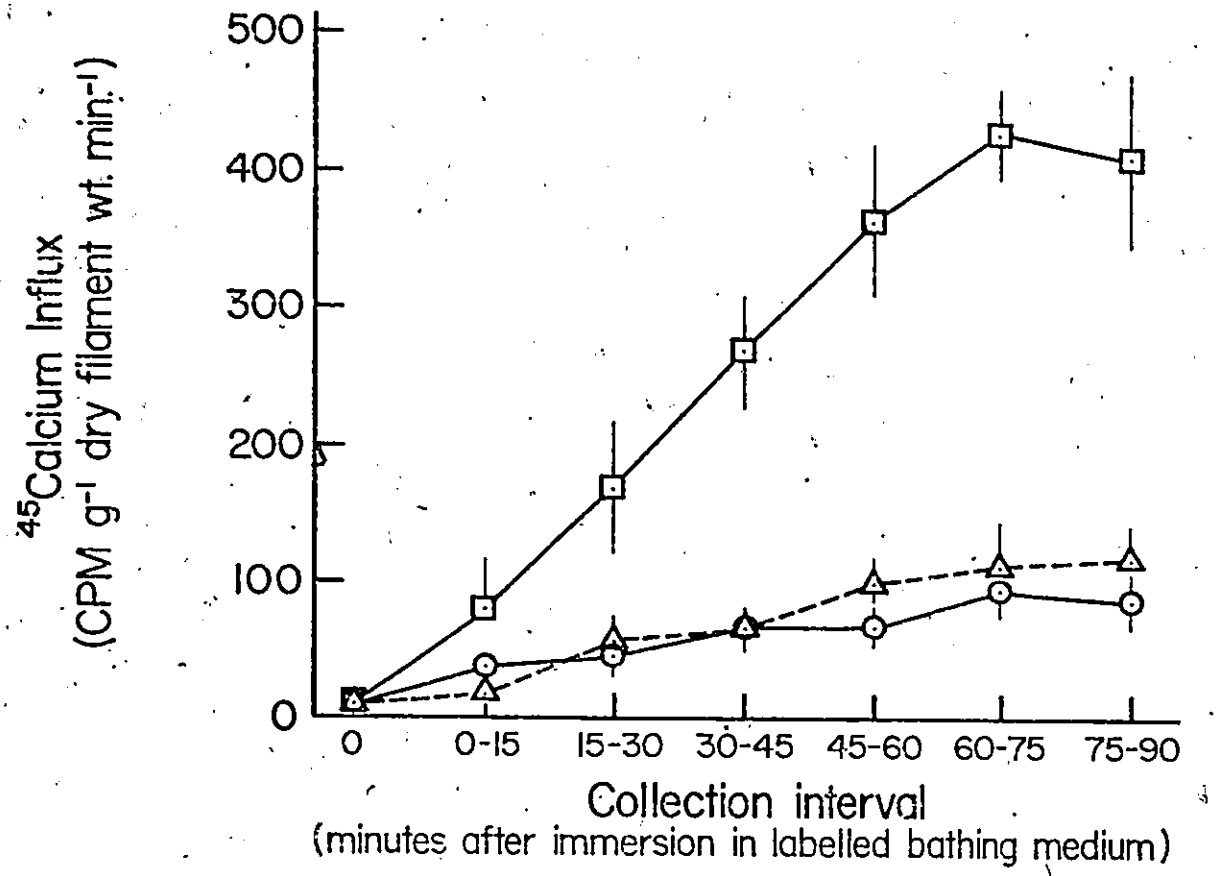
Figure 14

The effect of intraperitoneal injection of Stannius corpuscle extracts on the branchial ^{45}calcium influx.

The influx rates (expressed as CPM g^{-1} dry filament wt min^{-1}) were measured in the individual perfused isolated gills from two week stanniectomized eels which had received intraperitoneal injections of either previoulsy acidified (⊙—⊙), or unacidified (△—△) Stannius corpuscle extracts or perfusion fluid alone (□—□) on the last 6 days.

Samples of perfusion fluid were collected during 15 minute intervals after immersion of the perfused gills in bathing medium prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}).

Centers of the symbols are at the means; the vertical lines indicate the S.E.M. N = 6 in all experiments.


^{45}Calcium Influx
(CPM g^{-1} dry filament wt. min^{-1})
500
400
300
200
100
0
0
0-15
15-30
30-45
45-60
60-75
75-90
Collection interval
(minutes after immersion in labelled bathing medium)

ment wt hr^{-1}) into the isolated gills of the placebo injected stanniectomized fish between the 75th and 90th minutes of perfusion was 3 to 5 fold higher (significant at $P < 0.01$) than that in either of the extract injected stanniectomized groups (117 or 81 CPM g^{-1} dry filament wt min^{-1}; equivalent to 0.063 or 0.044 μmol Ca^{2+} g^{-1} dry gill filament hr^{-1})

C. Residual ^{45}Calcium in Gill Filaments

The residual ^{45}calcium in gill filaments from two week post-stanniectomized eels which had received six daily injection of either perfusion fluid, untreated or previously acidified Stannius corpuscle extracts is shown in Table VII. No statistically significant differences were seen ($P > 0.05$).

7. In Vitro Effect of Stannius Corpuscle Extracts, Kidney Extract and Liver Extract on Branchial ^{45}Calcium Influx

A. In Vitro Studies of Previously Acidified Extract of Stannius Corpuscles

The direct effect of previously acidified corpuscle extract on ^{45}calcium influx into perfused isolated gills from two week post-stanniectomized and sham-operated eels is shown in Figure 15. It is apparent from this

Table VII

Residual ^{45}Calcium in Isolated Perfused Gill Filaments[a] from two week Post-stanniectomized Eels which had received Daily Injection of Either Perfusion Fluid, Unacidified or Previously Acidified Stannius Corpuscle Extracts on the Last Six Days, measured as CPM g^{-1} dry gill filament wt.

Groups	Residual ^{45}Calcium[b] (x 10^4 CPM g^{-1} dry filament wt)
Perfusion Fluid Injection	5.83 ± 1.21 (3)
Previously Acidified Extract Injection	10.96 ± 4.59 (4)
Unacidified Extract Injection	12.11 ± 1.95 (5)

[a]The gills had been immersed in ^{45}calcium labelled bathing medium (5,000 CPM 0.1 ml^{-1}) and perfused for 90 minutes.

[b]Values represent means ± S.E.M. (N).

Figure 15

Effect of perfusion of previously acidified Stannius corpuscle extract on ^{45}calcium influx into individual perfused isolated gills from two week stanniectomized and sham-operated eels.

Samples of perfusion fluid were collected during 15 minute intervals after immersion of the perfused gills in a bathing medium prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}). After 45 minutes of perfusion, the isolated gills of sham-operated and one group of stanniectomized eels were perfused with fluid containing previously acidified Stannius corpuscle extract (2.5 mg ml^{-1}). Influx values are expressed as CPM g^{-1} dry filament wt min^{-1}.

Centers of the symbols represent the means, vertical lines are the S.E.M. and N = 6 in all experiments. S.E.M. are too small and therefore not shown for the sham-operated group.

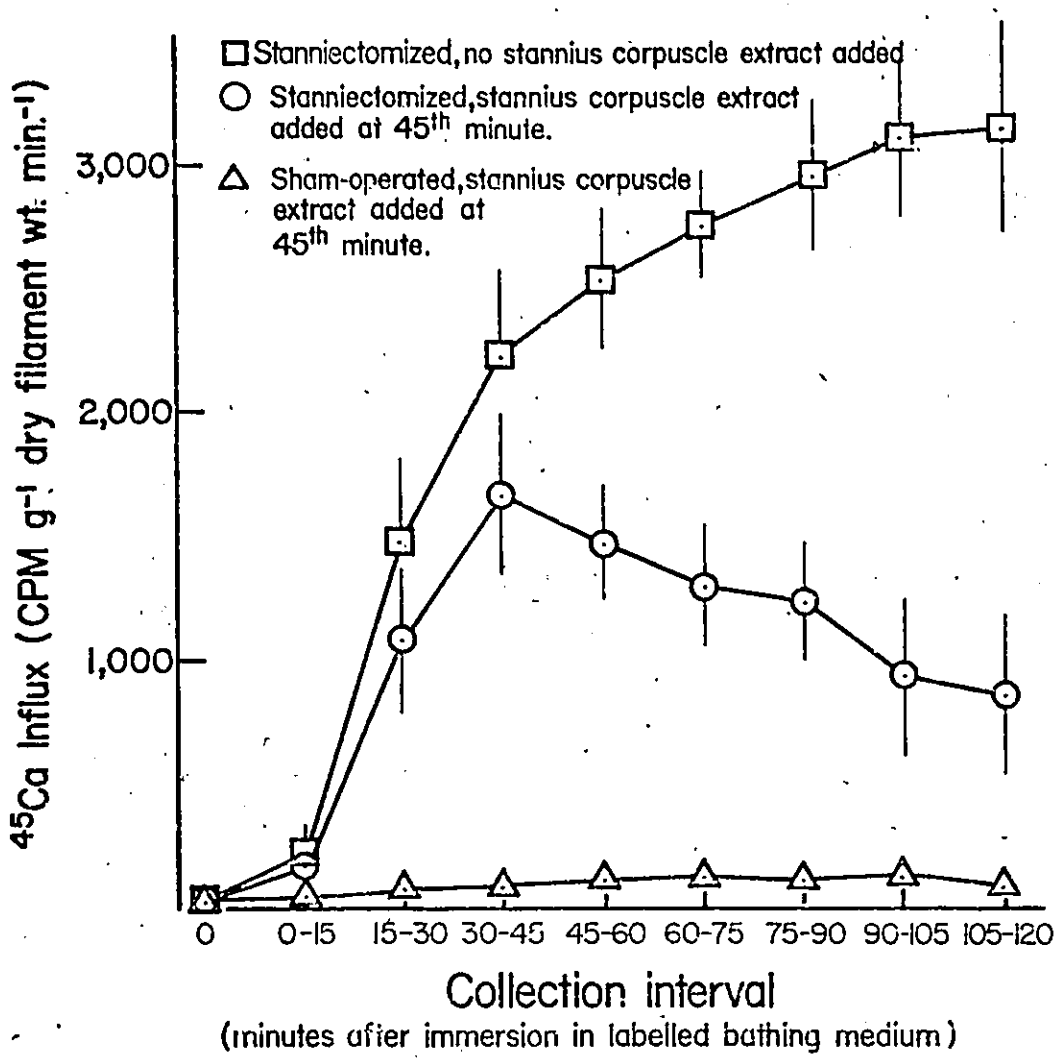

□ Stanniectomized, no stannius corpuscle extract added
○ Stanniectomized, stannius corpuscle extract added at 45th minute.
△ Sham-operated, stannius corpuscle extract added at 45th minute.
45Ca Influx (CPM g-1 dry filament wt. min.-1)
3,000
2,000
1,000
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
105-120
Collection interval
(minutes after immersion in labelled bathing medium)

figure that the addition of corpuscle extract to the perfusion system from the 45th minute of perfusion to the end of perfusion period brought about a significant reduction ($P < 0.01$) in ^{45}calcium influx into the gills of stanniectomized eels (869 CPM g^{-1} dry filament wt min^{-1}, equivalent to 0.469 µmol Ca^{2+} g^{-1} dry filament wt hr^{-1}) when compared to the gills perfused with perfusion fluid alone (3,156 CPM g^{-1} dry filament wt min^{-1}, equivalent to 1.704 µmol Ca^{2+} g^{-1} dry filament wt hr^{-1}). In other words, after perfusing the extract for 60 minutes, the Stannius corpuscle extract reduced the influx rate to 27.5% of that in control gills perfused with perfusion fluid alone. The effect of extract was apparent during the first 15 minutes after its addition.

Stannius corpuscle extracts which had not been previously acidified could not be used in the perfusion system as they consistently produced a precipitous drop in the effluent perfusion rate. As the affluent perfusion rate remained constant this could only have resulted from a drastic increase in vascular leakage. Previously acidified extracts produced no significant change in effluent perfusion rate.

B. In Vitro Studies of Previously Acidified Kidney and Liver Extracts on Branchial ^{45}Calcium Influx

To test for the specificity of the Stannius corpuscle extract the direct effect of previously acidified kidney and liver extracts on ^{45}calcium influx by gills from two week uninjected stanniectomized eels is shown in Figures 16 and 17 respectively. Extracts were added to the perfusion fluid at the 45th minute. In both cases, no significant difference was observed in ^{45}calcium influx between the control gills perfused with perfusion fluid alone and the control gills perfused with either kidney or liver extracts. The effluent perfusion rate was not altered during the extract perfusion.

8. Effects of Salmon Calcitonin administered in vivo on the Total Plasma Calcium and ^{45}Calcium Influx and Efflux across Perfused Isolated Gills from Stanniectomized Eels

A. Plasma Total Calcium

The effect of salmon calcitonin on the plasma calcium level of two week post-stanniectomized eel is shown in Table VIII. Plasma calcium levels in two week sham-operated and stanniectomized fish receiving control injections are also included. Both groups of stanniec-

Figure 16

Effect of perfusion of previously acidified kidney extract on ^{45}calcium influx into individual perfused isolated gills from two week stanniectomized eels.

Samples of perfusion fluid were collected during 15 minute intervals after immersing the perfused gills in bathing medium prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}). After 45 minutes of perfusion, the isolated gills from one group of post-stanniectomized eels were perfused with perfusion fluid containing previously acidified kidney extract (an equivalence of 2.5 mg tissue ml^{-1}). Influx values are expressed as CPM g^{-1} dry filament wt min^{-1}.

Centers of the symbols represent the means, vertical lines are the S.E.M. N = 3 and 5 in the control and extract perfused groups respectively.

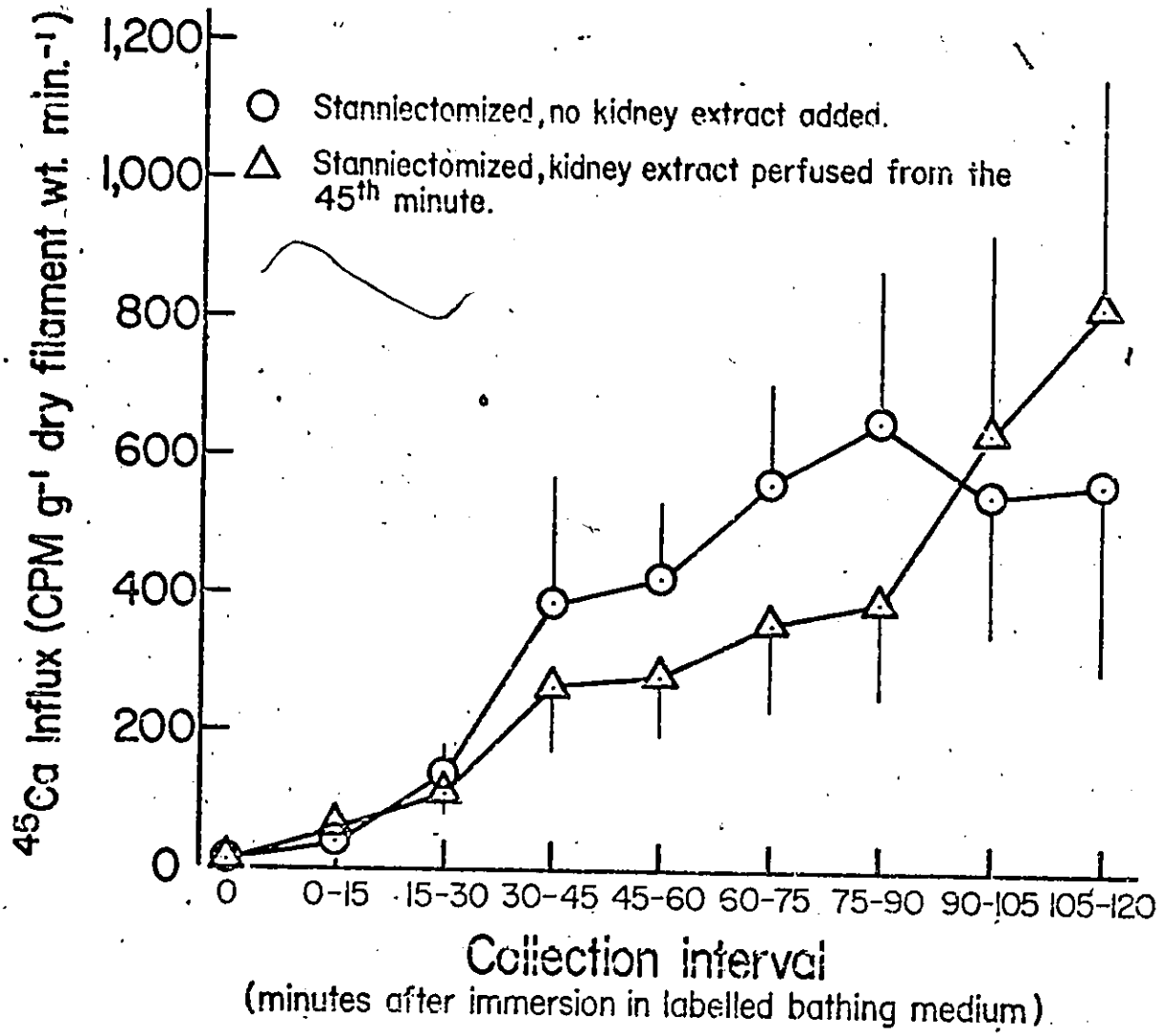

1,200
1,000
800
600
400
200
0
45Ca influx (CPM g-1 dry filament wt. min.-1)
Stanniectomized, no kidney extract added.
Stanniectomized, kidney extract perfused from the 45th minute.
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
105-120
Collection interval
(minutes after immersion in labelled bathing medium)

Figure 17

Effect of perfusion of previously acidified liver extract on ^{45}calcium influx into individual perfused isolated gills from two week stanniectomized eels.

Samples of perfusion fludi were collected during 15 minute intervals after immersing the perfused gills in bathing medium prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}). After 45 minutes of perfusion, the isolated gills from one group of post-stanniectomized eels were perfused with perfusion fluid containing previously acidified liver extract (an equivalence of 2.5 mg tissue ml^{-1}). Influx values are expressed as CPM g^{-1}dry filament wt min^{-1}.

Centers of the symbols represent the means, vertical lines are the S.E.M. N = 3 in both groups.

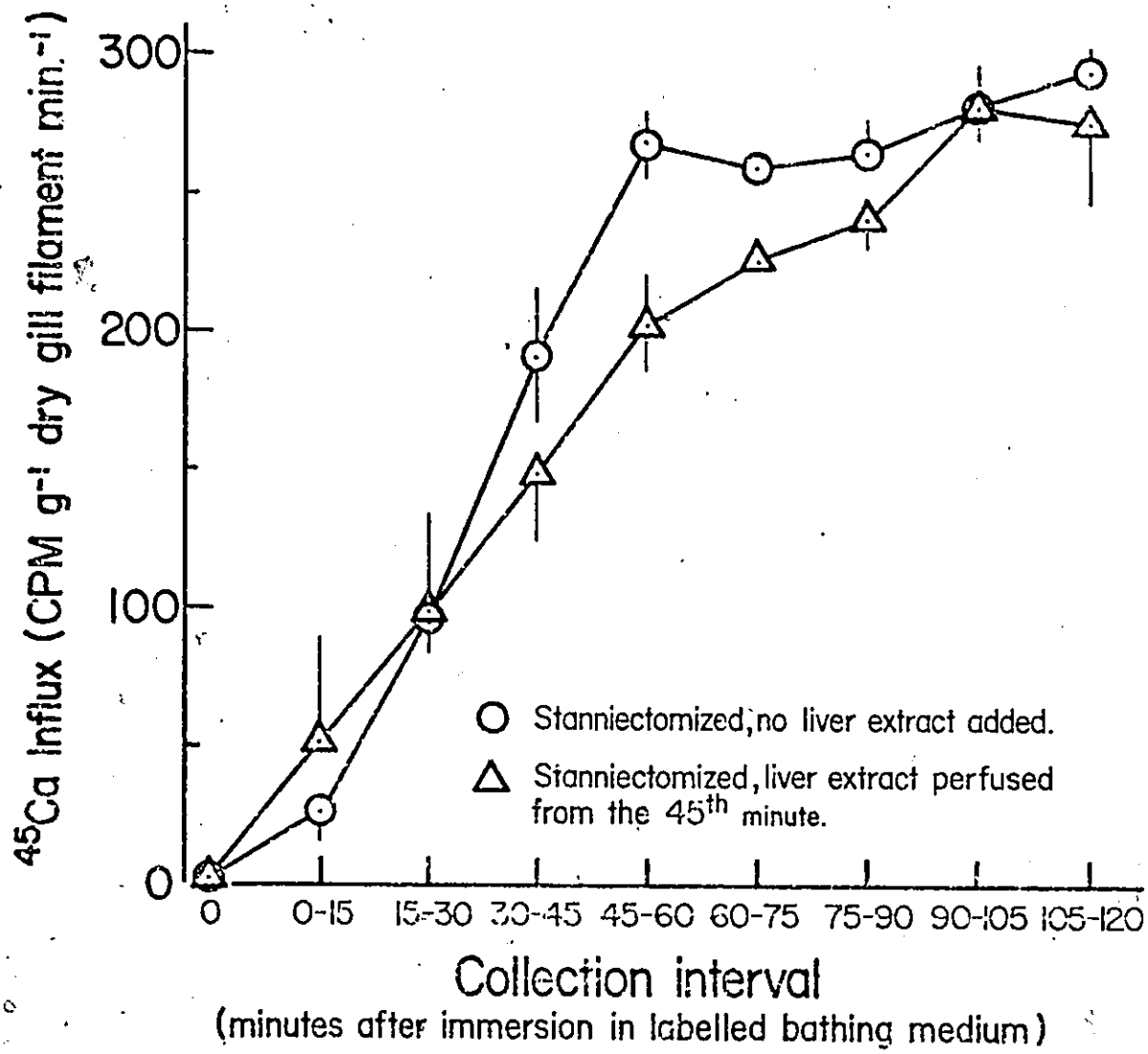

^{45}Ca influx (CPM g^{-1} dry gill filament min.$^{-1}$)
300
200
100
0
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
105-120
Collection interval
(minutes after immersion in labelled bathing medium)
Stanniectomized, no liver extract added.
Stanniectomized, liver extract perfused from the 45th minute.

Table VIII

Plasma Total Calcium Levels in Placebo injected Sham-operated, Placebo injected Stanniectomized, and Salmon Calcitonin injected Stanniectomized Eels [a]

GROUPS	Plasma Calcium Level (mM) [b]		
	0 day (before Operation)	8 days (before injection)	14 days (after injection)
Sham-operated with placebo injection	2.79 ± 0.13 (8)	2.90 ± 0.11 (8)	2.78 ± 0.18 (8)
Stanniectomized with placebo injection	2.94 ± 0.10 (6)	4.56 ± 0.24 (6)[c]	6.60 ± 0.32 (6)[ce]
Stanniectomized with salmon calcitonin injection	3.03 ± 0.18 (7)	5.21 ± 0.37 (7)[cd]	5.86 ± 0.27 (7)[cdf]

[a] Eels were injected daily on the last 6 days of the two week post-operative period with perfusion fluid or perfusion fluid containing salmon calcitonin (2 MRC units kg^{-1} day^{-1}).

[b] Values are means ± S.E.M. (Number of animals).

[c] Significantly different from sham-operated eels ($P < 0.01$) of the same period.

[d] Not significantly different from the placebo injected stanniectomized eels ($P > 0.05$).

[e] Significantly different from the same group on day 8 ($P < 0.01$).

[f] Not significantly different from the same group on day 8 ($P > 0.05$).

tomized fish demonstrated hypercalcemia on the 8th day ($P < 0.01$) and this trend towards hypercalcemia was not affected by the placebo injections. On the other hand the six daily injections of salmon calcitonin prevented any further significant ($P > 0.05$) increase in plasma calcium. The calcitonin injections, however, did not return the plasma total calcium levels to pre-operation or control levels.

B. Branchial Calcium Influx and Efflux

Branchial ^{45}calcium influx and efflux in two week sham-operated placebo injected eels, control injected stanniectomized eels and salmon calcitonin injected stanniectomized eels were measured and the equivalent calcium influx and efflux into the gills between the 105th and 120th minute of perfusion were calculated. The values are shown in Table IX.

Both groups of stanniectomized eels, control or salmon calcitonin injected, showed significantly higher calcium influx than the sham-operated fish ($P < 0.05$) but the injection of salmon calcitonin did not significantly ($P > 0.05$) affect the branchial calcium influx. Further, stanniectomy did not affect the branchial calcium efflux. However, the gills from stanniectomized fish which had received salmon calcitonin showed a significantly higher ($P < 0.05$) efflux rate than either of the other two groups.

From table IX it can be seen that the sham-operated fish had a very low net calcium efflux amounting

Table IX

Branchial Calcium Influx and Efflux in Control injected Sham-operated, Control injected Stanniectomized, and Salmon Calcitonin injected Stanniectomized Eels [a]

GROUPS	Calcium Fluxes during the 105th to 120th minute interval of perfusion, expressed as μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}	
	INFLUX [b]	EFFLUX [b]
Sham-operated with control injection	0.021 ± 0.004 (8)	0.024 ± 0.012 (8)
Stanniectomized with control injection	0.184 ± 0.035 (6)[c]	0.027 ± 0.021 (6)[dg]
Stanniectomized with salmon calcitonin injection	0.172 ± 0.039 (7)[ce]	0.124 ± 0.038 (7)[cf]

[a]Eels received daily injection of perfusion fluid or perfusion fluid contining salmon calcitonin (2 MRC units kg^{-1} day^{-1}) for the last 6 days of the two week post-operative period.

[b]Values are means ± S.E.M. (Number of animals).

[c]Significantly different from sham-operated eels ($P < 0.05$).

[d]Not significantly different from sham-operated eels ($P > 0.05$).

[e]Not significantly different from placebo injected stanniectomized eels ($P > 0.05$).

[f]Significantly different from placebo injected stanniectomized eels ($P > 0.05$).

[g]Significantly different from the equivalent influx group ($P < 0.05$).

to 0.003 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1} while the placebo injected stanniectomized eels had a net calcium influx of 0.157 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}. Due to the increased efflux rate subsequent to the injection of salmon calcitonin, the net calcium influx rate of the calcitonin injected stanniectomized fish was lower (0.048 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}) than in the control injected group.

C. Residual ^{45}Calcium in Gill Filaments

After two hours of perfusion the gill filaments used for influx and efflux studies from the above three groups of fish were digested to evaluate the presence of residual ^{45}calcium. These data are summarized in Table X.

In both stanniectomized groups which had been used for influx studies, the gill filaments contained more residual ^{45}calcium than the sham-operated fish ($P < 0.05$). Calcitonin did not significantly ($P > 0.05$) affect the amount of residual calcium. In the reverse experiment, that is when loading from the inside labelled perfusion fluid during efflux studies, all groups had the same amount of residual ^{45}calcium at the end of the perfusion period ($P > 0.05$).

Table X

Residual ^{45}calcium in perfused gill filaments[a] from control injected sham-operated, control injected stanniectomized and salmon calcitonin injected stanniectomized eels.[b]

GROUPS	Residual ^{45}Calcium [c]	
	Gills from Influx Studies (x 10^4 CPM g^{-1} dry filament wt)	Gills from Efflux Studies (x 10^6 CPM g^{-1} dry filament wt)
Sham-operated with control injection	3.51 ± 0.70 (5)	22.62 ± 3.34 (5)
Stanniectomized with control injection	10.71 ± 3.33 (5)[d]	22.56 ± 4.73 (5)[f]
Stanniectomized with salmon calcitonin injection	11.60 ± 0.62 (6)[de]	23.05 ± 2.77 (6)[f]

[a]The gills used for influx studies had been immersed in ^{45}calcium labelled bathing medium (5,000 CPM 0.1 ml^{-1}) and perfused for two hours; while the gills used for efflux studies had been perfused with ^{45}calcium labelled perfusion fluid (10^6 CPM 0.1 ml^{-1}) for two hours.

[b]Eels received daily injection of perfusion fluid or perfusion fluid containing salmon calcitonin (2 MRC units kg^{-1} day^{-1}) for the last 6 days of the two week post-operative period.

[c]Values are means ± S.E.M. (Number of animals).

[d]Significantly different from equivalent sham-operated group ($P < 0.05$).

[e]Insignificantly different from the placebo injected stanniectomized group ($P > 0.05$).

[f]Insignificantly different from equivalent sham-operated group ($P > 0.05$).

9. In Vitro Effect of Salmon Calcitonin on Branchial ^{45}Calcium Influx and Residual ^{45}Calcium in Gills from Stanniectomized Eels

A. Branchial ^{45}Calcium Influx

The direct in vitro effect of salmon calcitonin on the ^{45}calcium influx into the isolated perfused gills from two week post-stanniectomized eels is shown in Figure 18. The gill filaments that were perfused with salmon calcitonin from the 45th minute of perfusion did not show any significant change in ^{45}calcium influx rate ($P > 0.05$) when compared to the control gill filaments which were perfused with perfusion fluid alone. During the last perfusion period the control gills and calcitonin treated gills had calcium influx rates of 0.171 ± 0.029 µmol Ca^{2+} g^{-1} dry filament wt hr^{-1} and 0.188 ± 0.082 µmol Ca^{2+} g^{-1} dry filament wt hr^{-1} respectively.

B. Residual ^{45}Calcium in Gill Filaments

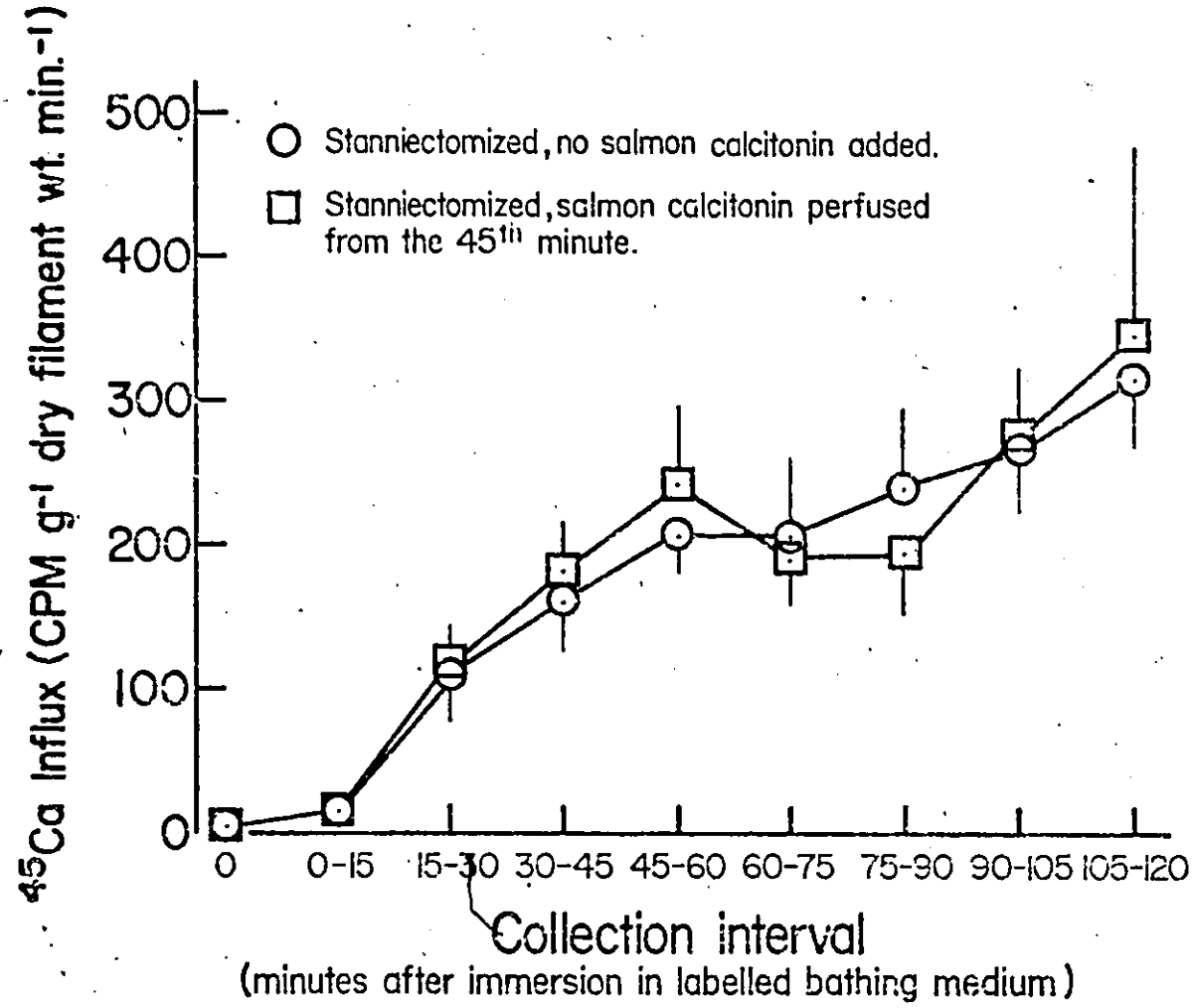
The isolated perfused gills from two week stanniectomized eels which were treated with either perfusion fluid or perfusion fluid containing salmon calcitonin (400 mMRC units ml^{-1}) possessed more or less similar amounts of residual ^{45}calcium. The calcitonin treated gills had $(6.56 \pm 1.22) \times 10^4$ CPM g^{-1} dry filament wt, a value which was not significantly different ($P > 0.05$) from the activi-

Figure 18

Effect of perfusion of salmon calcitonin on the ^{45}calcium influx into individual perfused isolated gills from two week stanniectomized eels.

Samples of effluent perfusion fluid were collected during 15 minute intervals after immersing the perfused gills in a bathing medium prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}). After 45 minutes of perfusion, one group of gills was perfused with perfusion fluid containing salmon calcitonin of 400 mMRC units ml^{-1}, while the other group continued to be perfused with perfusion fluid only. Influx values are expressed as CPM g^{-1} dry filament wt hr^{-1}.

Centers of the symbols represent the means, vertical lines are the S.E.M. and N = 7 in both cases.


45Ca influx (CPM g-1 dry filament wt. min.-1)
500
400
300
200
100
0
Stanniectomized, no salmon calcitonin added.
Stanniectomized, salmon calcitonin perfused from the 45th minute.
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
105-120
Collection interval
(minutes after immersion in labelled bathing medium)

ty in the gills perfused with perfusion fluid alone ({6.22 ± 11.5} x 10^4 CPM g^{-1} dry filament wt).

10. Effect of Calcium and Magnesium Ions on Branchial ^{45}Calcium Influx and Total Calcium Influx

A. Effect of High Concentration of Calcium in the Perfusion Fluid on Branchial ^{45}Calcium Influx

The influx rates of ^{45}calcium into gills from two week stanniectomized eels perfused with perfusion fluid containing either 5 mM calcium or 1.63 mM Ca^{2+} are shown in Figure 19. Although no significant differences ($P > 0.05$) were observed during any of the collection intervals there was a trend towards a higher ^{45}calcium influx in the gills perfused with the perfusion fluid of lower calcium concentration.

B. Effect of Seawater Bathing Medium on Branchial ^{45}Calcium Influx

Figure 20 compares the ^{45}calcium influx across isolated perfused gills placed in reconstituted 100% seawater bathing medium and normal freshwater bathing medium prelabelled with ^{45}calcium. The uptake rate of ^{45}calcium in gills submerged in seawater bathing medium seemed to be

Figure 19

Effect of high calcium concentration in the perfusion fluid on ^{45}calcium influx into isolated perfused gills from two week stanniectomized eels.

One group of gill filaments was perfused with perfusion fluid containing 5 mM Ca^{2+} while the other group was treated with perfusion fluid of 1.63 mM Ca^{2+} for the whole period of perfusion. Samples of effluent perfusion fluid were collected during 15 minute intervals after immersing perfused gills in different bathing media prelabelled with ^{45}calcium (5,000 CPM ml^{-1}). Influx values are expressed as CPM g^{-1} dry filament wt min^{-1}.

Centers of the symbols represent the means, vertical lines are the S.E.M. and N = 3 in both cases.

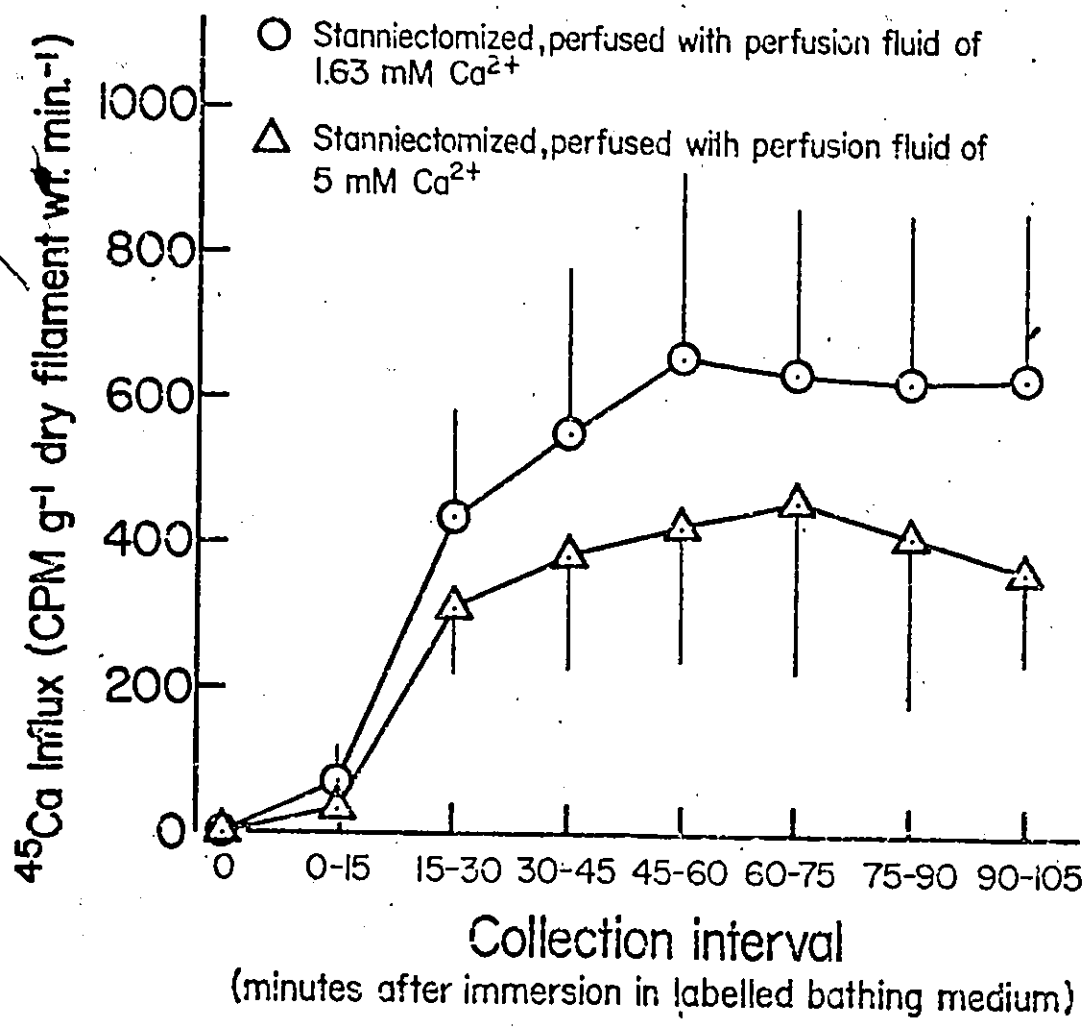
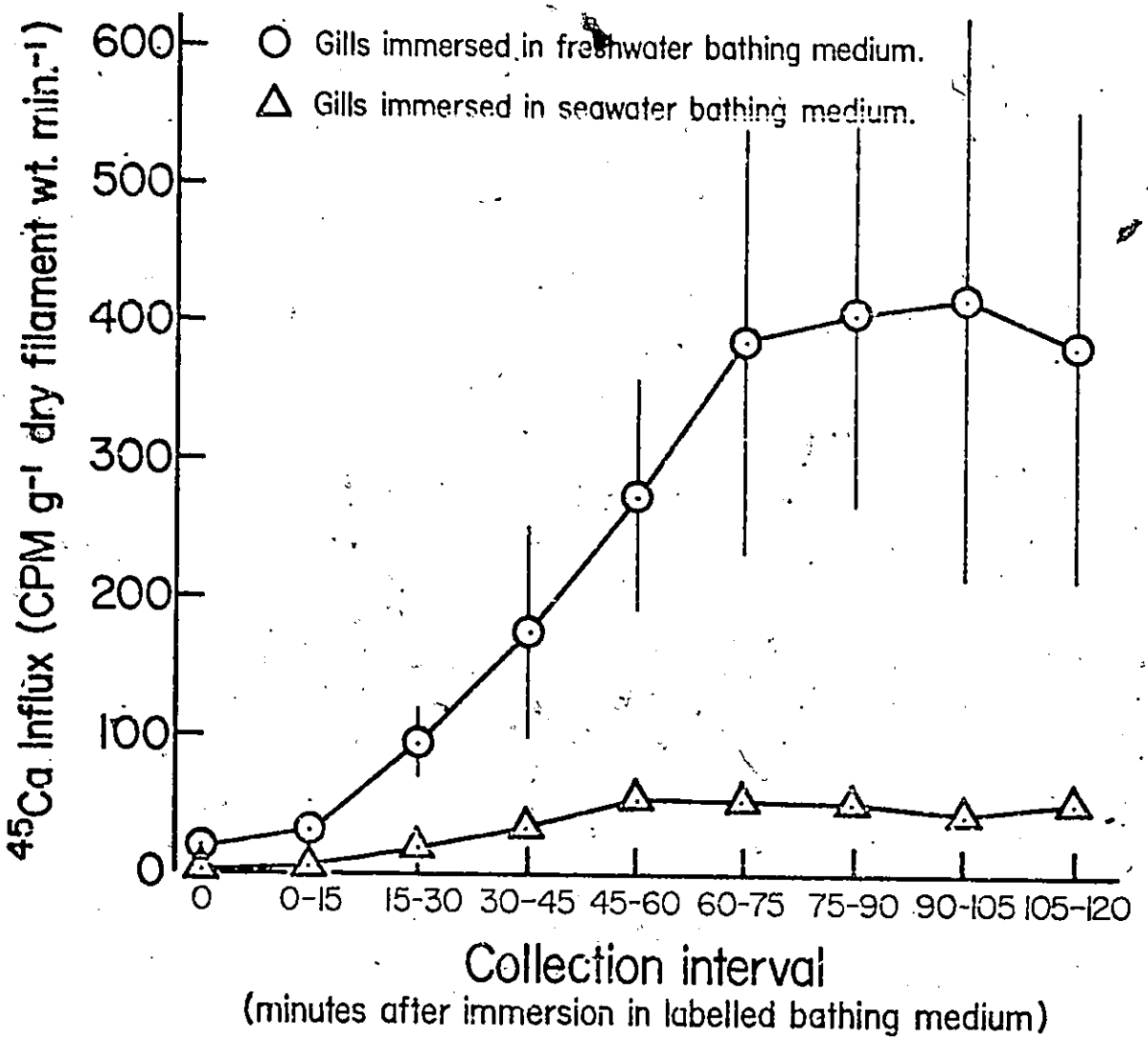

O Stanniectomized, perfused with perfusion fluid of 1.63 mM Ca^{2+}
Δ Stanniectomized, perfused with perfusion fluid of 5 mM Ca^{2+}
^{45}Ca influx (CPM g^{-1} dry filament wt. min^{-1})
1000
800
600
400
200
0
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
Collection interval
(minutes after immersion in labelled bathing medium)

Figure 20

Effect of seawater bathing medium on ^{45}calcium influx into isolated perfused gills from two week stanniectomized eels.

One group of gills was immersed in 100% reconstituted seawater bathing medium while the other group was placed in freshwater bathing medium during the whole period of perfusion. Samples of effluent perfusion fluid were collected during 15 minute intervals after immersing perfused gills in different bathing media prelabelled with ^{45}calcium (5,000 CPM ml^{-1}). Influx values are expressed as CPM g^{-1} dry filament wt min^{-1}.

Centers of the symbols represent the means, vertical lines are the S.E.M. and N = 4 in both cases.


600
500
400
300
200
100
0
45Ca Influx (CPM g-1 dry filament wt. min.-1)
Gills immersed in freshwater bathing medium.
Gills immersed in seawater bathing medium.
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
105-120
Collection interval
(minutes after immersion in labelled bathing medium)

low throughout the whole period of perfusion when compared to the control. However the seawater bathing medium had a high concentration of calcium ions (10.4 mM) resulting in a lower specific activity of ^{45}calcium in this bathing medium. Therefore by evaluating from the ^{45}calcium influx rate and its specific activity, during the period between the 105th and 120th minute of perfusion, the gills in seawater bathing medium attained a higher ($P < 0.05$) influx rate (0.619 ± 0.048 µmol Ca^{2+} g^{-1} dry filament wt hr^{-1}) than the gills submerged in freshwater bathing medium (0.206 ± 0.104 µmol Ca^{2+} g^{-1} dry filament wt hr^{-1}). The perfusion rates were not different in different media.

C. Effect of Seawater Concentration of Ca^{2+} and Mg^{2+} in Bathing Medium on Branchial ^{45}Calcium Influx

^{45}Calcium influx rate over two hours of perfusion into gills taken from stanniectomized eels and immersed in labelled bathing medium with calcium concentrations of either 10 mM or 0.45 mM are compared in Figure 21.

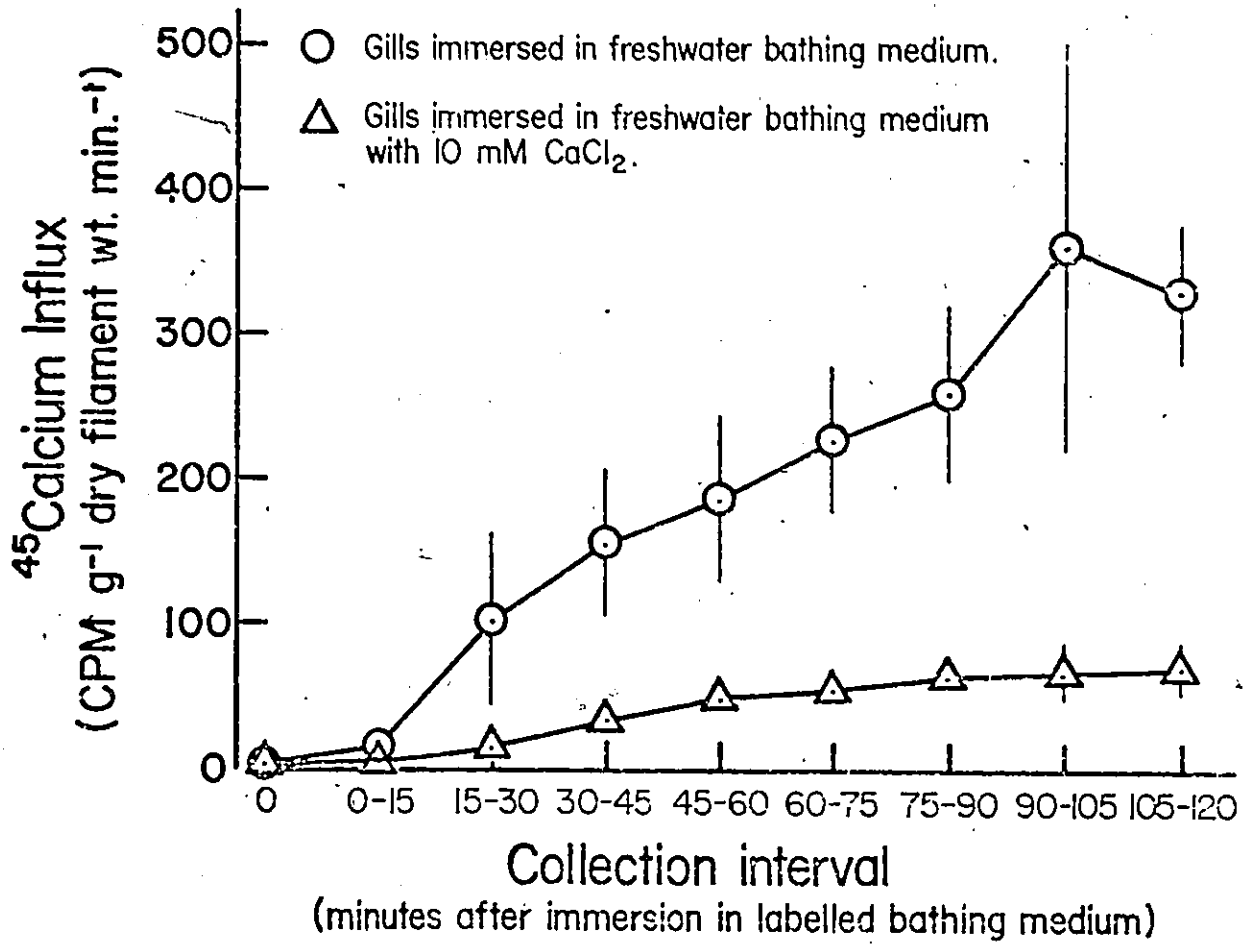
^{45}Calcium influx appeared to be low in the gills held in 10 mM Ca^{2+} bathing medium, and was below 69 ± 19 CPM g^{-1} dry filament wt min^{-1} at the end of the experiment as compared to that in normal freshwater bathing medium (329 ± 51 CPM g^{-1} dry filament wt min^{-1}). As the specific activity of ^{45}calcium in 10 mM Ca^{2+} bathing medium is 22.22 times lower than that in normal freshwater bathing medium,

Figure 21

Effect of 10 mM Ca^{2+} in freshwater bathing medium on ^{45}calcium influx into isolated perfused gills from two week stanniectomized eels.

One group of gills was immersed in freshwater bathing medium containing 10 mM calcium chloride (36 mOsm) while the other group was placed in freshwater bathing medium (0.45 mM Ca^{2+}) adjusted to the same osmolality with mannitol. Samples of effluent perfusion fluid were collected during 15 minute intervals after the gills were immersed in different bathing media prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}). Influx values are expressed as CPM g^{-1} dry filament wt min^{-1}.

Centers of symbols represent the means, vertical lines are the S.E.M. and N = 3 in both cases.


Gills immersed in freshwater bathing medium.
Gills immersed in freshwater bathing medium with 10 mM $CaCl_2$.
^{45}Calcium Influx
(CPM g^{-1} dry filament wt. min.$^{-1}$)
500
400
300
200
100
0
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
105-120
Collection interval
(minutes after immersion in labelled bathing medium)

the gills immersed in high Ca^{2+} bathing medium had a total calcium influx of 0.832 ± 0.230 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1} while it was 0.178 ± 0.028 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1} in the control medium. The perfusion rates were not significantly different.

Different results were obtained with 52 mM Mg^{2+} in the bathing medium (Figure 22). The gills immersed in bathing medium with high magnesium concentration (52 mM) had a significantly ($P < 0.05$) lower ^{45}calcium influx (174 ± 36 CPM g^{-1} dry filament wt min^{-1}, or 0.094 ± 0.019 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}) than the gills maintained in magnesium-free bathing medium (604 ± 237 CPM g^{-1} dry filament wt min^{-1} or 0.326 ± 0.128 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}). The perfusion rates were not significantly different.

D. Comparison of the Residual ^{45}Calcium present in Different Gill Filaments from the above Experiments in Different Bathing Media

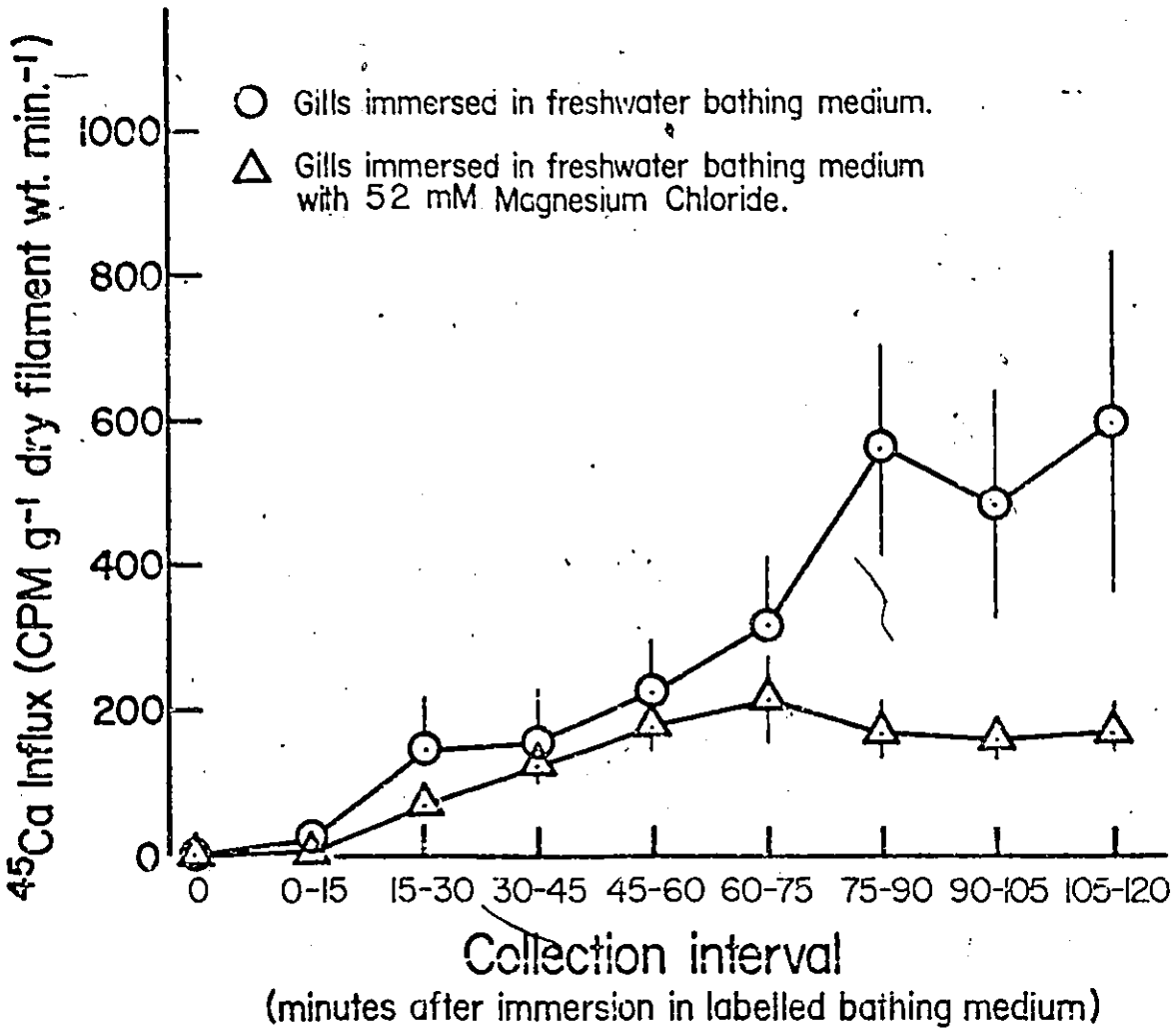
The residual ^{45}calcium present in the gill filaments taken from the above experiments was evaluated to see if the external ions affected the residual ^{45}calcium content. For this purpose, the ratios of the residual ^{45}calcium in gill filaments immersed in the different bathing media to that in the gill filaments immersed in the standard freshwater bathing medium were calculated and

Figure 22

Effect of 52 mM Mg^{2+} in freshwater bathing medium on ^{45}calcium influx into isolated perfused gills from two week stanniectomized eels.

One group of gill filaments were immersed in freshwater bathing medium containing 52 mM magnesium chloride (140 mOsm), while the other group was placed in freshwater bathing medium adjusted to the same osmolality with mannitol. Samples of effluent perfusion fluid were collected during 15 minute intervals after the gills were immersed in different bathing media prelabelled with ^{45}calcium (5,000 CPM 0.1 ml^{-1}). Influx values are expressed as CPM g^{-1} dry filament wt min^{-1}.

Centers of symbol represent the means, vertical lines are the S.E.M. and N = 5 in both cases.


○ Gills immersed in freshwater bathing medium.
△ Gills immersed in freshwater bathing medium with 52 mM Magnesium Chloride.
^{45}Ca influx (CPM g^{-1} dry filament wt. min.$^{-1}$)
1000
800
600
400
200
0
0
0-15
15-30
30-45
45-60
60-75
75-90
90-105
105-120
Collection interval
(minutes after immersion in labelled bathing medium)

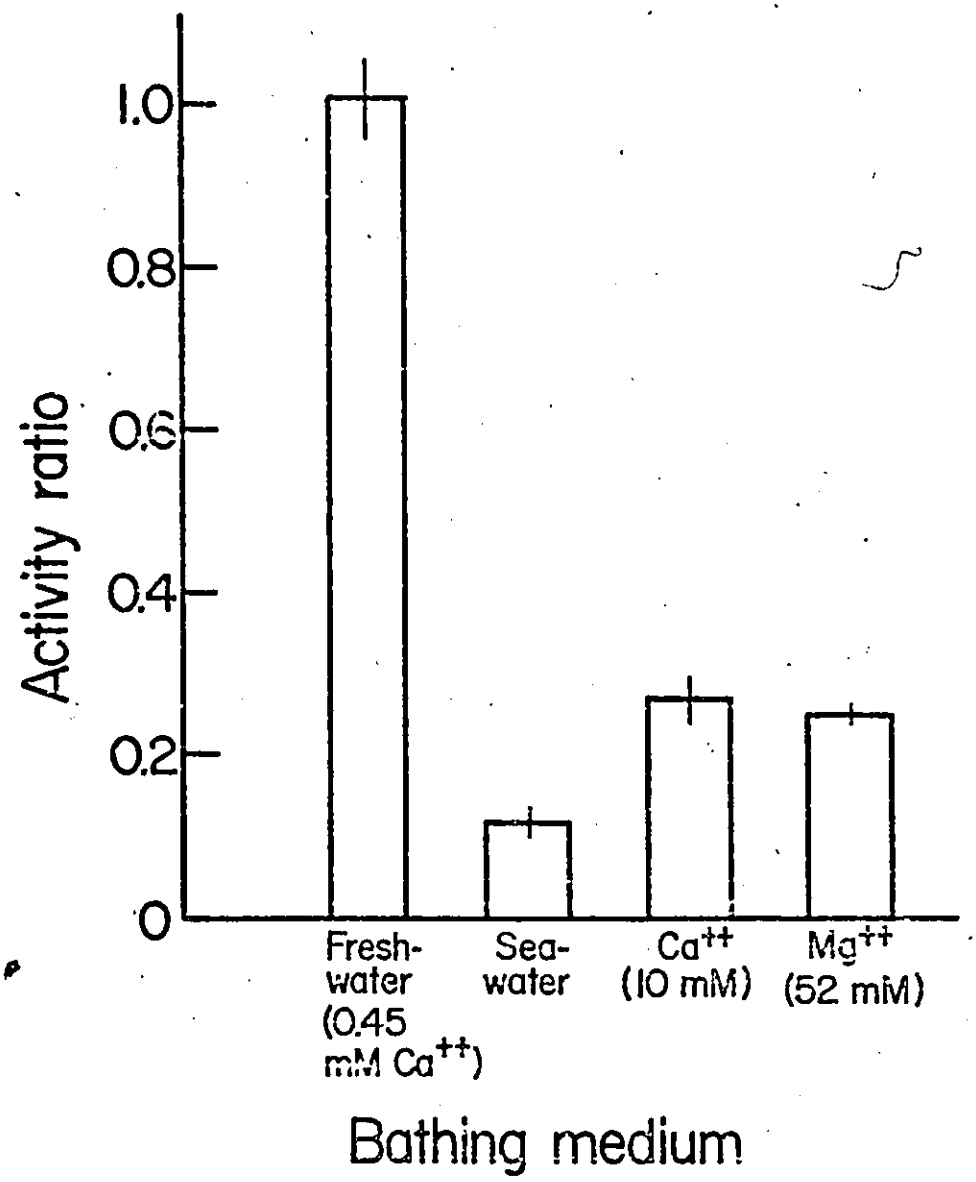
are shown in Figure 23. All the gills which were perfused in bathing media with 10 mM Ca^{2+} or 52 mM Mg^{2+} or both (seawater bathing medium) had a significantly lower residual ^{45}calcium ratio ($P < 0.01$) than the control gills. The presence of high calcium and magnesium ions in the bathing medium decreased the ratio to 25% of the control value.

Figure 23

Ratio of residual ^{45}calcium in gill filaments in different bathing media to the residual ^{45}calcium in gills bathed in the standard freshwater bathing medium.

The isolated gills from two week stanniectomized eels had been immersed in prelabelled bathing media (5,000 CPM 0.1 ml^{-1}) of either seawater (10.4 mM Ca^{2+} + 52 mM Mg^{2+}), 10 mM Ca^{2+} or 52 mM Mg^{2+}.

Values in histogram represent means ± S.E.M. and N = 3 in all cases.


Activity ratio
1.0
0.8
0.6
0.4
0.2
0
Fresh-water (0.45 mM Ca++)
Sea-water
Ca++ (10 mM)
Mg++ (52 mM)
Bathing medium

DISCUSSION

The primary objective of this thesis was to study the role of the gills in blood calcium homeostasis in the American eel, Anguilla rostrata (LeSueur), with special reference to the role played by the corpuscles of Stannius in the control of calcium movement across the branchial epithelium.

The data obtained, and reported in this thesis, confirmed earlier observations on the hypocalcemic action of Stannius corpuscles (Fontaine, 1967; Chester Jones et al., 1967; Chan, 1972; Fenwick & Forster, 1972; Pang et al., 1973). Further, and for the first time, the present data describe and perhaps even establish a cause-effect relationship between the surgical removal of the Stannius corpuscles and an increase in branchial calcium influx. Stanniectomy was consistently followed by an increase in plasma total calcium and a concomitant increase in branchial calcium influx into isolated perfused gills and both of these parameters could be normalized by replacement therapy with extract of Stannius corpuscles.

The branchial apparatus of the fish, the tool

utilized for the present studies, is anatomically composed of two main structures: the supportive gill archs and the primary and secondary lamellae which function as the sites for ionic and gaseous exchange (Hughes and Morgan, 1973). The secondary lamellae are lined by a single layer of flattened respiratory epithelial cells, while the primary lamellae are covered by multilayered epithelial cells together with the mucous cells and chloride cells. The chloride cells are abundantly localized in the interlamellar region, particularly at the base of the secondary lamellae. These cells cover and appear to be functionally connected to the central venous compartment (Girard and Payan, 1980). Chloride cells are also located on both edges of the primary lamella close to the afferent and efferent primary arteries ('afferent and efferent filamental arteries') but the cells are more numerous on the afferent side (Jozuka, 1966; Maetz, 1971). Physiologically, the afferent blood is pumped from the heart and through the ventral aorta to flow bilatero-anteriorly into the afferent branchial arteries serving the eight gill archs. After passing through the gill filaments, most of the blood leaves the gills by the same number of efferent branchial arteries which unite to form the dorsal aorta. Part of the efferent blood, however, enters the branchial venous system and passes through the branchial vein in the gill arch into the cardinal vein. These separate blood pathways have recently been described (Laurent and Dunel, 1976; Vogel et

al., 1976; Girard and Payan, 1980). The first is the arterio-arterial pathway where gaseous and passive ionic exchange may take place (Kerstetter et al., 1970; Wood and Randall, 1973a, 1973b). The second pathway is an arterio-venous pathway arising from the efferent primary arteries and in addition in the eel, from the afferent primary arteries (Steen and Kruysse, 1964; Laurent and Dunel, 1976). They are connected to the central venous sinuses of the primary lamella by arterio-venous anastomoses thereby permitting part of the blood flowing from the primary arteries to drain into the central venous sinus which then runs into the branchial vein through small veins. It was originally believed that the central sinus route was responsible for supplying nutrition to gill filaments, providing additional stiffness to the primary lamellae, diverting part of the oxygenated blood directly to the heart and acting as a reservoir for oxygenated blood (Laurent and Dunel, 1976). But because of the position of the chloride cells around the filamental sinus with their bases bathing in the extracellular space drained by the venous sinus, it has been suggested that this system functions as an important second pathway involved in active sodium and chloride transport (Laurent and Dunel, 1978; Girard and Payan, 1980).

In the isolated gill perfusion technique employed in the present studies as well as that performed earlier by Rankin and Maetz (1971), Shuttleworth (1972) and later by Ma (1976), the afferent and efferent branchial arteries

from the gill archs were cannulated for fluid perfusion. According to the previously mentioned nature of the intra-branchial circulation in gill filaments, it appears that by these techniques only the ionic transport through the secondary lamellae, that is, into the arterio-arterial pathway, were measured. In the present gill perfusion studies both ends of the gill arch were tied off completely and no leakage was seen when checked by comparing the affluent and effluent perfusion rates or when tested with dye perfusion. Leakage did not, therefore, occur through the arterio-venous pathway. Indeed, it is possible that the arterio-venous pathway was blocked by the cannulation technique. As it is not unreasonable to assume that the chloride cells along the arterio-venous pathway are also the sites for calcium transport, the ligation of this pathway may have rendered the central venous sinus non-functional so far as calcium transport was concerned. If this was true, the branchial calcium influx rates reported in the present or other studies may possibly be underestimated. On the other hand, the validity of the present gill perfusion technique can be supported indirectly by the following observations.

Influx studies on the relative permeability of the primary and secondary lamellae to ^{24}Na and ^{36}Cl have shown that in freshwater trout, the ratio of the venous and arterial radioactive concentration is equal to one for both isotopes (Girard and Payan, 1980). This implies that after passing through the secondary lamellae the fluid does not

receive any additonal isotopes (either ^{24}Na or ^{36}Cl). In other words, in freshwater fish, the arterio-venous pathway may not be so important in the transport of ions. If this is also true for the calcium ions, the activity of radioactive calcium from the effluent perfusion observed in the present perfusion studies may not necessarily be affected by the ligation of branchial veins and corresponds closely to the actual uptake rates in freshwater eels.

Additionally, even if the present perfusion technique confined the perfusion fluid to the arterio-arterial pathway in the secondary lamella calcium transport did occur, and in spite of the reported passive ionic exchange in the secondary lamellae (Kerstetter et al., 1970; Wood and Randall, 1973a, 1973b) the influx appeared to involve something more than simple diffusion. Indeed, in several of the fish studied the activity of ^{45}calcium in the effluent perfusion fluid was higher than that of the bathing medium so that calcium must have moved up a concentration gradient.

Of greater likelihood, however, is the possibility that the central flow remained patent. In addition to the connections between efferent primary arteries and the central venous sinus, the eel gills also have arterio-venous anastomoses joining the afferent primary arteries and the central venous sinus. Therefore in spite of the fact that the ligation of the branchial veins may have stopped the normal flow from the central venous sinus, the

constant pressure from the perfusion pump may have pushed the perfusion fluid into the sinus from the afferent primary arteries as well as out of the sinus space into the efferent primary arteries via the arterio-venous anastomoses. Therefore the calcium influx rates reported here may not be underestimated.

Lastly, it is also supported by some recent findings by Fenwick (personal communication) who used a whole eel head perfusion system in which arterial and venous effluents were collected separately. He showed that while 70% of ^{45}calcium taken up from the water irrigating the outside of the gills was indeed directed into the venous compartment, the total uptake as measured by both effluent routes was close to the predicted eight times that reported from the single hemibranch observed here.

The actual cardiac output was recently measured in short-finned eels (Anguilla australis schmidtii, Phillips) which had an average weight of 0.620 kg (Davie and Forster, 1980). It was observed to be 7.0 ± 1.2 ml min^{-1} when the fish were at rest. This means that the blood flow in each arch of the eels used in this study would possibly be 0.88 ml min^{-1}. In this and other isolated gill perfusion studies, the gill archs were perfused at a rate of 0.6 to 1 ml hr^{-1} (0.01 to 0.02 ml min^{-1}), a rate far below the expected flow rate in intact fish. However, this may not be unreasonable if one takes into consideration that the gill *in vitro* was removed from the normal neural and

humoral controls and that the gill membrane under such conditions becomes so delicate that higher flow rates and hence higher pressure may damage the membrane, the capillaries, or both. Indeed, it was observed early in this study that the gill arch became oedematous when prewashed at a rate of 0.5 ml min^{-1} as was suggested by Shuttleworth (1972). Besides, there was no reason to expect that the lower perfusion rates used in this study would affect the calcium influx rate as long as an adequate degree of mixing was maintained on the blood side of the branchial membrane. In spite of the above mentioned limitations, this technique was found to be satisfactory in evaluating calcium transport across the gills at least qualitatively.

In the present studies, by estimating the total calcium influx and efflux from the measured calcium influx and efflux rates in the eel gills, it was observed that a very small quantity of calcium ions was actually transported into the gills of the intact freshwater American eels. Further the rates varied with the season and with batches of fish used. Such differences have been observed previously by Fleming et al. (1973) who observed a similar pattern of seasonal variation in the rate of calcium uptake by male killifish, Fundulus kansae and by Pang (1973) who observed variation among batches of fish used. The latter author and I both circumvented this problem with the extensive use of control groups.

Since my initial publication (Ref. Fenwick and So, 1974), total branchial calcium influx and efflux rates have been reported in other laboratories. Ma (1976) working on the same American eel and using the same technique, obtained influx and efflux rates of 0.200 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1} and 0.005 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1} respectively. These values were close to those reported in the present study. Milet et al. (1979), however, reported that in the freshwater European eels, Anguilla anguilla, the branchial calcium influx rate was 0.009 μmol kg^{-1} body wt hr^{-1} while the efflux rate was up to 9.696 μmol kg^{-1} body wt hr^{-1}. In order to compare these values with those reported for the present study, it was assumed that a single dry gill in a one kilogram fish weighed 0.125 g (as previously calculated in this laboratory). Therefore, the calculated influx and efflux rates in the control freshwater European eels were 0.072 and 77.568 μmol Ca^{2+} g^{-1} dry gill filament hr^{-1} respectively. Though the influx value is close to those reported here for the American eel, the efflux rate is much higher. While this difference in efflux rates may represent a species or seasonal difference such an explanation seems unreasonable for a difference of this magnitude. On the other hand, Milet et al. (1979) claimed that their preparation did not leak, they did not adequately describe their method for testing for leaks. Further, as they

perfused the gill at a much higher rate (6 ml hr^{-1}) and with a peristaltic pump, they undoubtedly subjected the gills to pulses of high pressure during which they could have been a significant ultrafiltration of calcium through the branchial epithelium.

Because of the unfavourable concentration gradient aquatic organisms have a tendency to lose calcium in freshwater so that fish must have some mechanisms to reduce the loss of calcium ions and to extract calcium from the environment to make up for that which is lost. The fish gills, as observed in the present and other studies, are not sites of important calcium loss, but they do seem to perform a role in extracting this ion from the environment.

In the intact freshwater eel, however, the importance of the overall branchial calcium uptake is in some doubt. For example, Fenwick (1980) measured urinary calcium loss in freshwater eels and found that the normal loss rate was 1.24 ± 0.10 µmol kg^{-1} fish hr^{-1}. In the present study, as estimated from the single isolated perfused hemibranch preparation, net branchial calcium uptake rate in intact freshwater acclimated eels was 0.012 ± 0.003 µmol Ca^{2+} kg^{-1} fish hr^{-1}, a value which is only about 1% that of urinary calcium losses. This discrepency

probably indicates that the in vitro ^{45}calcium uptake rates by the perfused gills reported in this thesis are underestimated of what occurs in the intact animal.

In the present investigation on the freshwater American eel, the obtained data confirmed earlier observations that the removal of the corpuscles of Stannius resulted in a significant hypercalcemia in fish (Fontaine, 1967; Chan, 1972; Fenwick and Forster, 1972). The plasma calcium level in the present stanniectomized eels increased to a level about twice the pre-operative or control level (Table V). Furthermore, this occurred even though the eels were kept in Ottawa tap water with a calcium concentration as low as 0.45 mM and although this agrees with numerous reports of hypercalcemia in stanniectomized fish held in freshwater (Chan, 1972; Fenwick, 1974a) it is at variance with Pang's (1973) suggestion that the corpuscles of Stannius play a minimal hypercalcemic role in low calcium media.

More importantly, and for the first time, the present data showed that in addition to hypercalcemia, the removal of corpuscles of Stannius resulted in marked increase in ^{45}calcium influx across the gills (Figure 7). Indeed the ^{45}calcium influx rate increased from 11 (Figure

7) to 13 (Figure 8) fold relative to the rates in intact animals. This observation supported the earlier observation by Fontaine et al. (1972) on the increased net influx of environmental calcium into the European eel following stanniectomy. Furthermore, and more specifically, this result suggested that the gills did play some roles in the uptake of environmental calcium. However, stanniectomy did not affect branchial ^{45}calcium efflux (Figure 9) and the branchial ^{45}calcium efflux remained at very low rates (0.002 to 0.003 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}). Consequently these data imply that even at normal freshwater calcium concentration, the corpuscles of Stannius do exert an important effect on branchial calcium uptake. Further, these data suggest that a correlation may exist between the plasma calcium level and the branchial calcium influx rate.

Earlier studies have reported that the post-stanniectomy hypercalcemia is transient (Fontaine, 1967; Chan, 1972; Fenwick and Forster, 1972). That is, following the removal of the glands the plasma calcium level begins to rise within a few days, peaks after two or three weeks and returns towards normal values after four

to six weeks. Since branchial ^{45}calcium influx rates were shown to be related to post-stanniectomy hypercalcemia it was postulated that the influx rates would be reflected in the development of hypercalcemia in the fish following stanniectomy. Indeed, this was observed in the present study. That is, during the first two weeks after stanniectomy both the plasma calcium levels (Figure 10) and the rate of ^{45}calcium influx (Figure 11 and 12) increased simultaneously and this observation suggested that the development of post-stanniectomy hypercalcemia was closely related to the increased uptake of exogenous calcium. Furthermore, after the second week the branchial ^{45}calcium influx dropped precipitously back to control levels and this coincided with a levelling off of plasma calcium followed by a decline beginning at the fourth week.

The present data, as well as those reported by Ma (1976), indicate that the calcium ions are transported against a concentration gradient. In addition, the movement may have been against an electrical gradient since a number of authors have reported that the perfusion fluid is positive relative to the ambient fluid when gills are bathed in freshwater (Kerstetter et al., 1970; Maetz,

1974; Ma, 1976). Therefore, it is likely that the branchial influx of calcium involves some energy consuming process.

Despite the fact that the mechanism involved in the calcium uptake remains unclear, it has been suggested that a calcium activated adenosine triphosphatase in the gills is involved (Ma et al., 1974). Indeed, the activity of this enzyme was higher in the gills of stanniectomized freshwater eels than in sham-operated eels two weeks after surgery (Fenwick, 1976) and this agrees with the altered uptake rates obtained in the present study. Further, Chartier et al. (1975) provided some evidence for the participation of a branchial calcium binding protein which may account for some of the observations seen in the present study. Indeed, it may account for the fact that the calcium uptake system was readily saturated at the low calcium concentration found in the freshwater bathing medium. Further, the shape of the ^{45}calcium influx curve observed described essentially a sigmoid curve (Figures 7, 8 and 11) and the relatively slow increase in the rate of appearance of ^{45}calcium in the perfusion fluid was consistent with the assumption that during its passage through the branchial membrane, the ^{45}calcium passed through some internal calcium pool (Bygrave, 1978a). This could certainly account for the apparent lag phase in ^{45}calcium uptake seen during the first 15 to 45 minutes of perfusion. Indeed this must be true if our assumption that the total calcium uptake

(labelled plus unlabelled) by the perfused gills did not change during the course of perfusion with perfusion fluid alone. Further the similar time characteristics of the ^{45}calcium uptake curves in the different experimental groups and similarity in the amount of the residual ^{45}calcium remaining in the perfused gill filaments under all conditions (Table VI) suggest that the size of the internal calcium pool in the branchial cells is fairly constant. This suggests then that the mechanism involved in calcium uptake is not located within the branchial calcium pool.

Only one other study has reported on the effect of stanniectomy on branchial calcium influx in eels (Milet et al., 1979). These authors reported that following stanniectomy of European eels the fish had an influx rate of 0.26 μmol $Ca^{2+}g^{-1}$dry filament wt hr^{-1},a value significantly higher than the original normal value of 0.072 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}. These results agree closely with those reported here in American eels.

Though it is too early to relate the branchial calcium uptake with the general calcium turnover in the stanniectomized eels, an interesting point does emerge. From earlier measurements on the effect of stanniectomy on renal calcium excretion in the American eel, Fenwick(1974a) reported a net increase in the rate of urinary calcium excretion of 0.94 μmol Ca^{2+} kg^{-1} of fish hr^{-1} two weeks after the fish were stanniectomized (sham-operated: 1.77 μmol Ca^{2+} kg^{-1} hr^{-1}; stanniectomized: 2.71 μmol Ca^{2+} kg^{-1} hr^{-1}).

This increase was not apparent until after 10 days. In the present studies the net branchial calcium uptake two weeks after stanniectomy was calculated to be 1.24 μmol Ca^{2+} g^{-1} dry filament wt hr^{-1}. In other words, two weeks after stanniectomy the branchial calcium uptake exceeded about 0.3 μmol Ca^{2+} hr^{-1}. Further, and in view of the fact that the increase in branchial calcium influx became apparent within a few days while the increase in renal calcium excretion did not occur until after 10 days, the net effect would be one of the calcium loading which might be reflected in an increase in the plasma calcium concentration. Further, the excess calcium continuously entering the fish would also be available for distribution into the other body compartments. Indeed it was reported that stanniectomy increases the calcium in both the muscles (Chan, 1972) and the bone (Lopez, 1970). By the second week after stanniectomy, the branchial uptake decrease precipitously and as the renal calcium excretion became more important during and after the second week of stanniectomy, this may explain the slight drop in plasma calcium observed during the third and fourth weeks (Figure 10). The increased excretion of calcium from the kidney and the concomitant increase in bone mineralization (Lopez, 1970) would eventually bring the plasma calcium levels back towards the original control values and would explain the transient nature of post-stanniectomy hypercalcemia reported earlier (Fontaine, 1967; Chan, 1972; Fenwick and Forster, 1972).

Thus it appears that a cause-effect relationship exists between the surgical removal of the corpuscles of Stannius and an increase in branchial calcium influx. Stanniectomy is consistently followed by an increase in branchial calcium influx and this leads to the increase in plasma calcium level. Consequently, this suggest that the corpuscles of Stannius have important influences over the rate of calcium transport in the gills. Accordingly treatment of the fish with extracts of Stannius corpuscles should show some effect on branchial calcium movement.

Indeed the results obtained when the effect of Stannius corpuscle extracts were measured by either *in vivo* or *in vitro* studies on branchial ^{45}calcium uptake in post-stanniectomized eels support this hypothesis. The injection of Stannius corpuscle extracts corrected the state of hypercalcemia which invariably followed the removal of the Stannius corpuscles in the otherwise untreated teleosts (Figure 13). This observation confirms those previously reported studies (Fontaine, 1967; Fenwick and Forster, 1972; Pang *et al.*, 1973b, 1974). In fact, this response is so well established that it was used earlier by Pang *et al.* (1974) for the bioassay of the Stannius corpuscle hypocalcemic principle. In the present study the Stannius corpuscle extract was effective within 3 to 5 hours of the first injection. More importantly, it was found that the same injection also prevented the increase in calcium influx normally observed in gills taken from uninjected two week

stanniectomized eels (Figure 14). This finding was further substantiated by the observation that Stannius corpuscle extract was also effective in vitro. That is, the addition of Stannius corpuscle extract to the fluid used to perfuse the isolated gill archs taken from the stanniectomized eels significantly ($P < 0.001$) reduced branchial ^{45}calcium uptake within the first 10 minutes (Figure 15). This suggests that the Stannius corpuscle extract can exert a rather direct effect on the branchial calcium transport. This in vitro effect on the reduction of branchial ^{45}calcium uptake was also rather specific to the Stannius corpuscle extract as neither treated kidney (Figure 16) nor liver extracts (Figure 17) produced any significant effect on the branchial ^{45}calcium uptake. This further supports Pang's observation (Pang et al., 1974) that the hypocalcemic response was specific to the corpuscles of Stannius.

Since the initial publication on some of these data (Fenwick and So, 1974), only two other studies have been reported on the effect of Stannius corpuscle extracts on perfused gill calcium fluxes. In contrast to the present results, Milet et al. (1979) did not show any significant effect of the Stannius corpuscle extract on the calcium influx or efflux in perfused gills from stanniectomized European eels. While it is impossible to explain these discrepancies at the present time, it should be noted that they reported very high efflux rates so that the possibility of significant Ca^{2+} leaks cannot be ruled out. Ma and

Copp (1978) observed that partially purified Stannius corpuscle extract did lower branchial ^{45}calcium influx in the perfused gills from intact eels. Although their finding agreed with the present results their data may not be comparable to those presented here. In Ma and Copp (1978) the isolated gills were perfused for up to 240 minutes and no proper controls were used to check the functional state of the gills. Such checks would be especially important during the last 120 minutes.

While it is too early to postulate a precise mechanism by which the Stannius corpuscle extract exerts their inhibitory effect, there is at least some evidence to suggest that the Stannius corpuscle extract exhibits the action on a branchial calcium activated adenosine triphosphatase (Ma, 1976). This suggestion is supported by three observations: the activity of branchial Ca^{2+}-ATPase in killifish Fundulus heteroclitus was inhibited by injecting extract of Stannius corpuscles into the animal (Burdick et al., 1976), Stannius corpuscle extract directly inhibited in vitro the activity of Ca^{2+}-ATPase isolated from eel gills (Ma, 1976), and the total activity of this enzyme was significantly increased in the gills of stanniectomized eels (Fenwick, 1976). This is, however, only circumstantial evidence, and although it indicates a correlation, a correlation in no way proves a cause-effect relationship.

The present study employed previously acidified corpuscle extracts to measure their in vitro effects on

branchial ^{45}calcium influx. Unacidified extracts always produced, within a few minutes, a marked drop in the effluent perfusion rate presumably due to the presence of the acid-labile pressor substance described by Chester Jones et al. (1966). While such acid treatment of the preparation probably destroyed the renin-like activity found in the Stannius corpuscles (Pang et al., 1974; Nishimura, 1978; Ogawa et al., 1978) it would not destroy the activity of teleocalcin described by Ma and Copp (1978). On the other hand, angiotensinase has been found in the Stannius corpuscles (Chester Jones and Henderson, 1965) and because of the anatomical arrangement of the Stannius corpuscle vascular system (Ogawa and Oguri, 1978), it is possible that some Stannius corpuscle angiotensin-like material remained in the corpuscles (Ogawa, personal communication). Such angiotensin-like compounds are stable in acid media and may have an effect on the branchial calcium movements when applied to the gill preparation. Whether it could directly effect the activity of branchial Ca^{2+}-ATPase has not been reported. But one has to bear in mind that the crude extracts of Stannius corpuscles that Ma (1976) used during her incubated Ca^{2+}-ATPase studies were prepared in eel saline without any special treatment. Hence the possibility of contamination by Stannius corpuscle renin-like or angiotensin-like substances cannot be eliminated.

If some Stannius corpuscle angiotensin-like substance remained in the extracts used in the present study,

what might the effects be? The carp Stannius corpuscle angiotensin-like material and the goosefish kidney angiotensin-like material show similar characteristis on S.E.-Sephadex columns (Nakajima et al., 1971) so it is possible that any eel Stannius corpuscle angiotensin-like material may have the same pressor activity as the fish renal angiotensin. If so, it can be speculated that the application of vasoactive CS angiotensin-like substance to the gill could alter the direction of blood flow within the pathway in the gill filaments and thus reduce the flow of perfusion fluid perfusing the areas where the most important uptake of calcium takes place. This hypothesis seems not too unreasonable as the extract was observed to be very effective in vitro within a few minutes (Figure 15) while the residual ^{45}calcium remained unchanged. Accordingly, and based on the previous discussion on the blood pathways in the eel gill, the hypocalcemic factor described here may have diverted the flow away from the arterio-venous pathway. However, as discussed earlier, the rate of ^{45}calcium influx into the perfused gill preparation was similar to the rate found by Fenwick in whole perfused heads. In other words, even if a vasoactive substance could alter the direction of flow such alterations did not affect ^{45}calcium uptake rates in this preparation.

Whatever the mechanism, the increase in ^{45}calcium influx and the hypercalcemia following stanniectomy was corrected by replacement therapy with Stannius corpuscle

extracts and this suggested that the corpuscles of Stannius exert their hypocalcemic role in fish mainly by attenuating the calcium uptake across the gills. If this is true, what then causes the reduction in branchial ^{45}calcium influx seen in stanniectomized eels during the third and fourth weeks after the operation and what causes the return towards normocalcemia?

Fish calcitonin has a well defined hypocalcemic action in mammals (Copp et al., 1970) but does not consistently show a similar effect in fish (Pang, 1973; Pang et al., 1974; Pang et al., 1980). While the failure to show overt hypocalcemic action of calcitonin in fish indicates that calcitonin does not lower plasma calcium levels, plasma calcium concentration may not be an appropriate parameter for detecting a possible effect of calcitonin on calcium turnover (Chan, 1972). Indeed there is evidence to suggest that calcitonin does perform some regulatory function in calcium homeostasis. For example, it was shown that endogenous calcitonin levels increase when fish encounter a high calcium challenge (Dacke et al., 1971; Deftos et al., 1972). Further, it exerts a calciuretic action on the kidney (Fenwick, 1978) and increases calcium efflux from the head region of eels (Leung, 1979). On the other hand Dacke (1975) showed that salmon calcitonin decreased the total ^{45}calcium efflux from intact freshwater silver European eels. All these observations suggest that

calcitonin can effect calcium homeostasis possibly by acting on such target organs as the kidney and the gills. For these reasons an *in vivo* study on the effect of salmon calcitonin on calcium exchange across the gills from hypercalcemic stanniectomized eels was carried out. The results of this indicated that 6 daily injections at a dose of 2 MRC units kg^{-1} of fish day^{-1} during the second week after stanniectomy prevented any further increase in plasma calcium (Table VIII). On the other hand, the stanniectomized fish which received only placebo injections showed a continued increase in the plasma calcium levels during the second week. These results indicate that whilst calcitonin did not exert a frank hypocalcemic action, it did have an anti-hypercalcemic effect. Further this anti-hypercalcemic effect of salmon calcitonin was accompanied by a 360% increase in branchial calcium efflux from the perfused gills (Table IX). This latter observation corroborates that of Leung (1979) who observed that salmon calcitonin caused a net increase in calcium efflux from the head region of intact calcium loaded eels. Conversely in the present study, the branchial calcium influx was unaffected by the 6 daily injections of salmon calcitonin. This inability of calcitonin to reduce branchial ^{45}calcium influx was confirmed *in vitro* (Figure 18) as salmon calcitonin did not significantly alter the ^{45}calcium influx rate of the perfused gill.

The *in vitro* effect of salmon calcitonin on

branchial calcium influx has been studied by various authors. In contrast to the present study, calcitonin was reported to decrease calcium influx in perfused isolated pacific salmon gills (Milhaud et al., 1977). Peignoux-Deville et al. (1978) and Milet et al. (1979) also observed that calcitonin decreased branchial calcium influx across perfused isolated European eel gills by 26%. But they also reported that calcitonin had an even more marked effect on efflux as efflux rates increased by 80%. However the difference reported by these investigators when compared to the present study may be explainable on the basis that they used ultimobranchialectomized eels. That is, the ultimobranchialectomized eels may have had more active Stannius corpuscles (Lopez et al., 1976) which may have changed the response of the gills to salmon calcitonin. Another possibility is that gills deprived of their normal titer of calcitonin may have become less sensitive to the hormone. This requires further study. In the present study the hypercalcemia subsequent to stanniectomy may have increased endogenous calcitonin secretion and this may have partially masked the effect of the exogenous calcitonin (Chan, 1972).

It appears then that calcitonin did exert an anti-hypercalcemic action following stanniectomy and that at least in part this action is accomplished by enhancing the calcium efflux across the gills.

The alkaline earth ions, especially calcium ions, exert major influences over membrane permeability. They also affect the transport of ions across a number of epithelia (Cuthbert and Wong, 1971) and both calcium and magnesium have significant effects on ion transport and water permeability of the fish branchial epithelium (Pott and Fleming, 1970, 1971; Cuthbert and Maetz, 1972; Bornancin et al., 1972; Fleming et al., 1974; Maetz, 1974; Isaia and Masoni, 1976). Since in the stanniectomized eels the plasma calcium levels rose to 5.32 mM (Table V), it is possible that this high concentration of plasma calcium may have itself affected the calcium influx across the gills. This led to the current in vitro study on the effect of high calcium perfusion fluid on the branchial calcium uptake (Figure 19). When the calcium concentration of the perfusion fluid was raised to 5 mM, calcium uptake in the perfused gills was not significantly altered relative to that seen when the perfusion fluid contained the normal amount of calcium (1.63 mM). This observation is at variance with that of Ma (1976) who reported a gradual increase of calcium influx across the gill membrane of the intact eel when the calcium concentration of the perfusion fluid was raised from 0.75 mM to 2.25 mM. While there is no obvious explanation for this discrepancy, it is important to note that in the present study an increase in the concentration of calcium in the perfusion fluid did not increase ^{45}calcium influx. This is important as this

observation tends to rule out the possibility that ^{45}calcium uptake was occuring simply by exchange diffusion. If this was the case, the higher concentration of calcium in the perfusion fluid should have elevated ^{45}calcium influx. Further, it provides evidence that the concentration of calcium in the perfusion fluid does not have to be changed for specific experimental animals subject to various degrees of hypercalcemia.

It has been reported that when euryhaline fish move from freshwater to seawater, the magnitude and direction of monovalent ion movement in gills are altered (Maetz, 1976) because of changes in membrane characteristics. As the gills appeared to be important sites for calcium exchange it did not seem unreasonable to assume that changes in ionic concentrations in the environment would affect the exchange of calcium ions as well. Subsequently it was observed (Figure 20) that the isolated perfused gills of stanniectomized eels in reconstituted seawater bathing medium had calcium influx rates which were significantly higher (greater than 200%) than gills in the freshwater bathing medium as far as the total calcium uptake was concerned. The increase was almost immediate and this raised the question of which ions in the marine environment contributed to this change. External calcium ions have been shown to have important influences on the change in the branchial permeability and also the active

transport of monovalent ions (Potts and Fleming, 1970, 1971; Bornancin et al., 1972; Cuthbert and Maetz, 1972; Fleming et al., 1974; Eddy, 1975), and magnesium ions can replace the calcium ions in some of their effects upon ionic transport across the gills (Pic and Maetz, 1975). As seawater contains considerable quantities of both calcium (10.4 mM) and magnesium (52 mM) it is possible that the increased branchial calcium influx in seawater resulted from the effect of one or both of these ions. For this reason the effect of these ions was tested separately. When the calcium concentration of the freshwater bathing medium was raised to that of seawater (a 22 fold increase) the calcium influx increased by 4.67 fold (see calculated results on p. 87). On the other hand, when the magnesium concentration of the freshwater bathing medium was raised to that of seawater, the calcium influx decreased by 72% (Figure 22). These changes were not simply due to changes in the osmolality of the bathing media as equivalent increases in the osmolality of the bathing medium with mannitol did not affect ^{45}calcium uptake rates. These observations indicated several possibilities. Firstly, as the calcium uptake rate did not increase in proportion to the increase in the calcium concentration gradient, this suggested that the mechanism by which calcium moved across the membrane was saturated or that the high calcium concentrations reduced the overall permeability of the branchial membrane to calcium. Secondly, the reduced calcium

uptake rate in the presence of seawater concentration of magnesium ions suggested that magnesium ions competed for the transport mechanism or that the magnesium ions reduced the overall permeability of the branchial membrane to calcium ions. But by whatever mechanism, seawater concentrations of calcium ions and magnesium ions on the mucosal side of the branchial epithelium reduced the rate of calcium influx as predicted by the concentration gradient.

It is interesting to note that Fenwick (1979), also working on American eels, observed that the gills of seawater acclimated fish had lower Ca^{2+}-ATPase activity than the gills of freshwater acclimated eels. Since the activity of this enzyme seems to be related to the transport of calcium ions (Cha et al., 1971; Ma et al., 1974), it suggests that less calcium is actively transported across the gills after adaptation to seawater. Further, the enzyme activity was inhibited in vitro by the presence of seawater concentration of magnesium ions and calcium ions (Fenwick, 1979). These observations concur with the present suggestion that calcium ions and magnesium ions can affect the branchial calcium movements.

Thus it appears that when fish are challenged with a high external concentration of calcium ions and magnesium ions, as occurs when they enter seawater, the calcium ions and magnesium ions in the external environment can directly decrease the branchial permeability to the calcium ions, as well as possibly reducing the acti-

vity of branchial Ca^{2+}-ATPase. It is also possible that the corpuscles of Stannius become activated during the high calcium challenge and release some hypocalcemic factor(s) which facilitates this reduction in branchial calcium uptake.

To recapitulate, the present investigation provides some information on the relationship between the corpuscles of Stannius and the calcium handling by the fish gills. The data obtained confirmed the earlier observation on the hypocalcemic action of the corpuscles of Stannius even when the fish are in freshwater. These data suggest also a cause-effect relationship between the surgical removal of the corpuscles of Stannius and an increase in the branchial calcium influx. Stanniectomy was consistently followed by an increase in the plasma total calcium and an increase in calcium influx. Calcium efflux rates were not affected. Both elevated plasma calcium and the increased branchial calcium influx could be corrected by treatment with extracts of Stannius corpuscles. The in vitro effect on the calcium influx in the gills was specific to the extract of Stannius corpuscles and kidney and liver extracts did not produce a significant effect. The corpuscles of Stannius appeared to contain an active, acid-stable hypocalcemic factor(s) which could effect the gills. The post-stanniectomy hypercalcemia did not appear

to affect the rate of calcium influx in the gills whereas high concentrations of calcium or magnesium ions in the external environment prevented the branchial calcium influx rates from rising to its predicted level. Calcitonin appears to play primarily an anti-hypercalcemic role in fish possibly by increasing branchial calcium efflux.

The results shown in this thesis clearly indicate that the gills are involved in calcium exchange between the fish and its environment. Moreover, both the rate and direction of this exchange are subject to environmental and hormonal influences. This leads me to the conclusion that the gills are involved in calcium homeostasis and that they are a target organ for the hypocalcemic hormone(s) of Stannius corpuscle origin. On the other hand, the amount of calcium ions moving across the gills is only a small portion of the total movement of calcium between the animal and its environment. This suggests then that while the gills may perform a significant role in the fine control of plasma calcium concentration, they probably do not play more than a supportive role in the regulation of total body calcium.

SUMMARY

1. Stanniectomy of freshwater adapted American eels, Anguilla rostrata (LeSueur), resulted in a simultaneous increase in plasma calcium level and calcium influx into the isolated perfused gills during the first two weeks.

2. No significant change in the branchial calcium efflux was observed after the removal of corpuscles of Stannius.

3. Both the elevated plasma calcium level and the branchial calcium influx were corrected by the injection of either untreated or previously acidified extract of corpuscles of Stannius.

4. Perfusion of previously acidified Stannius corpuscle extract into the gill of stanniectomized eels resulted in a decrease in calcium influx. This effect was specific to the Stannius corpuscle extract.

5. Calcitonin exerted an anti-hypercalcemic effect in two week stanniectomized eels but did not lower plasma calcium.

6. Exogenous calcitonin treatment of stanniectomized eel increased calcium efflux from the gills; influx

rate was not affected.

7. Perfusion of calcitonin into gills from stanniectomized eels did not affect branchial calcium influx.

8. Perfusion of the gills from stanniectomized eels with perfusion fluid containing 5 mM Ca^{2+} did not affect the branchial calcium influx.

9. Branchial calcium influx rate increased when the gills from the stanniectomized eels were placed in the 100% reconstituted seawater. In freshwater bathing medium with high (10 mM Ca^{2+}) calcium concentration, branchial calcium influx increased while high concentration of magnesium ions (52 mM) present in the freshwater bathing medium prevented the increase of calcium influx into the gills.

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