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STUDIES ON THE STRUCTURE AND FUNCTION OF THE TELEOST PSEUDOBRANCH

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STUDIES ON THE STRUCTURE AND FUNCTION OF THE TELEOST PSEUDOBRANCH

by

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DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted for any other award.

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This is to certify that the work presented in this thesis was carried out by the candidate himself.

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Studies on the Structure and Function of the Teleost Pseudobranch

by

D. L. Mattey

Abstract

Four main types of pseudobranch were distinguished on the basis of their epithelial covering. The terms 'free', 'semi-free', 'covered' and 'buried' are used to describe these types. All pseudobranchs possess specific 'pseudobranch type' cells, characterised by an orderly arrangement of tubules around closely packed mitochondria. They also contain vacuoles which may play a role in osmoreception by causing changes in the size and shape of the cells. Another specialised cell type, similar to the gill 'chloride' cell was found in 'free' and 'semi-free' pseudobranchs of salt water fish. They are associated with smaller 'accessory' cells from which they are separated by 'leaky' junctions which may provide a structural basis for the proposed ion secretory nature of 'chloride type' cells. Ultrastructural changes in 'chloride type' and 'pseudobranch type' cells were noted under osmotic stress but the cells still remained distinguishable from each other, giving no reason to suggest that they were different forms of the same cell. The two other main cell types found in the pseudobranch epithelium were mucous and rodlet cells. The epithelial surface possesses numerous microridges which are thought to aid anchorage of mucous.

The vascular system of the pseudobranch shows close similarities to that of the gill and contains a well developed arterio-venous pathway as well as an arterio-arterial system. Arterio-venous anastomoses were found between the efferent filament artery and the central venous sinus of the bass pseudobranch.

The pseudobranch innervation is extremely complex. Morphological and denervation studies suggest an autonomic innervation and physiological evidence indicates the presence of at least two and possibly four types of receptor.

The results of this study indicate that the pseudobranch has a number of inter-related functions associated with the development of specific cell types and a complex innervation linked directly or indirectly to the vascular system.

INTRODUCTION

The pseudobranch of teleost fish shows a considerable degree of structural variation ranging from a 'free' gill-like structure projecting into the buccal cavity to a 'glandular' structure buried in a mass of connective tissue (Granel 1927). Harb & Copeland (1969) suggested that the descriptive designations of the different types of pseudobranch may need to be revised. This was based on their discovery of two distinct cell types in the pseudobranch of the marine teleost Paralichthys lethostigma; one type similar to the 'chloride' cell of the gill and one specific to the pseudobranch. Confusion has arisen in the literature due to the failure of many authors to differentiate between 'chloride' and 'pseudobranch type' cells and their relevance to 'free' or 'covered' pseudobranchs. Misconceptions about the pseudobranch structure have led to the production of various, often contradictory, theories on the function of this organ. Because of its general resemblance, macroscopically and microscopically with the 'true' gills its possible role in the physiology of fish was first thought to be respiratory. However, it was soon realized (Hyrtl 1838) that in the adult animal the pseudobranch is supplied with arterial blood, already oxygenated after passing through the capillary network of the gills. More recent theories suggest that the organ is involved in enzyme production (Leiner 1938; Maetz 1956); endocrine functions (Holliday & Parry, 1960); regulation of blood pressure in the eye (Granel 1927) or modification of blood flowing to the eye (Wittenberg & Haedrich 1974); salt regulation similar to that described for the gills (Bertin 1958), or chemoreception (Laurent and Dunel 1964, 1966; Laurent & Rouzeau 1972). Vervoort (1958) suggested that the pseudobranchs may be important only at certain stages of embryonic or post embryonic life. It is possible that the pseudobranch may fulfil one or

several of these functions depending on the species and the type of pseudobranch.

In this study these theories are examined in the light of new structural and functional evidence, relationship of the pseudobranch to habitat and species, and association with the gills, choroid rete mirabile, blood circulation and innervation.

REVIEW

The pseudobranch was first described by Broussonet (1782, 1785) when he discovered a pair of gill-like organs on the inner surface of the opercular fold in teleosts.

Distribution

Early anatomical studies reported the existence of a pseudobranch in Elasmobranchs (J. Muller, 1839; Hyrtl, 1858, 1872; Virchow 1890a, 1890b; Carazzi, 1904; Allis 1911a, 1912b, 1912c; Vialli, 1924a; Granel, 1924) and Actinopterygians (J. Muller, 1840, 1844; Ramsay Wright, 1885; Dohrn, 1886; F. W. Muller, 1897; Allis 1900, 1911b; Vialli 1924a, 1925a, 1925b), but its major evolution has taken place in association with the teleost respiratory system, with a maximal development in Euteleosts (Hyrtl, 1838; J. Muller, 1839; Maurer, 1884; Dohrn, 1886; Allis, 1912a; Grassi, 1914; Vialli, 1924b, 1926a; Granel, 1922a, 1922b, 1923a, 1923b, 1926, 1927), and especially in Acanthopterygians which display the most highly developed branchial respiration (Laurent 1974). It is absent in Cyclostomata, the Holocephali, Dipnoi and a few teleosts (Goodrich, 1930; Wittenberg and Haedrich, 1974). The adult Holocephali and Dipnoi have lost both the pseudobranch and spiracular slit (Goodrich 1930), but in the Teleostomi an open spiracle persists in Polypterus, Acipenser and Polyodon (Muller, 1840). In all other adult Teleostomes the spiracle is closed although a distinct vestige may remain of the pharyngeal pouch in Amia, Lepisosteus and some teleosts (Ramsay Wright 1885; Sagemehl, 1884a, 1884b, 1891). A pseudobranch is developed here in Acipenser, Polyodon, Lepisosteus, Amia and the majority of teleosts.

It is the absence of the pseudobranch in some teleosts that has made the physiological importance of this organ a controversial matter.

J. Müller (1839, 1840, 1844) investigated a large number of teleosts and found it to be present in the adult stage of many species but to be completely absent from others. More recently Wittenberg andHaedrich (1974) carried out an extensive study on the association of the pseudobranch with the choroid rete mirabile in teleosts. The principal groups lacking a pseudobranch are the Anguilliformes, Osteoglossiformes, Siluriformes and a few Salmoniformes. In the Leptocephalus larva of Anguilliformes (eels) the pseudobranch is a true gill with a respiratory function, but it disappears in the adults and the existence in them of any vestigeal organ is questioned (Grassi 1913, 1914).

Circulation and Development

Most of the early work on the pseudobranch was concerned with its development and the relationship of its blood supply with that of the primary aortic arches. For some time it was disputed whether the pseudobranch represented the mandibular (spiracular) or hyoideal gill arch. In 1839 J. Muller established the homology of the pseudobranch of Teleostomes with that of Selachians, where it represents the spiracular or mandibular hemibranch. This followed the discoveries of V. Baer (1827) and Valentin (1831) that the pseudobranchs of Acipenser (sturgeon) and Lepisosteus (gar) occur along with opercular gills which undoubtedly represent the hyoideal hemibranchs, indicating that the pseudobranchs belong to the mandibular arch.

The pseudobranchial circulation is dealt with in studies by Hyrtl (1838, 1858, 1872), J. Müller (1839), Maurer (1884), F. W. Müller (1897), and especially Allis (1900, 1908a, 1908b, 1911a, 1911b, 1912a, 1912b, 1912c).

In the Selachians, lower Teleostomes and many Teleosts (e.g. Salmo) the pseudobranch receives blood from the efferent vessel of the hyoid arch via an anastomosis (afferent pseudobranchial vessel). The ventral region

of the mandibular arch is interrupted and the more dorsal region of the arch becomes the efferent pseudobranchial vessel. This vessel joins the lateral dorsal aorta (internal carotid) in Selachians, whilst in Amia (bowfin) and most Teleosts the efferent pseudobranchial artery separates from the internal carotid and passes directly into the ophthalmic artery (Fig 1) (Allis, 1900, 1911a, 1911b, 1912a, 1912b, 1912c; Goodrich 1930). In Teleosts this is the usual definitive condition; the two internal carotids come close together and fuse to complete the circulus cephalicus, while the separated efferent pseudobranchial arteries continue forward as the ophthalmics. A slender cross vessel unites them in front of the circulus (Allis, 1912a).

The cross commissural vessel from the hyoidean to the mandibular arch always seems to be developed in the embryo, though it may disappear later (Amia and most Teleosts). The pseudobranch then receives blood from the mandibular arch, when the ventral mandibular supply persists (e.g. Polyodon, Allis 1911b), and a secondary afferent vessel coming from the circulus cephalicus (e.g. Gadus, Allis 1912a). In Amia and such Teleosts as Esox (pike) the secondary vessel (which may be derived from the orbital artery) alone supplies blood to the pseudobranch (see Fig 2a - e).

Studies on the morphology and development of the pseudobranch in different forms are extensively reviewed by Vialli (1926a) and Granel (1927).

Selachians usually have a well developed pseudobranch lodged in the spiracular slit and reaching to near its external opening. Although gill-like, it possesses only one row of filaments, unlike the typical branchial arch which has filaments on both anterior and posterior surfaces (holobranch). The pseudobranch filaments may be numerous but bear few secondary lamellae covered by a thick layer of cubical epithelial cells. They do not contain the acidophil cells typical of higher forms (Vialli 1926a).

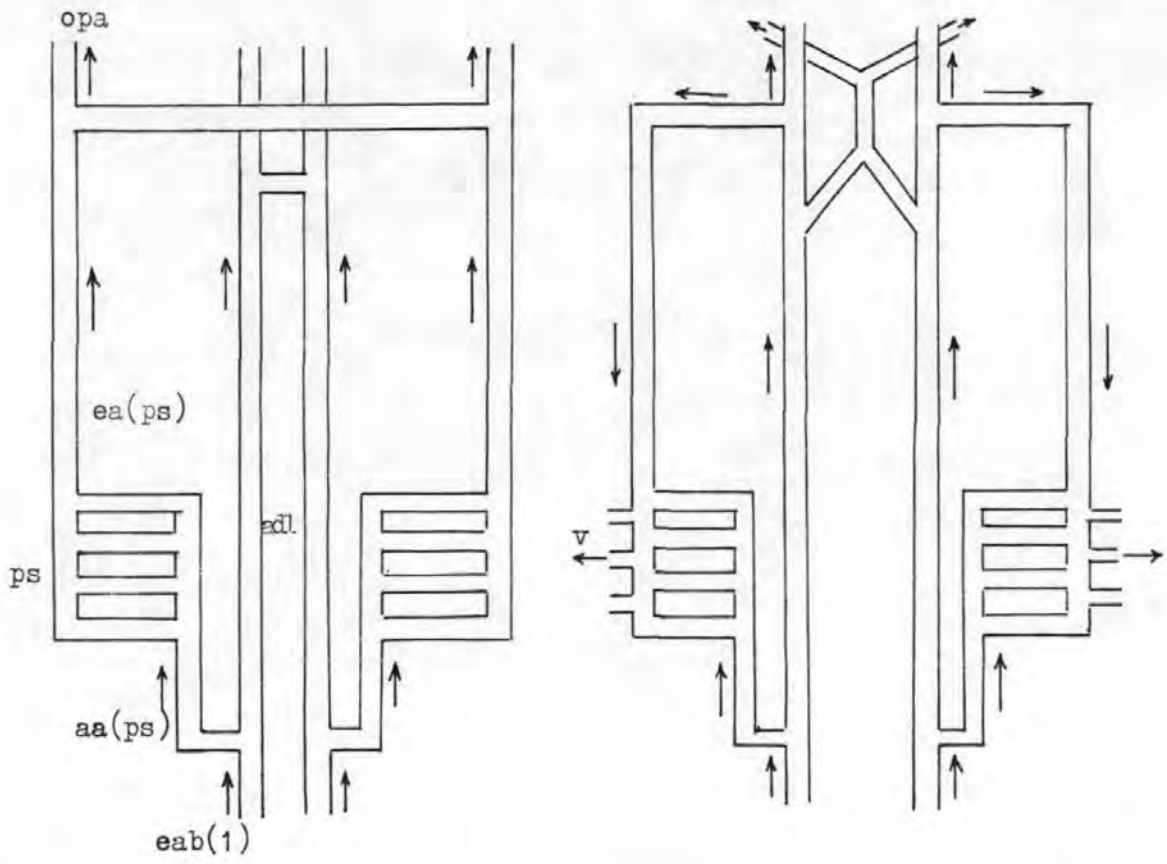


Fig 1

Diagrammatic representation of the cephalic arterial circulation in the teleosts (left) and in Torpedo marmorata (right). Note the presence of two independent circles in teleosts in contrast with the selachians. In the teleosts the blood flows through the pseudobranch (ps) from the afferent pseudobranch artery aa(ps) to the ophthalmic artery (opa). The blood originates from the efferent artery of the first gill arch (eab(1)) : adl : lateraldorsal aorta.

In Torpedo, the blood flows through the pseudobranch from the afferent pseudobranchial artery aa(ps) and a branch of the anterior carotid to the venous system (v). The vascularisation is arteriovenous, the blood originating from the efferent hyoid artery. (After Laurent, 1976)

Fig 2

Development of aortic arches and pseudobranch circulation (after Kryzanovsky 1934)

aab(1)	afferent branchial artery (1st gill arch)
eab(1)	efferent branchial artery (1st gill arch)
DA	dorsal aorta
VA	ventral aorta
m	mandibular arch
ma	mandibular artery
h	hyoid arch
ps	pseudobranch
aa(ps)	afferent pseudobranchial artery
ea(ps)	efferent pseudobranchial artery
oa	orbital artery

The pseudobranch circulation may arise from one of 3 sources

Fig c) ventral origin - from efferent branchial artery and mandibular artery

Fig d) mixed origin - cephalic circle and mandibular artery

Fig e) dorsal origin - cephalic circle.

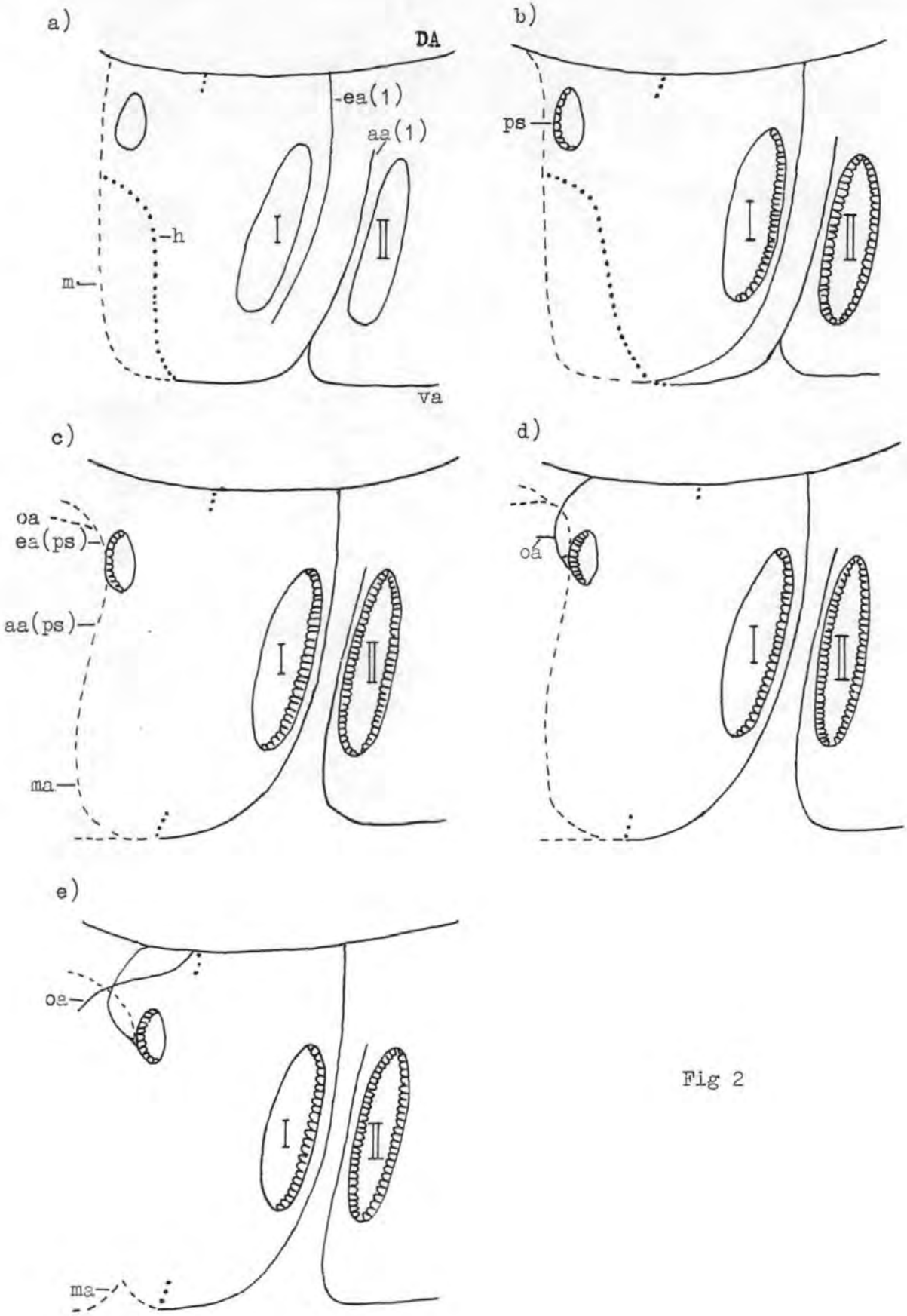

a)
DA
ea(1)
aa(1)
h
m
I
II
va
b)
ps
I
II
c)
oa
ea(ps)
aa(ps)
ma
I
II
d)
oa
I
II
e)
oa
ma
I
II

Fig 2

Virchow (1890b) described three groups of Selachians on the basis of their pseudobranch or lack of it:

1. Notidanidi, which possess a pseudobranch.
2. Squalidi, which do not have a pseudobranch but only an arterial network connecting the afferent to the efferent spiracular artery (Selache maxima).
3. A third group which possesses neither arterial network nor pseudobranch, so blood flows directly from the afferent to the efferent artery via an anastomosis (e.g. Callorhynchus).

Recent studies on the pseudobranch of the Selachian Torpedo marmorata, by Barets et al (1976 in preparation) showed the presence of a deeply modified gill with 7 to 10 primary lamellae as slightly prominent folds inserted on a common base. Small, often abortive secondary lamellae are sparse and insert laterally and obliquely on these folds. The study showed the presence of two parts in the circulatory system : the corpus cavernosum located at the base of each primary lamella and the pseudobranch parenchyma located at the top (Fig 3). Previous papers already demonstrated the presence of these two structures (Vialli 1924a, Granel 1924), but the vascular connection between them was not understood. The blood gets into the parenchyma by two pathways:

1. the blood flows through the corpus cavernosum, penetrates the secondary lamellae and finally enters the parenchyma. There is no direct access between the two parts as previously described. The sparse secondary lamellae are nearly always abortive, the vasculature consisting only of a marginal vessel.
2. as an alternative pathway the blood has direct access to the parenchyma via a small dorsal branch of the afferent arterial plexus. This access is controlled, as in the preceding case, by sphincters in the parenchyma.

The parenchyma is a very complex structure composed of several types of vessels : the efferent vessels (distal part of the secondary lamellae)

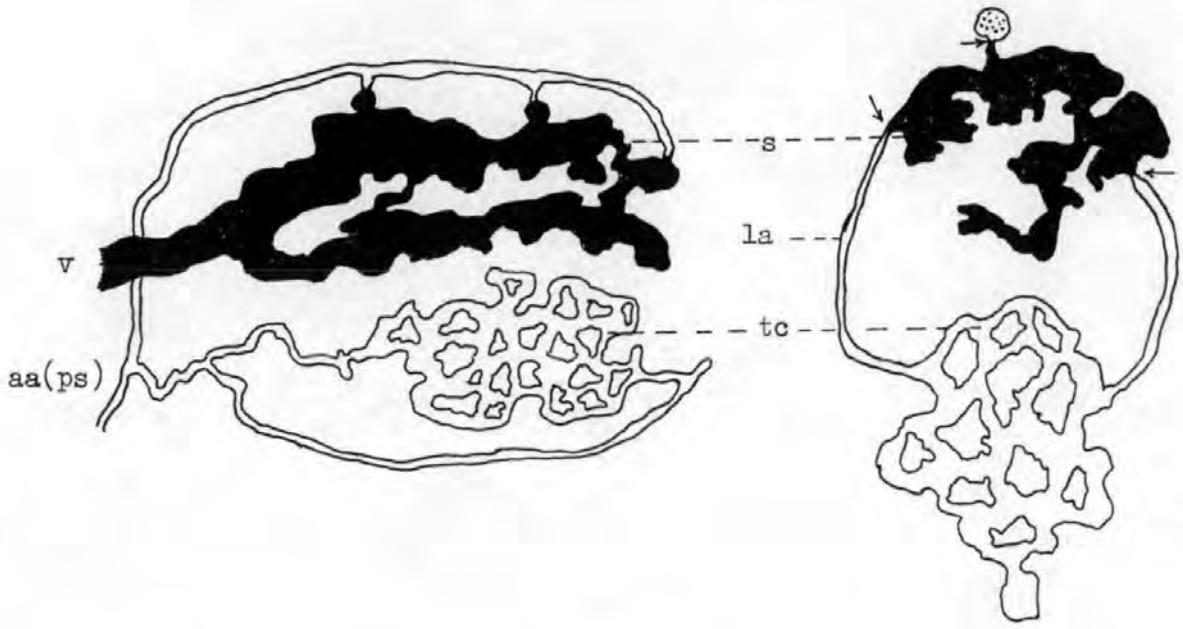


Fig 3

Schematic representation of the primary lamella vascularisation in Torpedo. Branches of the afferent pseudobranch artery aa(ps) run into the corpus cavernosum (tc) which communicates with the pseudobranch parenchyma (s) by means of the marginal vessels of the abortive secondary lamellae (la). A dorsal branch of the aa(ps) is directly connected with the parenchyma but sphincters (arrows) are interposed between the anterior part of the system and the parenchyma sinus. The sinus communicates with the venous system. (After Laurent, 1976)

are connected with a large sinus by way of narrow channels and sphincters. Finally the sinus blood is conveyed by veins towards the jugular system. This venous outflow is necessary because in Torpedo marmorata and many other Selachians there is a connection between the cephalic circle and ophthalmic circle (unlike teleosts where the circles are not connected) so there is no hydrostatic pressure gradient between input and output of the pseudobranch rete. Consequently the blood could not flow through the rete unless a venous outflow drains it off (Laurent 1976). (Fig 1)

The pseudobranchs of Acipenser and Polyodon are similar to those of Selachians, but are more deeply set in the spiracular slit (Vialli 1924a, 1925a, 1926a; Granel 1927). In Lepisosteus where this slit is closed externally but widely open internally the pseudobranch migrates in development into the branchial cavity to a position on the under surface of the operculum (Ramsay Wright, 1885; Müller, 1897; Vialli, 1925b; Granel, 1927). The pseudobranch of Amia preserves the essential gross structure of a gill with lamellae supported by branchial rays as in the previously described forms, but it is larger and bulges outwards on the wall of a saccular diverticulum of the spiracular slit (Ramsay Wright, 1885; Vialli 1925b, 1926a). It also resembles that of Teleosts in the appearance of a layer of acidophil cells (Vialli 1926a; Granel 1927).

In the Teleostei the spiracle closes early, the mandibular pseudobranch arises near the internal opening of the spiracular slit, but migrates and spreads into the subopercular cavity (Dohrn 1886). It shows progressive structural modification from the respiratory branchial arches. At first it is a 'free' gill-like organ with projecting vascular lamellae possessing secondary lamellae, but it tends to sink below the covering epithelium and loses its primitive structure in later stages of more specialised cases (Maurer 1884).

In 1839 J. Müller described two kinds of Teleostean pseudobranch:

1. free, with gill-lamellae projecting into the sub-opercular cavity;
2. glandular, sunk below a covering epithelium.

However Granel (1927) made it clear that all possible transitions occur between 'free' and 'enclosed' pseudobranchs, although four main adult types could be distinguished (Fig 4).

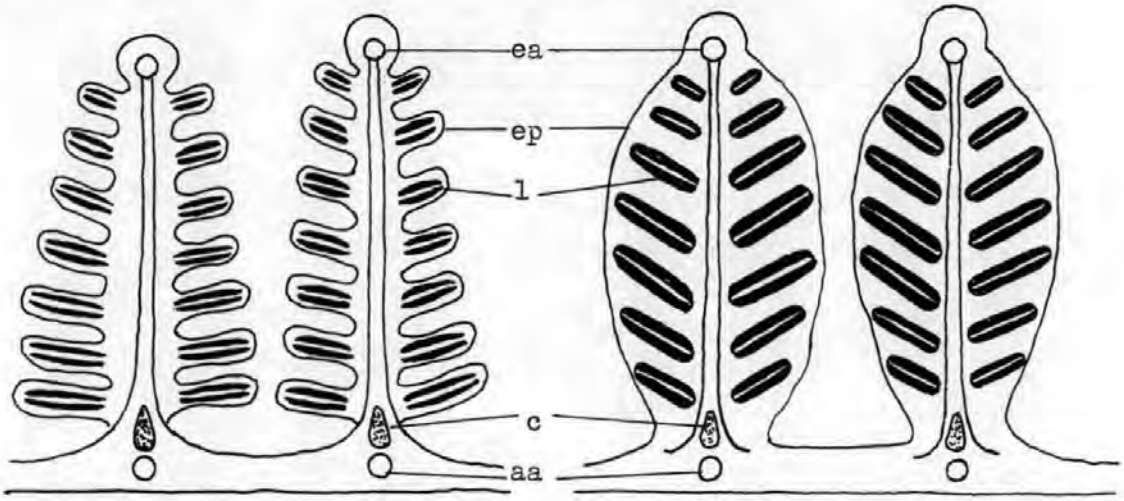
1. a 'free' pseudobranch with lamellae projecting into the buccal cavity and bearing secondary lamellae each covered by buccal epithelium (e.g. Trachinus);
2. the lamellae are still free but are shorter and less freely exposed to the water flow; the buccal epithelium covers each lamellae without sinking between the secondary lamellae (e.g. Chrysophrys);
3. the lamellae no longer project, and are covered by a common veil of buccal epithelium (e.g. Phoxinus);
4. the whole organ is not only buried but has become more or less separated from the superficial epithelium by an overgrowth of connective tissue (Gadus, Cyprinus and others with a 'glandular' pseudobranch).

In those pseudobranchs which are covered by a continuous layer of epithelium the secondary lamellae fuse together down the length of the filament and also between adjacent filaments. The covered or 'enclosed' pseudobranch is completely isolated from the water.

Although progressively modified the histological structure of the teleost pseudobranch remains essentially the same in all types. It still retains the gill-like structure of filaments with secondary lamellae disposed perpendicular to the filament axis, and containing a flat capillary system strengthened by pillar cells. The pseudobranch of Amia and the Teleostei is distinguished by the conversion of the subepithelial cells

Type I

Type II

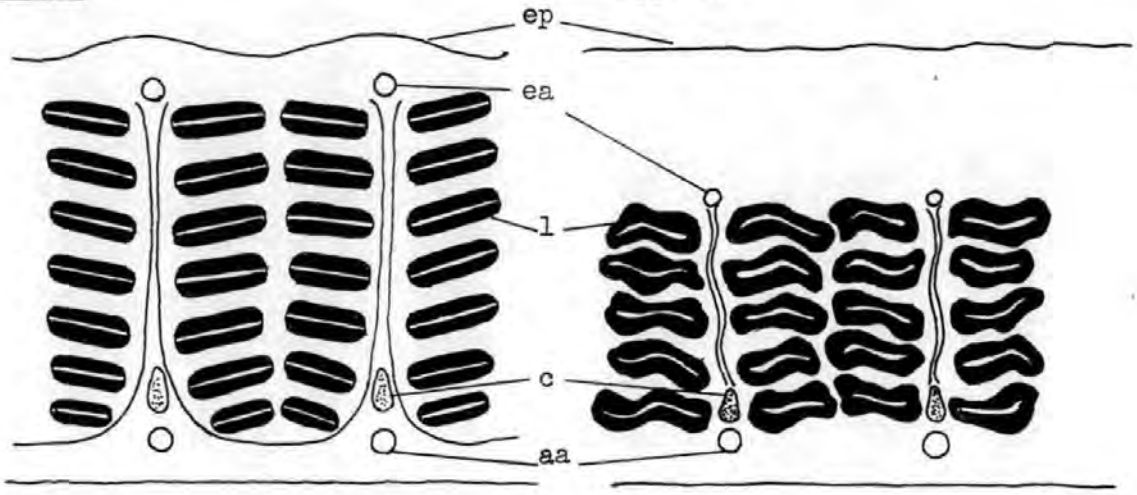


Transverse section of the pseudobranch of Trachinus

Transverse section of the pseudobranch of Chrysophrys

Type III

Type IV



Transverse section of the pseudobranch of Phoxinus

Transverse section of the pseudobranch of Gadus

Fig 4

The four types of teleost pseudobranch according to the classification of Granel (1927)

(ep epithelium: aa afferent artery: ea efferent artery: c cartilage: l pseudobranch lamellae.)

covering the middle vascular layer into large granular secretory acidophil cells (Granel, 1922a, 1922b, 1927; Vialli, 1924b). They are thought to arise from the mesenchyme cell layer which also gives rise to the pillar cells (Granel 1923a).

The acidophil cells of the pseudobranch were thought to be identical or similar to the proposed chloride secretory cells found in the gills by Keys and Willmer (1932), (Leiner 1938; Copeland 1951; Crasse 1958). Holliday and Parry (1962) stated that at the electron microscopic level the structure of the specific cells in both pseudobranch and gill is identical in the flounder Pleuronectes flesus. However their interpretations were not supported by Harb and Copeland (1969) who found two distinct cell types in the pseudobranch of the flounder Paralichthys lethostigma. They described one cell type comparable in all essential details with the chloride cell in the gill of the same fish, and that described in the gills (Doyle and Gorecki 1961; Philpott and Copeland 1963) buccal epithelium (Burns and Copeland 1950) and surface epithelium of other fish (Hendrikson and Matoltsy 1968). The 'chloride type' cells are characterized by their highly branched tubular network, randomly distributed mitochondria, and the presence of an apical pit by which the cell makes direct contact with the water. The other cell type is specific to the pseudobranch ('pseudobranch type' cell) and is identified by the orderly arrangement of parallel tubules around the closely packed mitochondria as described by Copeland and Dalton (1959) for the glandular pseudobranch. The 'pseudobranch type' cell does not possess an apical pit and is isolated from the water.

Further electron microscope studies by Dunel and Laurent (1973) and Mattey, Moate & Morgan (1978) confirmed the presence of two cell types distinguished by their ultrastructural characteristics.

Some of the initial confusion as to the identity of pseudobranch acidophil cells can be explained by the fact that both cell types do not occur in all pseudobranchs. Their presence and distribution within the organ depends on the type of pseudobranch and the habitat of the fish species (Dunel and Laurent 1973). 'Chloride' cells (Type I cells of Dunel and Laurent (1973)) are present when the lamellae are free or partially free (Fig 5a, b, c). This is the usual case for salt water teleosts when the chloride cells are located in regions where they have easy access to the external enviornment through the apical pits in the layer of overlapping epithelium. 'Pseudobranch type' cells (Type II cells of Dunel and Laurent (1973)) are also present in salt water teleosts but are isolated from the water by a layer of epithelium. In fresh water teleosts, the pseudobranchial epithelium, always far from the external environment due to the fusion of the secondary lamellae, only includes 'pseudobranch type' cells (Fig 4d). Only one exception (Perca) has been found to this rule (Dunel and Laurent 1973) where the pseudobranchial epithelium, in contact at a particular point with the water, contains some 'chloride' cells. In all teleosts, whether fresh or salt water, the 'pseudobranch type' cells are always isolated from the external environment. 'Chloride type' cells show the same structural differences between fresh-water and seawater teleosts as those described for the 'chloride' cells of the gill (Philpott and Copeland 1963; Threadgold and Houston 1964; Shirai and Utida 1970; Fearnhead and Fabian 1971). On the contrary, 'pseudobranch type' cells which are identical in fresh water and salt water teleosts, can be considered as morphologically and functionally independent of the external environment (Dunel and Laurent 1973; Mattey et al 1978).

Newstead (1971) in a study of the gills of a tidepool sculpin (Oligocottus maculosus) claimed that under osmotic stress the 'chloride type'

Fig 5

Relationship of 'chloride type' cells (Type I) 
and 'pseudobranch type' cells (Type II)  in 'free', 'semi-free' and 'covered' pseudobranchs. After Dunel & Laurent (1973).

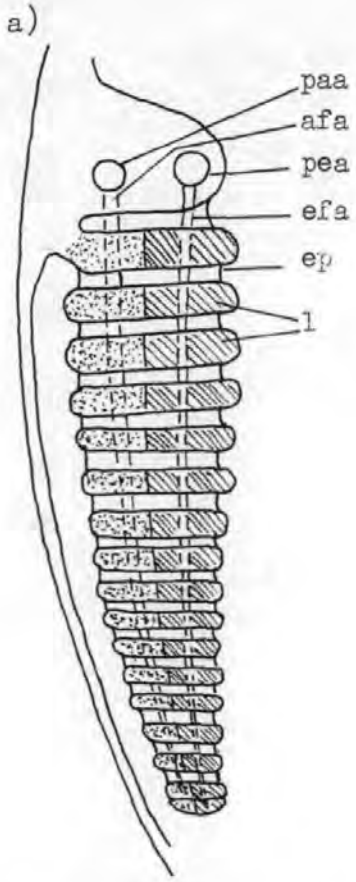
a) Sagittal section of the pseudobranch of a marine teleost (Gobius)

b) Sagittal section of the pseudobranch of a marine teleost (Morone)

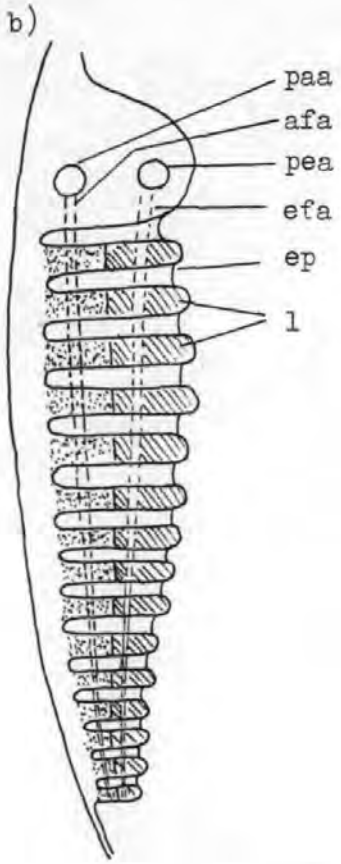
c) Sagittal section of the pseudobranch of a marine teleost (Spondyliosoma)

d) Sagittal section of the pseudobranch of a freshwater teleost (Salmo)

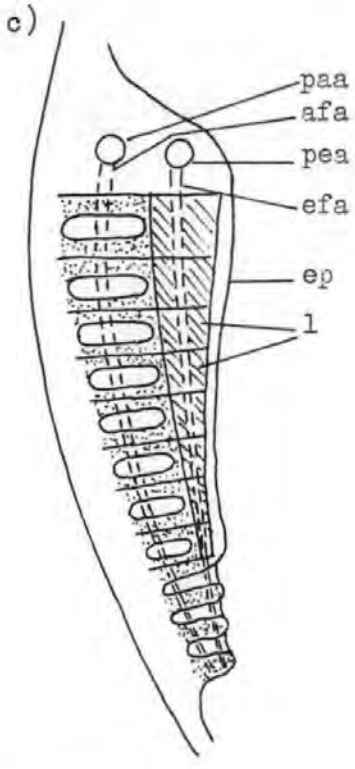
paa	primary afferent artery
pea	primary efferent artery
afa	afferent filament artery
efa	efferent filament artery
ep	epithelium
l	lamellae



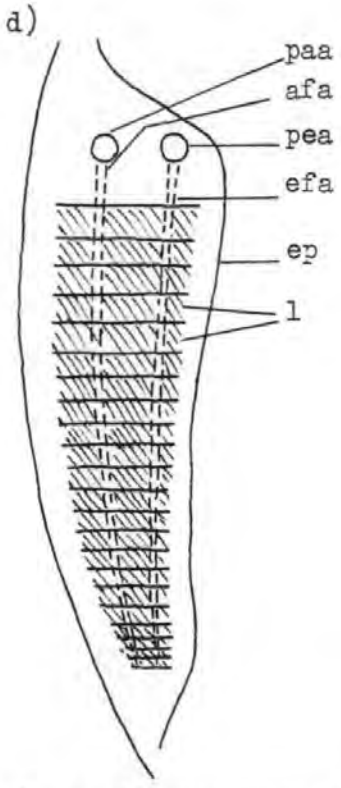
Free pseudobranch (I)



Free pseudobranch (II)



Semi-free pseudobranch



Covered pseudobranch

Fig 5

cells normally found in the gill epithelium could undergo rearrangement of their cytoplasmic components into the pattern characteristic of 'pseudobranch type' cells. He suggested that 'the more intimate association of mitochondria and tubule membrane in the 'pseudobranch type' cells may reflect a shift toward an arrangement more effective in ion transport than in the unorientated arrangement found in 'chloride' cells of unstressed animals'. Mattey et al (1978), in a study of the pseudobranchs of marine euryhaline and freshwater species, noted that although ultrastructural changes occured under osmotic stress, the 'chloride type' and 'pseudobranch type' cells remained distinguishable from each other. They concluded, in agreement with Harb and Copeland (1969), and Dunel and Laurent (1973), that 'chloride type' and 'pseudobranch type' cells are distinct cell types, performing different, though possibly related functions.

Functions

The 'chloride' cell of teleost gills is commonly regarded as the site of extrarenal salt regulation (Smith 1930; Keys and Willmer 1932; Copeland 1948; Philpott and Copeland 1963; Threadgold and Houston 1964; Fearnhead and Fabian 1971; Maetz and Pic 1977; Sardet, Pisam, Maetz 1979). The definitive proof of this assumption is lacking but an abundance of histological data (reviewed by Conte, 1969) and biochemical data (reviewed by Maetz & Bornancin, 1975) strongly suggest that these cells are actively engaged in ion transport. The chloride cells of marine teleosts are thought to actively excrete sodium and chloride ions, whilst in freshwater teleosts net absorption of Na^+ and Cl^- occurs to compensate for the urinary and diffusional loss of these ions (Krogh, 1937; Pettengill and Copeland 1948; Maetz and Romeu 1964; Romeu and Maetz 1964; Maetz 1971). Histochemical evidence has been presented to show accumulation of chloride in 'chloride' cells, particularly in the region of the apical pit (Copeland

1948; Datta Munshi 1964; Philpott 1966; Petrik 1968; Fearnhead and Fabian 1971). Masoni and Garcia-Romeu (1973) used an autoradiographic technique to show the localisation of Cl^- and Na^+ ions in the chloride cells of sea water eel gills, particularly in the apical pole. Shirai (1972) found a difference in the localisation of Na^+ ions between the chloride cells of Japanese eels adapted to seawater and freshwater. It is well known that major changes occuring in the gills of eels transferred from freshwater to seawater include the appearance of increased numbers of chloride cells (Shirai and Utida 1970; Thomson & Sargent 1977) and a parallel increase in the activity of Na^+/K^+- ATPase (Epstein, Katz & Pickford 1967; Utida, Kamiya & Shirai 1971; Thomson & Sargent 1977). There is also an increase in sodium efflux (Forrest, Cohen, Schon & Epstein 1973) and in the Na^+/K^+ exchange which parallels the net Na^+ extrusion rate in seawater adapted eels (Bornancin & de Renzis 1972). Such findings support the idea that the net outflux of Na^+ in seawater eels is due to the presence of chloride cells containing an active Na^+ pump that is manifested as a Na^+/K^+- ATPase in biochemical assays. This supposition is supported by histochemical evidence indicating that Na^+/K^+- ATPase is located in the plasma membranes and smooth endoplasmic reticulum of chloride cells (Mizuhira, Amakawa, Yamashina, Shirai & Utida 1970; Shirai 1972; Hootman & Philpott 1979) and, further, by the demonstration that chloride cells, isolated after treatment of gill tissue with elastase, contain the bulk of the Na^+/K^+-ATPase activity of the gill (Kamiya 1972). Karnaky, Ernst & Philpott (1976a) found that increases in Na/K-ATPase activity roughly parallels the proliferation of chloride cell basal lateral surface membrane. Using an autoradiographic localisation technique with tritiated ouabain, Karnaky, Kinter, L., Kinter, W. & Stirling (1976b) found unexpected increases in basal-lateral enzyme at higher salinities and questioned the long postulated role of the Na^+ pump in Na Cl transport.

Motais and Garci-Romeu (1972) surmised that most gill Na/K-ATPase was associated with the tubular system of the chloride cell and suggested that its function in high salinity environments was solute recycling and water absorption rather than active Na^+ secretion. Moreover Kirschner, Greenwald & Sanders (1974) concluded on the basis of available electrophysiological data that the primary osmoregulatory mechanism in gills of seawater adapted fish is a secretory Cl^- pump and that it may not even be necessary to postulate a secretory Na^+ pump.

Sexton and Russell (1955) suggested that succinic dehydrogenase, found in goldfish gills, was involved in active sodium transportation. However, the direct involvement of succinic dehydrogenase in ion transport was not supported by Banerjee and Mittal (1975) who pointed out that the high activity of this enzyme in the chloride cells is an indication of their high metabolic role which is essential for the efficient functioning of the cells i.e. active transportation of ions. Sargent, Thomson & Bornancin (1975) found that the specific activity of succinic dehydrogenase was 2.5 times greater in cells from seawater adapted eels than in those from freshwater adapted fish. They concluded that the adaptation of the fresh water eels to seawater involves the elaboration not only of a Na^+ pump expressed biochemically as Na^+/K^+-ATPase, but also the energy producing apparatus to drive the pump, expressed biochemically as mitochondrial enzymes including succinic dehydrogenase. This is in accord with the proliferation of chloride cells with their numerous mitochondria in seawater fish.

Apart from changes in enzyme activity and chloride cell number, many studies have also reported cytological changes in response to salt or freshwater adaptation of fish (Getman, 1950; Kessel & Beams 1962; Philpott and Copeland 1963; Shirai & Utide 1970; Fearnhead & Fabian 1971; Olivereau,

1971; Karnaky et al 1976a). The function of the 'chloride type' cell in the pseudobranch has been assumed to be the same as that of the gills (Harb & Copeland 1969; Dunel & Laurent 1973).

The function of the 'pseudobranch type' cell, however, remains uncertain. It appears to have no direct osmoregulatory function since it is isolated from the external environment. Parry and Holliday (1960) objected to the pseudobranch having any osmoregulatory role since fish that had undergone pseudobranchectomy were apparently able to osmoregulate quite normally and showed no significant difference in survival time to control fish, when exposed to various seawater dilutions. However they believed that all the cells of the pseudobranch were the same or similar to the 'chloride' cells of the gill when in fact their experimental fish Salmo gairdneri and Salmo trutta contain only 'pseudobranch type' cells. It would appear, therefore, that these cells, and pseudobranchs containing only these cells, have no direct role in osmoregulation.

As in the chloride cell of the gill there are significant amounts of Na^+/K^+ -ATPase in 'pseudobranch type' cells (Laurent, Dunel & Barets 1968; Bonnet, Bastide & Laurent 1973; Dendy, Deter & Philpott 1973a, 1973b). Laurent et al (1968) found that enzyme activity was located on the inward membrane of the 'pseudobranch type' cells facing the extracellular spaces of the intralamellar areas. These intralamellar areas contain numerous afferent nerve endings. Laurent (1969) found large variations in the membrane potentials of the 'pseudobranch type' cells associated with PO_2 or pH changes, which indicated an ionic redistribution between the intra - and extracellular milieu. He suggested that the extracellular K^+ concentration, a possible activation factor for the adjacent endings is linked to the PO_2, and that the passage of ions at the level of the membrane is easily reversible. He postulated that the most probable mechanism is an ionic

pump, an argument supported by the cytochemical (Laurent et al 1968) and biochemical (Bonnet et al, 1973) confirmation of a very large Na^+/K^+-ATPase activity of a particular type in this membrane. Bonnet et al (1973) reported that the Na^+/K^+-ATPase of 'pseudobranch type' cells was of a different type to that in the chloride cells of the gills. The Na^+/K^+-ATPase from the pseudobranch of trout had a maximum activity at pH 6.4, whilst the maximum activity of this enzyme from the gills of the same fish occured at pH 7.4. They found two different types of enzyme in the pseudobranch of Scorpaena porcus (small scaled scorpion fish) which contains both 'pseudobranch type' and 'chloride type' cells. One type had maximum activity at pH 6.4 whilst the other occured at pH 7.6. They attributed this to Na^+/K^+-ATPase from the 'pseudobranch type' cells and 'chloride type' cells respectively.

Following fractionation by differential centrifugation and rate sedimentation centrifugation, and the use of negative staining of fractions, Dendy et al (1973a, 1973b) concluded that the Na^+/K^+-ATPase of the pseudobranch is localised in plasma membranes. However they assumed that it was associated with the tubular plasma membrane invaginations and did not give a specific location on the membranes of the antivascular pole of 'pseudobranch type' cells as found by Laurent et al (1968). Also, Dendy et al used the brackish water Pinfish, Lagodon rhomboides, which possesses a semi-free pseudobranch so it is likely that their subcellular fractions contained plasma membranes of 'chloride type' cells as well as 'pseudobranch type' cells. Epstein et al (1967) reported 6-fold and 10- fold increases in Na^+/K^+-ATPase activity in microsomal fractions of the gills and pseudobranch respectively, after adaptation from fresh to salt water. This was thought to reflect on increased transport activity.

Copeland and Dalton (1959) expressed the view that the structural specialisation of the 'pseudobranch type' cell is possibly related to the

production of carbonic anhydrase, high levels of which have been described in the pseudobranch by Leiner (1940), Sobotka and Kann (1941), and Maetz (1956). The histochemical localisation of carbonic anhydrase in pseudobranch has been reported by Vervoort (1958) and Laurent, Dunel & Barets (1969). Vervoort found that the carbonic anhydrase activity was restricted to the granular fraction of the acidophil cell cytoplasm and the nuclei of the erythrocytes. Laurent et al (1969) reported localisation of the enzyme at the vascular pole of the pseudobranch cells. They suggested that the carbonic anhydrase activity is related to the tubules opening on the vascular side of these cells.

It has been proposed that a functional relationship between the pseudobranch and the swimbladder may exist via carbonic anhydrase (Fange 1950; Copeland 1951). Fange (1950) reported that carbonic anhydrase inhibitor depresses swimbladder gas release, whilst Copeland (1951) showed that ablation of the pseudobranchs of Fundulus caused inhibition of the swimbladder's ability to regenerate gas by about 50%. However, Laurent and Rouzeau (1972) pointed out that the operation causes such serious circulatory disturbances that it can influence the mechanism of gaseous secretion by affecting the acid-base balance of the animal, this balance being involved in circulatory and respiratory functions. Also, the pseudobranch is not essential for oxygen secretion per se in the swimbladder, since many fish (e.g. the eels, Anguilla, Conger, Muraena) (see Wittenberg and Haedrich 1974) lack pseudobranchs, yet have well developed retia and can secrete gas into the swimbladder.

Fairbanks, Hoffert & Fromm (1969) suggested that concentration of oxygen in the fish eye by the choroid rete mirabile (Wittenberg and Wittenberg 1962) is dependent on carbonic anhydrase. Since the pseudobranch vessels are in series with the choroidal gland (J. Müller 1839) any carbonic

anhydrase secreted by the pseudobranch could be available for the oxygen concentrating mechanism of the eye. However Maetz (1956) reported no evidence of a difference in the concentration of carbonic anhydrase in afferent and efferent blood going to or from the pseudobranch and concluded that it does not secrete carbonic anhydrase. Wittenberg and Haedrich (1974) suggest that the pseudobranch's function in relation to the choroid rete mirabile is to maintain low bicarbonate levels in the blood supply to the choroid rete. This would be important in preventing a rise in carbon dioxide tension in the eye during counter-current multiplication in the choroid rete. They offer the hypothesis that 'the pseudobranch acts to modify the incoming arterial blood in such a way that the choroid rete may concentrate oxygen without simultaneously concentrating carbon dioxide, which when hydrated becomes a strong acid'.

Maetz (1956) measured the pH in the vitreous humor near the retina of the perch (Perca fluviatilis) following injection of the carbonic anhydrase inhibitor acetazolamide, and found decreases in pH from 7.6 to 6.9 in an hour, and to 6.6 after 12 hours. Ballintijn, Beatty & Saunders (1977) found that injections of acetazolamide caused a marked decrease in ocular PO_2 and the amount of visual pigment, as well as body darkening and blindness in Salmo salar (Atlantic Salmon). However these effects are not permanent unlike the similar effects caused by bilateral pseudobranchectomy (Parry and Holliday 1960; Denton and Saunders 1971; Ballintijn et al 1977). Unilateral pseudobranchectomy does not produce these effects because there is an interconnection between the ophthalmic arteries (Ballintijn et al 1977). Thus, the choroid rete of the ipsilateral eye can receive blood from the contralateral pseudobranch.

It is unusual for the pseudobranch to be well developed in teleosts which lack a choroid rete, this condition being found only in certain marine

teleosts such as members of the Myctophidae which have conspicuous gill-like pseudobranchs (Wittenberg & Haedrich 1974). The elasmobranchs which also lack choroid retia have pseudobranchs which do not possess specific 'pseudobranch type' cells (Leiner 1939). This might explain the absence or carbonic anhydrase activity in these pseudobranchs (Leiner 1939). The Holostean, Lepisosteus, which lacks a choroid rete, also possesses a pseudobranch without specific 'pseudobranch type' cells. However, occasional occurrence of a pseudobranch without an accompanying choroid rete led Wittenberg and Haedrich (1974) to postulate the possibility of multiple functions for the pseudobranch.

All fish which lack a pseudobranch but have a well developed choroid rete, inhabit freshwater e.g. Osteoglossiformes (Wittenberg & Haedrich 1974). The Elopiformes, all of which have well developed choroid retia, show a striking gradation in the size of the pseudobranch, from a large, conspicuous organ in marine species to a small or minute organ in brackish or freshwater species. Wittenberg & Haedrich (1974) inferred that the role of the pseudobranch in the choroid rete function of marine species, may be dispensed with in freshwater species.

Parry and Holliday (1960) suggested that the pseudobranch is concerned in some way with the maintenance of the pale colour phase in normal teleost fishes. They found that bilateral pseudobranchectomy or ligation of the pseudobranch blood vessels quickly caused darkening of the fish due to chromatophore expansion and caused degeneration of the choroid gland after some weeks. They also found that injection of extracts of homogenised pseudobranchs into pseudobranchectomised fish caused various degrees of temporary local paling around the site of injection, and isolated chromatophores contracted when placed in saline extracts of horse mackerel and cod pseudobranchs. They concluded that the pseudobranch produces or activates a hormone affecting the chromatophores and that entry of this

hormone into the general circulation is controlled by the choroid gland.

The investigations of Laurent and Dunel (1964, 1966) and Barets, Dunel & Laurent (1970) showed that the pseudobranch is richly innervated and they suggest a functional relationship between afferent type nerve endings and the pseudobranch cell. The discovery of a group of receptors in the pseudobranch (Laurent 1967, 1969; Laurent and Rouzeau 1969, 1972a) has led to the proposal that the pseudobranch is involved in chemoreception and baroreception. These studies, reviewed by Laurent 1974, show the presence of neural plexuses located within the secondary lamellae. These networks are formed by the dendritic systems of many small bipolar neurons located at the bases of the secondary lamellae whose axons converge towards large multipolar association neurons located in the axis of each filament. The axons of these association-neurons pass toward the central nervous system via the glossopharyngeal or facial nerve according to the species. The neural plexuses organised at the level of the primary efferent vessel are at the origin of several types of innervation. Up to now, four types have been distinguished (Fig 6) (Laurent and Rouzeau 1972a).

The most important innervation is the interlamellar plexus located at the antivascular pole of the pseudobranch type cells (Laurent and Dunel 1964, 1966; Barets et al 1970). It consists of nonmyelinated fibres and in the case of pseudobranchs with fused lamellae is common to two continguous lamellae being located within the lacunar tissue which separates two neighbouring layers of pseudobranch type cells. This network represents the dendritic system of bipolar neurons located at the base of the lamellae in the filament axis, and is thought to be afferent. Two other types of afferent innervation have been distinguished - aborescent endings in the wall of the primary afferent artery and specialised neurons in the wall of the primary efferent artery in marine teleosts. Finally, an efferent

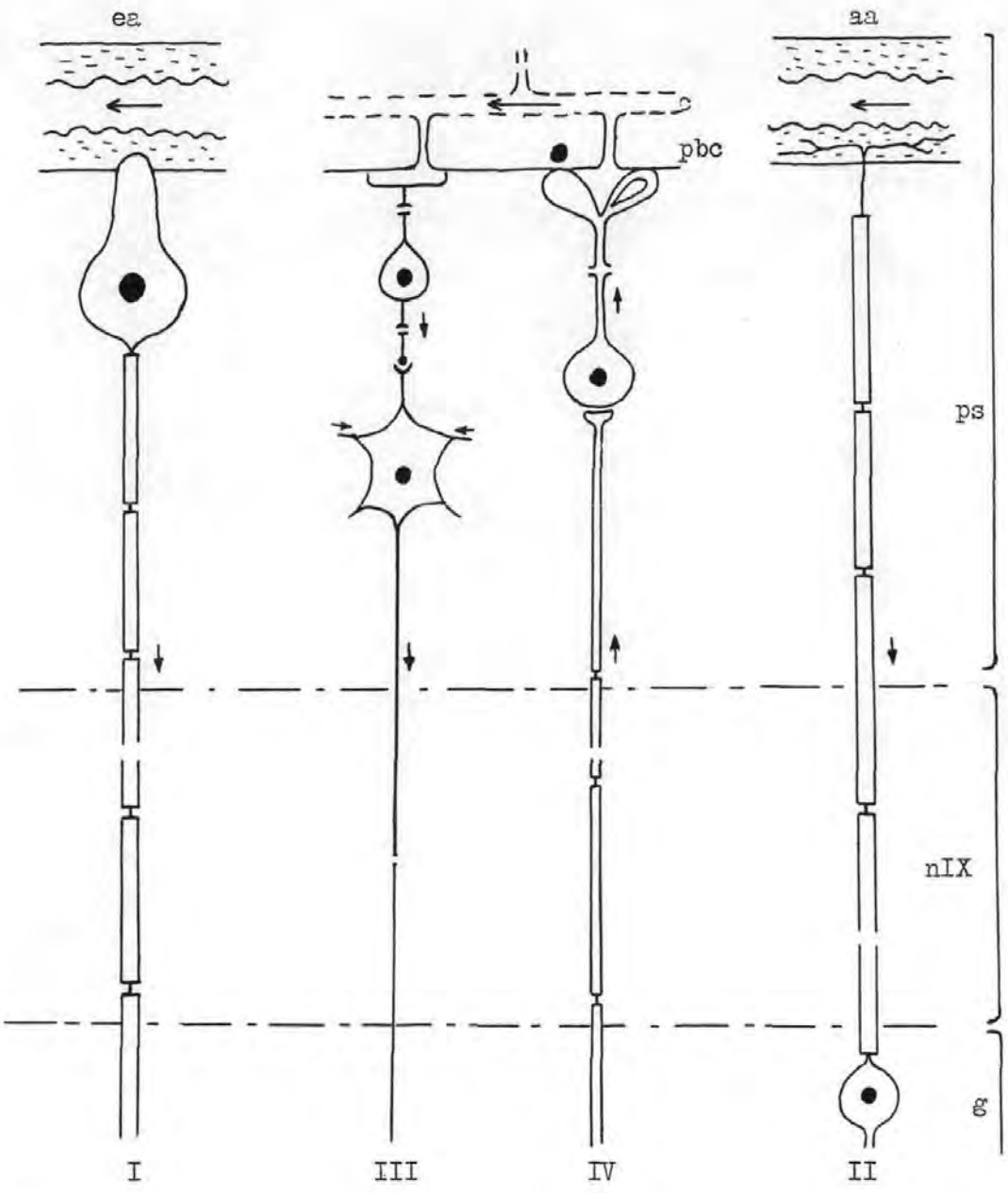


Fig 6

Diagram of the pseudobranch innervation in teleosts. aa afferent artery, ea efferent artery, c capillary, pbc pseudobranch cell, ps pseudobranch, nIX glossopharyngeal nerve, g glossopharyngeal ganglia. (After Laurent & Rouzeau, 1972). I. Afferent possible sodium receptor system. II. Afferent baroreceptor system. III. Afferent probable O_2, H^+ and osmotic receptor. IV. Efferent neural system.

innervation possibly controls the functioning of the 'pseudobranch type' cells as is suggested by the presence of endings filled with cholinergic-type vesicles in the interlamellar region.

In the Selachian, Torpedo marmorata, the large sinuses are bordered by characteristic cells named "chief cells" near which large neurons and nerve fibres are located (Delage 1974; Laurent 1976). These "chief cells" are fairly large clear cells up to 15 microns across with rounded nuclei. They are separated from the blood by only the thin endothelium of the sinus wall (0.1 µm) and contain glycogen granules and dense cored vesicles (1000 Å diameter). The "chief cells" send long processes which make synaptic contacts with dendrites of large neurons. The synaptic junctions show large presynaptic membrane densities associated with dense-cored vesicles and small clear vesicles. The neuron overlaps the "chief cell" process but the reverse situation can be observed. The neurons are of multipolar type and are often linked together by 'gap junctions'.

Nerve bundles originating from the facial nerve penetrate the pseudobranch parenchyma. The course of these nerves is parallel to the longitudinal axis of the primary lamellae. Degeneration experiments show that a part of these axons are afferent and consequently constitute the axons of intramural neurons (Laurent, pers. comm.).

The association of this innervation with a specialized vascular system constitutes an arterial receptor structurally somewhat different from the corresponding system of the teleost pseudobranch but possibly equivalent from a physiological point of view. Neurophysiological data is not yet available for the Selachian pseudobranch.

In the teleost pseudobranch it has been confirmed that a centripetal neuronal activity originates in the organ (Laurent & Rouzeau 1972a; Laurent 1974). The overall afferent activity can be differentiated into two types

characterized by the amplitude of their compound action potentials. The first type (Type A) has spike potentials of relatively large amplitude (from 100 - 200 microvolts), the second type (Type B), has spikes of lower amplitude (50 microvolts down to noise level).

Abrupt changes in hydrostatic pressure in perfused pseudobranch influence type A activity but not type B. Type A activity is thought to emanate from afferent vessel baroreceptors on the basis of the relationship between fibre diameters and spike potential amplitudes.

Decrease of PO_2 in the perfusing fluid causes type B activity to increase in a hyperbolic manner, but type A activity is unaffected. The change in B activity appears rapidly (less than one second) and disappears as rapidly when normoxic perfusion is restored. Other parameters such as pH and osmotic pressure also influence type B activity. Type B activity is thought to be related to the terminal plexus receptors of the secondary lamellae.

The results obtained on isolated perfused pseudobranch have been confirmed by experiments made on "in situ" pseudobranch of anaesthetized rainbow trout (Laurent & Rouzeau 1972b; Laurent 1974). Activity from such a preparation appeared to be much more clearly influenced by PO_2 than under in "vitro" conditions, due to closer physiological conditions, with the blood carrying the hypoxic stimulus.

Laurent (1974) suggested that the functional concept of the pseudobranch as a chemoreceptor (Laurent 1967) is very close to that proposed for the carotid body (Biscoe 1971), a mammalian arterial chemoreceptor. Granel (1927) noted that the clusters of acidophil cells found in the pseudobranchs of teleosts resemble the glomus tissue and chemoreceptor cells of the carotid body. However later studies with the electron microscope have shown that the ultrastructure of these cells are very different (c. f. Dunel & Laurent

1973: Biscoe 1971). Despite this Laurent (1974) believes the pseudobranch and carotid body to be closely related physiologically.

The main controversy in the study of fish oxygen receptors has always been that of a central (i.e. brain or aorta) versus a peripheral (i.e. gills or pseudobranch) site for the receptors.

Randall and Jones (1973) found that deafferentation of the pseudobranch in rainbow trout had no significant effect on either the magnitude or rate of response to hypoxia or hyperoxia. They concluded that chemoreceptors in the pseudobranch, innervated by the glossopharyngeal nerve, play little or no part in the regulation of ventilation in fish during hypoxia or hyperoxia. The experiments of Bamford (1974) also failed to support the view of Laurent and Rouzeau (1972) that the pseudobranch is an important oxygen receptor. Bamford (1974) found that bilateral removal of the pseudobranchs of rainbow trout did not affect the response of these animals to hypoxia. He suggested that the pseudobranch's other functions make it oxygen sensitive, but this sensitivity appears to have little physiological significance. Satchell (1961) found that the response of dogfish to anoxia was unimpaired after the pseudobranchs were: (a) surgically removed; (b) extensively cauterized; (c) frozen; (d) rendered ischaemic by cutting or clipping the afferent pseudobranchial artery.

In support of the pseudobranch's role as an oxygen receptor Davis (1971) showed that ligation of the first gill arch caused a greater drop in arterial PO_2 than when other gill arches were ligated and the first gill arch left intact. Fish with blood flow to arches 1 and 3 only could maintain arterial PO_2 at 90 - 100 mm Hg whereas in those with blood flow to 3 and 4 only, arterial PO_2 fell to around 40 mm Hg. Since the pseudobranch receives its blood supply from an extension of the first

efferent artery (Goodrich 1930) bilateral ligation of arch 1 would rob both pseudobranchs of their blood supply and lead to degeneration. Those fish with arch 1 ligated had no functional pseudobranch and were unable to maintain arterial PO_2 levels during reduction of gill surface area. This led Davis (1971) to suggest that the pseudobranch may well serve as an important site in the regulation of arterial oxygen tension.

The existence of four types of pseudobranch, and of marine and freshwater fish with and without choroid retes, as well as the absence of the pseudobranch in some species, all combine to offer a range of "natural experiments" from which it should be easy to deduce the role of the pseudobranch. However this has proved to be far from the case, and many questions still remain to be answered.

The present picture of the pseudobranch, is that of an organ with a number of possible functions, related to the habitat of the fish and the structural modification of the pseudobranch. These functions may include a role in osmoregulation, monitoring and regulation of blood flow and composition with respect to the gills and/or eye, and receptor systems linked directly or indirectly to the vascular system. The possibility of an endocrine function should also be considered.

MATERIALS AND METHODS

Fish Species

The pseudobranchs of ten species of teleost fish were examined. Four freshwater species were used:-
rainbow trout (Salmo gairdneri Richardson), carp (Cyprinus carpio L.), roach (Rutilus rutilus L.) and perch (Perca fluviatilis L.); and six marine species: bass (Dicentrarchus labrax L.), grey mullet (Mugil capito Wheeler), smelt (Osmerus eperlanus L.), pollack (Pollachius pollachius L.), garfish (Belone belone L.) and mackerel (Scomber scombrus L.) The fish were identified with the aid of Wheeler (1969).

Collection

Rainbow trout (250 - 350 g) and carp (500 - 1200 g) were obtained from commercial fish suppliers. Roach (35 - 45 g) and perch (25 - 40 g) were collected from Slapton Ley lake by electrofishing whilst grey mullet (10 - 25 g) and smelt (8 - 15 g) were caught using a seine net at St. John's Lake (Fig 7). Bass (60 - 180 g) were caught with a rod and line at Warleigh Point whilst pollack (110 - 160 g), garfish (95 - 200 g) and mackerel (200 - 400 g) were caught in the same way at Devil's Point. Fish lightly hooked in the mouth were returned to the aquarium whilst badly damaged specimens were killed, the pseudobranchs were removed and placed in bottles of fixative.

Maintenance of Fish

Rainbow trout, carp, roach and mullet were fed on a variety of commercially available dried foods, usually in the form of pellets. Bass smelt and perch were fed on macerated fish (usually coalfish or whiting) which was stored deep frozen until needed, and occasionally on small fish, crabs or shrimps when available.

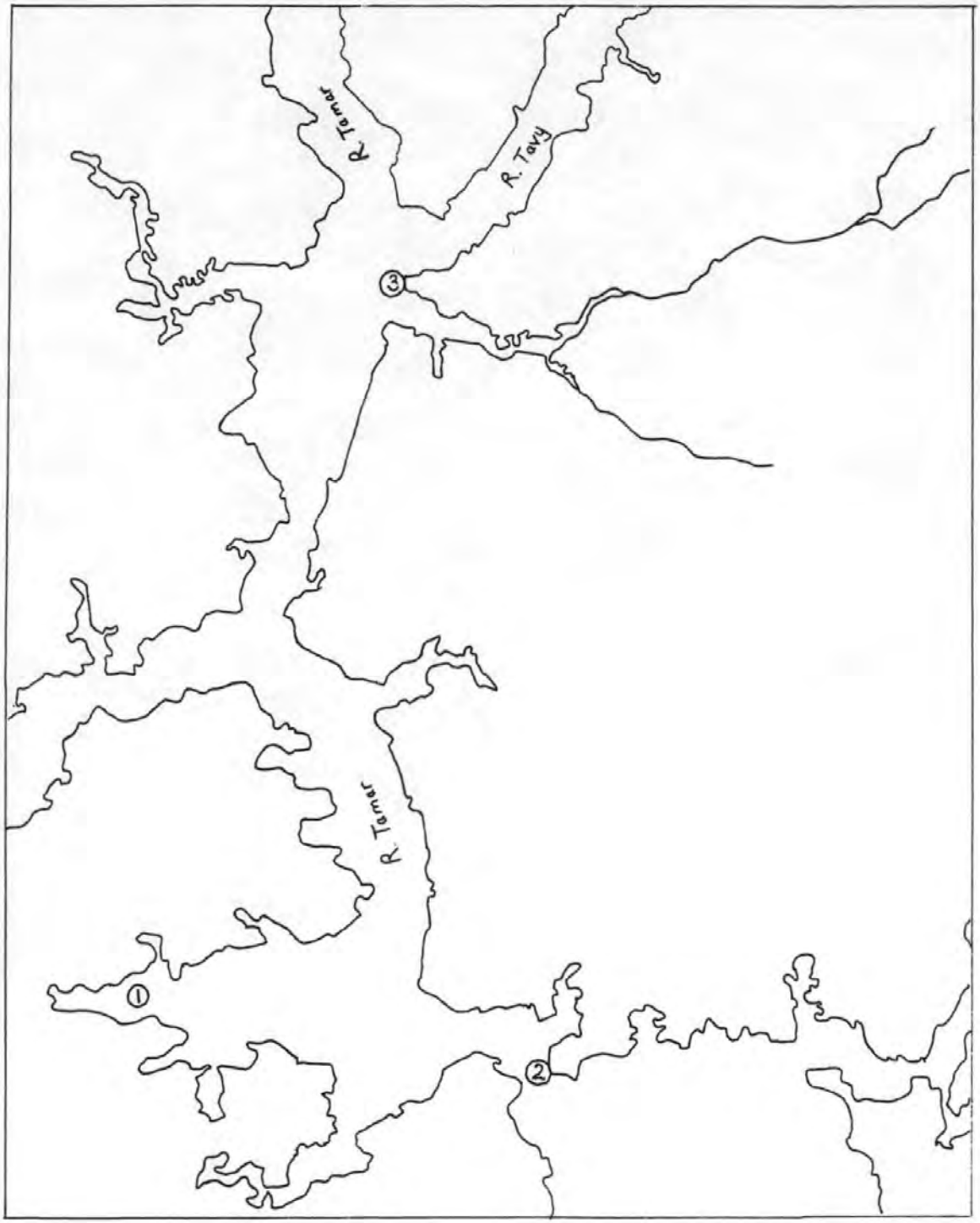


Fig 7 Collecting sites near Plymouth

Key

(1) St. John's Lake.

(2) Devil's Point.

(3) Warleigh Point.

Morphological and Cytological Investigations

Microfil injection

Vascular casts of the pseudobranch were made using a silicon rubber compound, Microfil, obtained from Canton Medical Products, Colorado, U.S.A. Two methods were used.

1. The fish was anaesthetized with MS222 (Sandoz) and the heart and ventral aorta were exposed through a ventral incision. A catheter was inserted in the end of the bulbus arteriosus and the auricle incised. The catheter was connected to a syringe containing heparinised saline. The gills and pseudobranchs were perfused at 5 - 10ml/min until devoid of blood. The catheter was then connected to a syringe filled with Microfil which was perfused at the same rate until the Microfil exuded from the auricle, and the pseudobranchs were seen to be filled. The perfusion was stopped and the auricle ligated to prevent Microfil from flowing out of the injected vessels. In some cases especially in small fish, it was difficult to fill the pseudobranchs completely due to the resistance of the gills and a build up of back pressure causing blockage or bursting of the vessels. A second method was tried by filling the pseudobranch directly.

2. After anaesthesia and bleeding of the fish, the pseudobranchs were carfully removed, taking care to leave the afferent and efferent vessels intact. A catheter was placed in the afferent artery and the organ was perfused with Microfil until filled, when the efferent artery was ligatured. In some cases the pseudobranch was perfused via the efferent artery.

Polymerisation of the Microfil injected organs was carried out by leaving them in a refrigerator overnight. They were then carefully dissected and cleared by immersion for 24 hour periods in successive mixtures of glycerol/water; $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$ and finally pure glycerol. The tissues were

dissected, studied and photographed with a Zeiss Tessovar low power photomicroscope using Ilford Pan F film at A.S.A. 50.

Histological Methods

Light Microscopy

For routine histological examination whole pseudobranchs were fixed in Bouin's fixative (24 - 48 hours) or neutral buffered formalin (24 hours - 1 week), washed in 70% alcohol, dehydrated in a graded series of alcohols, cleared in xylene and embedded in paraffin wax (melting point 56°C). Sections were cut using a Leitz rotary microtome at 6 - 10 µm, dewaxed in xylene and stained with Mayers haematoxylin and eosin or Mallory's triple stain, to show general structure.

Histochemistry

The periodic acid Schiff's technique (P.A.S.) (Pearse 1968, after McManus) was used to test for polysaccharides, while acid mucins were differentiated by the alcian blue stain at pH levels 0.2 - 2.5 (Pearse 1968) and by the alcian blue critical electrolyte concentration method (C.E.C.) (Scott and Dorling 1965) using increasing concentrations of magnesium chloride. Schiff's reagent without oxidation was used to test for free aldehyde (Pearse 1968). The Sudan black B technique (Pearse 1968, after McManus 1946) was used for lipids.

Fresh material was frozen using solid CO_2 and cut at 10 µm on a cryostat. The calcium cobalt method of Gomori (1952, see Pearse 1968) was used for the demonstration of alkaline phosphatase. Succinic dehydrogenase activity was examined using the nitro - B.T. method (Pearse 1972).

Sections were examined and photographed using a Zeiss Photomicroscope II.

Nerve Histology

Pseudobranchs were fixed in three different fixatives depending on the silver staining technique to be used.

10% formalin, (Bielchowsky 1904, 1909; block staining. See McClury Jones 1929).

10% formol saline (Gros-Bielchowsky; frozen or paraffin sections. See Carleton & Drury 1957).

50 ml absolute alcohol + 2 - 3 drops of ammonia (Cajal formula 3, 1910; block staining. See McClury Jones 1929).

Osmium tetroxide vapour was used to stain myelin of the pseudobranch nerve and its branches.

Electron Microscopy

Transmission Electron Microscopy

Material was routinely fixed by immersion in a solution of 2.5% glutaraldehyde in either 0.1M Sorensen's phosphate buffer (Gomori 1955) or 0.1M cacodylate - HCl buffer (Gomori, 1955; Sabatini et al 1963) at pH 7.2 - 7.4 and 4°C for 1 hour. This was followed by three washes in buffer containing 4 - 5% glucose and post fixation for 1 hour at 4°C in 1% buffered osmium tetroxide. The fixative and buffer solutions were calculated to be slightly hypertonic to the fish tissues (Holmes & Donaldson 1969) using graphs relating molarity with pH and osmolality (Maser, Powell and Philpott, 1967). For freshwater fish the final osmolality of the fixative and buffer solutions was 375 - 500 milliosmols whilst for seawater fish it was 410 - 550 milliosmols.

Tissues were dehydrated in a graded series of alcohols, followed by propylene oxide, and embedded in either Spurr (Spurr 1969) or Epon resin. Sections 1 µm thick were cut on a Porter-Blum MTB - 2 Ultramicrotome, and

stained with 1% methylene blue in 1% borax for examination in the light microscope. Thin sections (60 - 90 nm thick) displaying gold or silver interference colours were expanded with chloroform vapour and collected on uncoated copper or nickel grids. The grid mounted sections were stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963; Sato 1967) and examined in a Philips E.M. 300 transmission electron microscope at 80 KV. Observations were also carried out at lower accelerating voltages (40 and 60 KV) in cases where contrast was low or when unstained sections were used (e.g. in some histochemical techniques).

Scanning Electron Microscopy

Intact pseudobranchs were rinsed in distilled water and fixed for 2 days - 1 week at 4°C in 2.5% glutaraldehyde in 0.1M phosphate or 0.1M cacodylate buffer (pH 7.4). They were given three washes of 30 minutes each in buffer and dehydrated in a graded alcohol series, followed by immersion in amyl acetate. The tissues were critically point dried in a Samdri PVT3 using carbon dioxide as the transitional fluid, and mounted on brass stubs with double sided tape. They were sputter coated with gold in a Polaron E5100 Coating Unit and examined in a Jeol 35C scanning electron microscope.

Histochemistry

(a) Positive Staining

For the localisation of chloride ions, tissues were fixed in 1% silver acetate in 1% osmium tetroxide (Philpott 1966) before normal processing for electron microscopy. Sodium ions were localised following fixation in 1 volume of 4% osmium tetroxide and 2 volumes of 2% potassium pyroantimonate (after Shirai 1972). Some thin sections were stained with uranyl acetate and lead citrate while others were left unstained to avoid confusing reaction product with artefacts that might arise from uranyl or

lead staining.

Thin sections were stained with phosphotungstic acid (P.T.A.) at pH 1 - 2 to stain for acid mucopolysaccharides (Marinozzi 1967, 1968; Pease 1970) or at pH 0.3 to stain for glycoproteins (Tsuchiya and Ogawa 1973). The periodic acid-chromic acid-silver method of Rambourg, Hernandez & Leblond (1969) was used to stain thin sections for polysaccharides.

Whole pseudobranchs were immersed in unbuffered 2% osmium tetroxide for 48 hours to stain the Golgi apparatus (selectively) and endoplasmic reticulum (Friend & Brassil 1970).

(b) Enzyme Histochemistry

Carbonic anhydrase activity was demonstrated using a modified Hansson (1967) method (see Hayat 1974). Alkaline phosphatase was located using the lead citrate method of Mayahara, Hirano & Saito (1967) (see Pearse 1972). Na^+/K^+ ATPase was detected using the modified method of Shirai (1972) based on Farqhar & Palade's (1966) modification of the original Wachstein & Meisel (1957) method.

Experimental Investigations

Effect of Salinity

To ascertain if drastic osmotic stress could cause rearrangement of 'chloride' cell structure into that characteristic of 'pseudobranch type' cells a similar experiment to that of Newstead (1971) was carried out on bass, Dicentrachus labrax (L). Fish measuring 20 - 25 cm were exposed to 0.34‰ salinity without previous acclimation to dilute sea water, for periods of 5, 10, 24 and 48 hours. Control fish were kept in full sea water of 34‰ salinity. All fish were held at 18 - 20°C in continuously aerated water.

More gradual acclimation to different salinities was carried out with smelt, Osmerus eperlanus L., and rainbow trout, Salmo gairdneri Richardson. Bass were not used because of unavailability at the time.

Smelt, approximately 12 cm long were held in a closed circuit seawater (34‰) tank in the laboratory at 18 - 20°C. Groups of fish were acclimated to salinities of 24‰, 16‰, 8‰ and fresh water. Each group was acclimated gradually (over 15 hours at each stage) to their respective target salinities where they were held for up to 36 hours.

Rainbow trout approximately 20 cm long were maintained in the laboratory in a closed circulation system of freshwater at 10-12°C. Groups of fish were acclimated (over 15 hours at each stage) to salinities of 10‰, 20‰, 34‰, 42‰ and 60‰ salinity, where they were held for 36 hours.

All fish were killed by a blow on the head. The pseudobranchs were removed intact and processed for light and electron microscopy as previously described.

Denervation Studies

Rainbow trout were chosen for denervation studies because of the easy accessibility of the pseudobranch nerve (Plate 2-1). For nerve section an anaesthetized fish was approached ventrally. The operculum was pulled back to expose the pseudobranch and gill arches. The IX nerve was located running ventro-medially in a groove formed at the anterior margin of the opercular opening and the body wall. The nerve supply to the pseudobranch leaves the IX nerve peripheral to the ganglion and near the pseudobranch runs close to the efferent artery (Fig 8). The pseudobranch nerve was exposed by a 2 mm incision and sectioned close to its point of entry to the pseudobranch. Bilateral section of the nerve was completed within a few minutes. Fish were allowed to recover from the operation and replaced in the aquarium. Pseudobranchs were subsequently

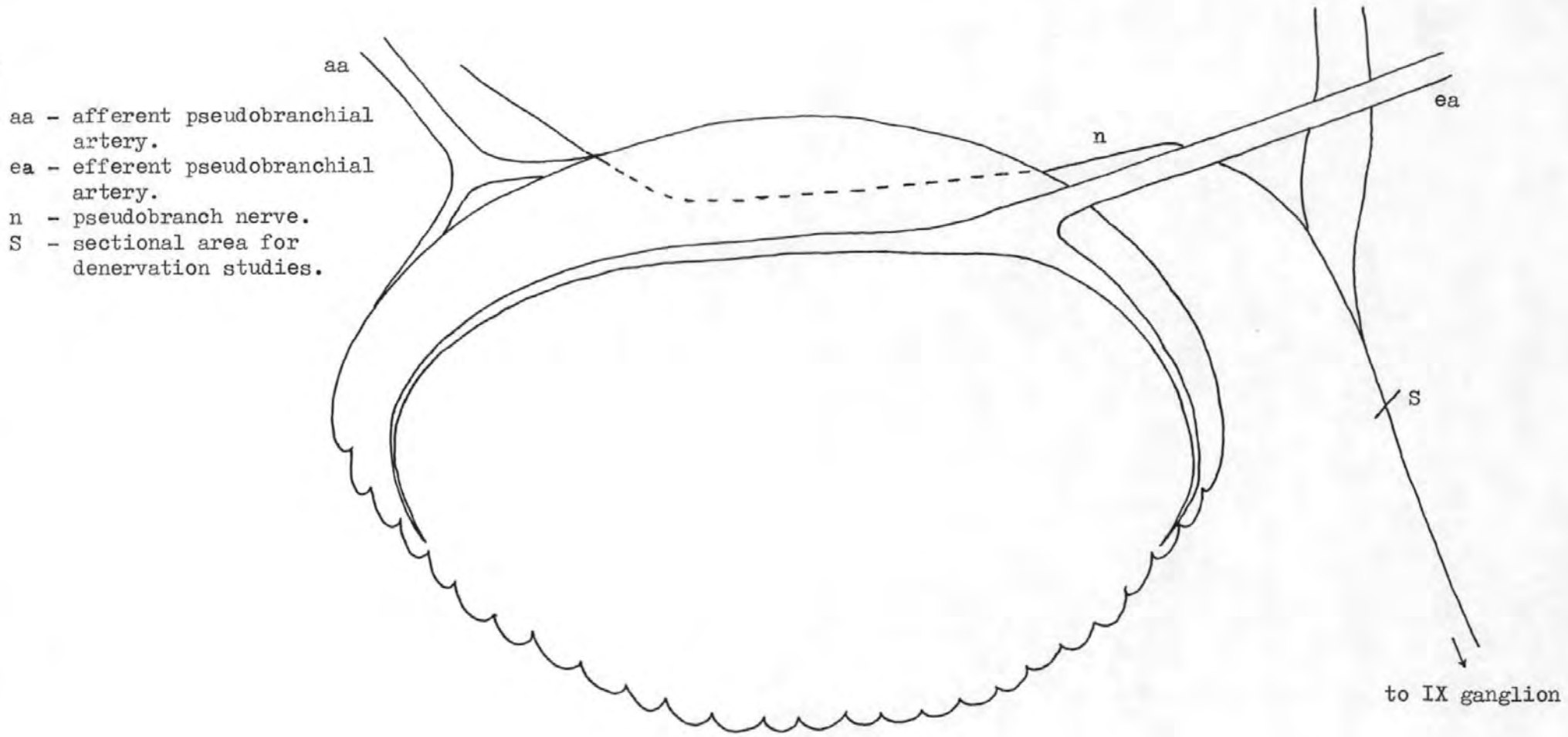


Fig 8 Schematic representation of trout pseudobranch innervation.

removed from fish at time intervals ranging from 24 hours to 15 weeks and processed for electron microscopy as previously described.

Effect of Salinity Changes on Denervated Trout

Preliminary experiments by Laurent (see Laurent & Rouzeau 1972) indicated that the survival of trout, normally indefinite in 50% seawater, does not exceed several hours after bilateral sectioning of the pseudobranch nerves. In the present study 3 rainbow trout were acclimated to 50% seawater for 48 hours before denervation whilst 3 others were transferred directly from freshwater to 50% seawater after denervation. After 7 days, denervated fish in 50% seawater were transferred to either 100% seawater or back to freshwater.

Effect of Pseudobranchectomy on Rainbow Trout

Both pseudobranchs were removed (bilateral pseudobranchectomy) from fish under anaesthetic and the fish were allowed to recover after the operation. Operated fish were either kept separated from stock fish or returned to the stock tank after allowing a period of recovery (1 - 2 weeks). In some cases only one pseudobranch was removed (unilateral pseudobranchectomy). Observations were made on the effect of bilateral and unilateral pseudobranchectomy on the sight, colour, ventilation rate, feeding and activity of the fish.

Acetazolamide Injection

Five rainbow trout (250 - 300 g) were injected with the carbonic anhydrase inhibitor, acetazolamide, at concentrations ranging from 1 to 5 mg/kg. The sodium salt of acetazolamide (Serva Biochemicals Ltd.) was dissolved in sterile teleost Ringer (Wolf 1963) and injections of 0.2 ml were given intramuscularly in the caudal region of anaesthetized trout. Fish were revived and replaced in the aquarium. Pseudobranchs were

subsequently removed from fish at regular intervals between 4 hours and 96 hours after injection and processed for electron microscopy.

Injection of 5-hydroxydopamine and 6-hydroxydopamine

To examine the possibility of a sympathetic innervation of the pseudobranch a number of rainbow trout were given injections of either 5-hydroxydopamine (5-OHDA) or 6-hydroxydopamine (6-OHDA). 5-OHDA has been used as a specific E.M. marker of adrenergic nerve endings where it accumulates in the form of large dense vesicles (Tranzer & Thoenen 1967; Gordon-Weeks 1977). The compound 6-OHDA is known to promote a specific and reversible degeneration of nerve terminals of the sympathetic nervous system (Krostrzewa & Jacobowitz 1974) and has been extensively used in various species to promote a 'chemical sympathectomy'.

Rainbow trout (250 - 300 g) were given i.p. injections of 5-OHDA HCl or 6-OHDA HCl (Sigma, 80 mg/kg in 0.2 ml teleost ringer). Control fish were injected with teleost ringer only. The pseudobranchs of fish injected with 5 OHDA were removed after 4 hours and 24 hours and processed for E.M. examination. 6-OHDA injected fish were given a second injection after 16 hours and the pseudobranchs were removed after 24 hours and 48 hours.

Electrophysiological Studies

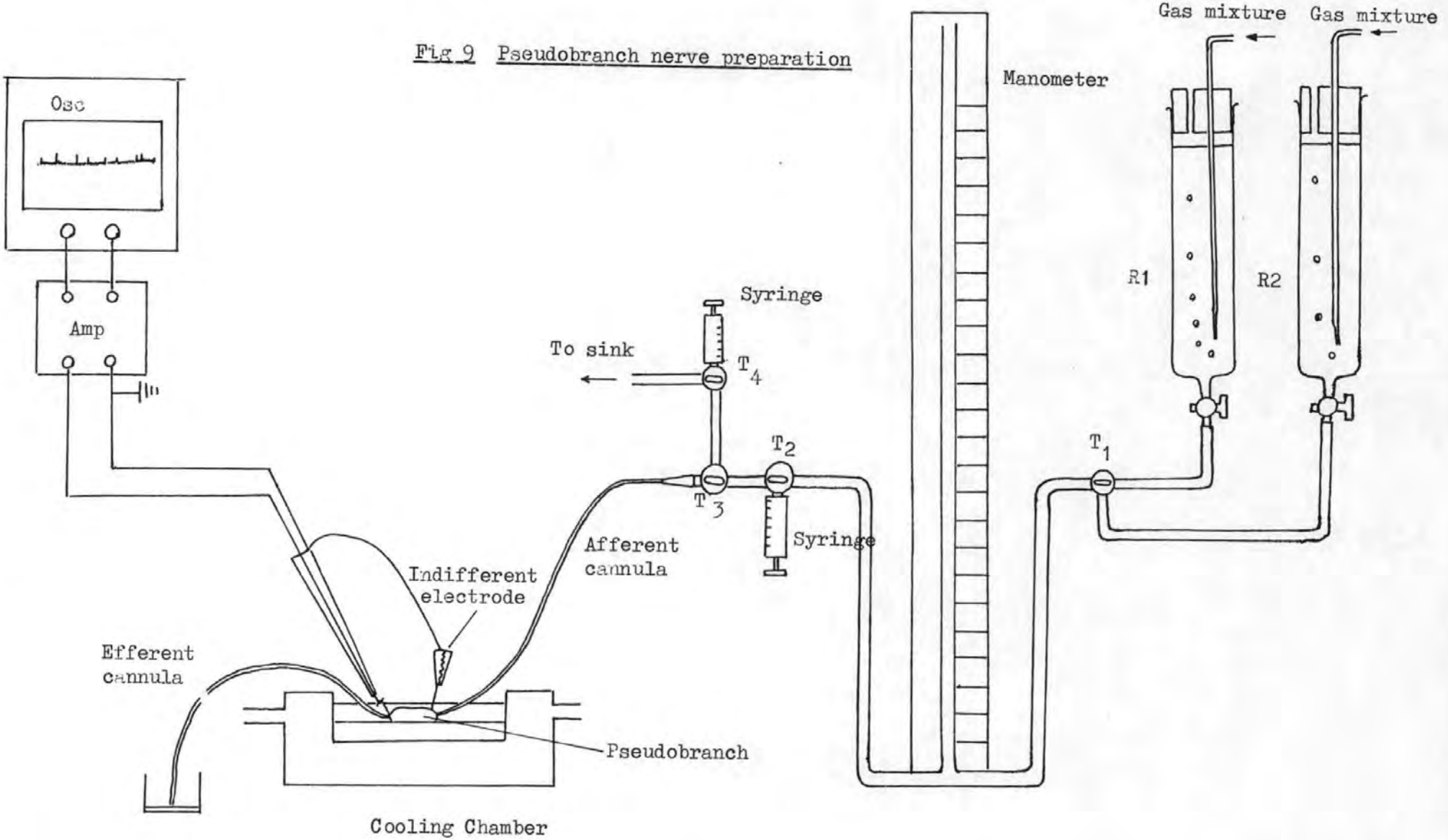
Rainbow trout (250 - 300 g) were chosen as the experimental fish for recording from the pseudobranch nerve. Besides the easy accessibility of the pseudobranch nerve the afferent and efferent pseudobranch vessels are large enough for cannulation with a 27 gauge hypodermic needle.

Pseudobranch Preparation

Before an experiment, anaesthetized fish were given an intracardial injection of heparin (0.1 ml of 5000 U.S.P. units/ml). After a few minutes the blood was removed by opening the body cavity and sectioning the dorsal aorta. The fish was laid on its side and the operculum held back. The sub-opercular epithelium was cut away to within 2 or 3 mm of the edges of the pseudobranch. The epithelium was detached from the operculum taking the pseudobranch with it. The afferent artery was sectioned at the point where it disappears into the cartilage of the operculum. The pseudobranch nerve was sectioned as near as possible to the glossopharyngeal ganglion leaving up to 8 mm of nerve attached to the pseudobranch. The pseudobranch was quickly excised by cutting the efferent vessel and its surrounding tissue.

After removing the pseudobranch (4 - 5 minutes) with its nerve and blood vessels, it was transferred to a temperature regulated chamber (Fig 9 , Plate 1-1.2). The perfusion apparatus is shown in Fig 9 . Reservoirs 1 and 2 contain perfusion solutions which can be gassed with different gas mixtures. The preparation can be perfused from either reservoir and a 3 way tap is inserted (T_1.) to allow change over from one reservoir to another. To flush the tubing with fresh perfusion fluid another tap can be opened at T_4. Samples of perfusion fluid for analysis can also be taken from T_4 using a syringe inserted into the tap. A manometer tube (M) is incorporated into the system to measure the pressure of solution perfusing the preparation. Another tap (T_3) controls the entry of perfusion fluid into the catheter tubing. Perfusion solution samples were monitored using an IL.413 pH/blood gas analyser modified for measurement at various temperatures.

Fig 9 Pseudobranch nerve preparation



The afferent artery of the pseudobranch was cannulated with a 27 gauge hypodermic needle attached to a length of polyethylene tubing. The organ was initially perfused with teleost Ringer (after Laurent & Rouzeau 1972, see Perfusion solutions) gassed with 30% O_2 in N_2, at a pressure of 26 cm of water (Laurent & Rouzeau 1972). When all traces of blood had been washed out of the organ, an exit catheter was introduced into the efferent artery and connected to a short length of tubing with its opening 5 cm below the preparation. The pseudobranch was covered with teleost Ringer and chilled to the required temperature. Experiments were carried out at 0, 5, 10, 15 and 20°C.

Nerve Preparation

The epithelium surrounding the pseudobranch nerve was cut away to a point very near the pseudobranch. Small branches of the nerve running away from the pseudobranch were drawn aside and the main nerve drawn up into paraffin oil and hooked over the electrode(s). Because the preparation of isolated units, especially the smallest fibres (0.1 µm) was very difficult, all the experiments were carried out using multifibre recordings. Sensory activity was recorded from the whole nerve or groups of fibres by means of a monopolar platinum electrode attached to a differential preamplifier (Tektronix 5113). Recordings were stored on magnetic tape on a Sony stereo cassette recorder (TC-131SD). They were photographed with a C50/70 series Polaroid camera (Tektronix), at various scanning speeds, making use of the oscilloscope storage facility.

Measurement of conduction velocity was based on the simultaneous monopolar recording of spontaneous activity at two points on the nerve by silver electrodes connected through preamplifiers to each of the beams on the oscilloscope.

Perfusion Solutions

The standard teleost Ringer used was based on that of Laurent & Rouzeau (1972) for rainbow trout (Table 1)

Table 1

Composition of Ringer Solution

Na Cl	125 mM
K Cl	3.45mM
$Mg Cl_2$	1.5 mM
$Ca Cl_2$	0.45mM
Glucose	1.0 g l^{-1}
Adrenaline	10^{-6}M (optional)

The solution was buffered to pH 7.80 by a mixture of $Na_2 HPO_4$ and $KH_2 PO_4$ (4.8 mM). It was gassed with 30% O_2 in N_2 to give a PO_2 of 220 $\pm$ 10 torr (at 20°C).

Test Solutions

PO_2

Test solutions in the PO_2 ranges 5 - 10, 20 - 30, 40 - 50, 70 - 80 and 90 - 100 were prepared by bubbling the appropriate mixtures of oxygen and nitrogen through the standard solution.

PCO_2

Various solutions were made up by replacing the $Na_2 HPO_4/KH_2 PO_4$ mixture with different concentrations of Na HCO_3 ranging from 4 - 20 m M . The pH of each solution was adjusted to 7.8 by bubbling the appropriate gas mixture of $CO_2/O_2/N_2$ through them to give PCO_2 values ranging from 2.5 - 12.3 torr.

pH

The standard solution was adjusted to pH values ranging from 6.5 to 8.0 by varying the proportions of $Na_2 HPO_4$ and K $H_2 PO_4$ but without changing

the total concentration of the solution (4.8 m M).

Osmotic Pressure and Ionic Concentration

The standard solution had an osmotic pressure of 268 - 272 m Osm and a Na Cl concentration of 124 - 126 m M . Several series of test solutions were used in order to observe independently the effects of osmolarity and ionic changes (both Na^+ and Cl^-).

(1) Osmolarity Changes With Ionic Concentration Variation

The osmolarity of the standard solution was varied between 154 and 498 m Osm by varying the Na Cl concentration between 62.5 and 250 m M.

(2) Osmolarity Changes Without Ionic Concentration Variation

Mannitol was added to the standard solution to obtain osmotic pressures between 270 and 500 m Osm .

(3) Ionic Concentration Changes Without Osmolarity Variation

Mannitol was added to solutions containing varying concentrations of Na Cl (62.5 - 100 m M) to maintain the osmotic pressure at 270 m Osm .

(4) Separate Effects of Na^+ and Cl^-

To study the separate effects of $[Na^+]$ and $[Cl^-]$ in hyperosmotic solutions the hyperosmotic Na Cl fraction was substituted with either choline chloride (35 mM) or sodium propionate (35 m M).

Effect of Acetazolamide

The carbonic anhydrase inhibitor, acetazolamide (sodium salt, 10^{-4}M) was added to the standard solution buffered at either pH 7.8 or 7.0 with $Na_2 HPO_4/K H_2 PO_4$.

Effect of 2 - 4 Dinitrophenol

Various concentrations of 2 - 4 dinitrophenol ranging from 10^{-4} to 10^{-9}M

were made up in standard solution at pH 7.8.

All solutions were filtered through a 0.22 μm Millipore filter before perfusion of the pseudobranch.

RESULTS

The position and gross morphology of the pseudobranch in ten species of teleost fish were examined (see Materials and Methods). More detailed structural studies were made on pseudobranchs representative of the four main pseudobranch types from the following species: Dicentrarchus labrax (free pseudobranch), Mugil capito (semi free), Salmo gairdneri (covered) Cyprinus carpio (buried).

Pseudobranch Position

In all species except Cyprinus carpio and Pollachius pollachius the pseudobranchs were clearly visible as dark red organs on the underside of the operculum in the cranial part of the sub-opercular cavity, where they are supported by the internal opercular surface (Plates 2-1, 2, 3). The position of the pseudobranch in relation to the rest of the head is shown in Fig 10. The pseudobranch is situated on a base of loose connective tissue containing many fat cells and covering a layer of muscle (Plate 2-3). The pseudobranchs of Cyprinus and Pollachius are not visible since they have moved mediocranially in the course of development and are buried within loose connective tissue beneath a layer of stratified epithelium and mucous cells. The surrounding layer of connective tissue contains many fat cells and is separated from the covering epithelium by a well developed basement membrane.

Macroscopic Appearance

The 'free' or 'semi-free' pseudobranchs of Dicentrarchus labrax, Scomber scombrus, Mugil capito and Osmerus eperlanus were more gill like 'in situ' than the compressed, covered pseudobranchs of Salmo gairdneri, Rutilus rutilus, Perca fluviatilis and Belone belone. The pseudobranchs of Cyprinus carpio and Pollachius pollachius were revealed by dissection to be small, compressed ovoid bodies.

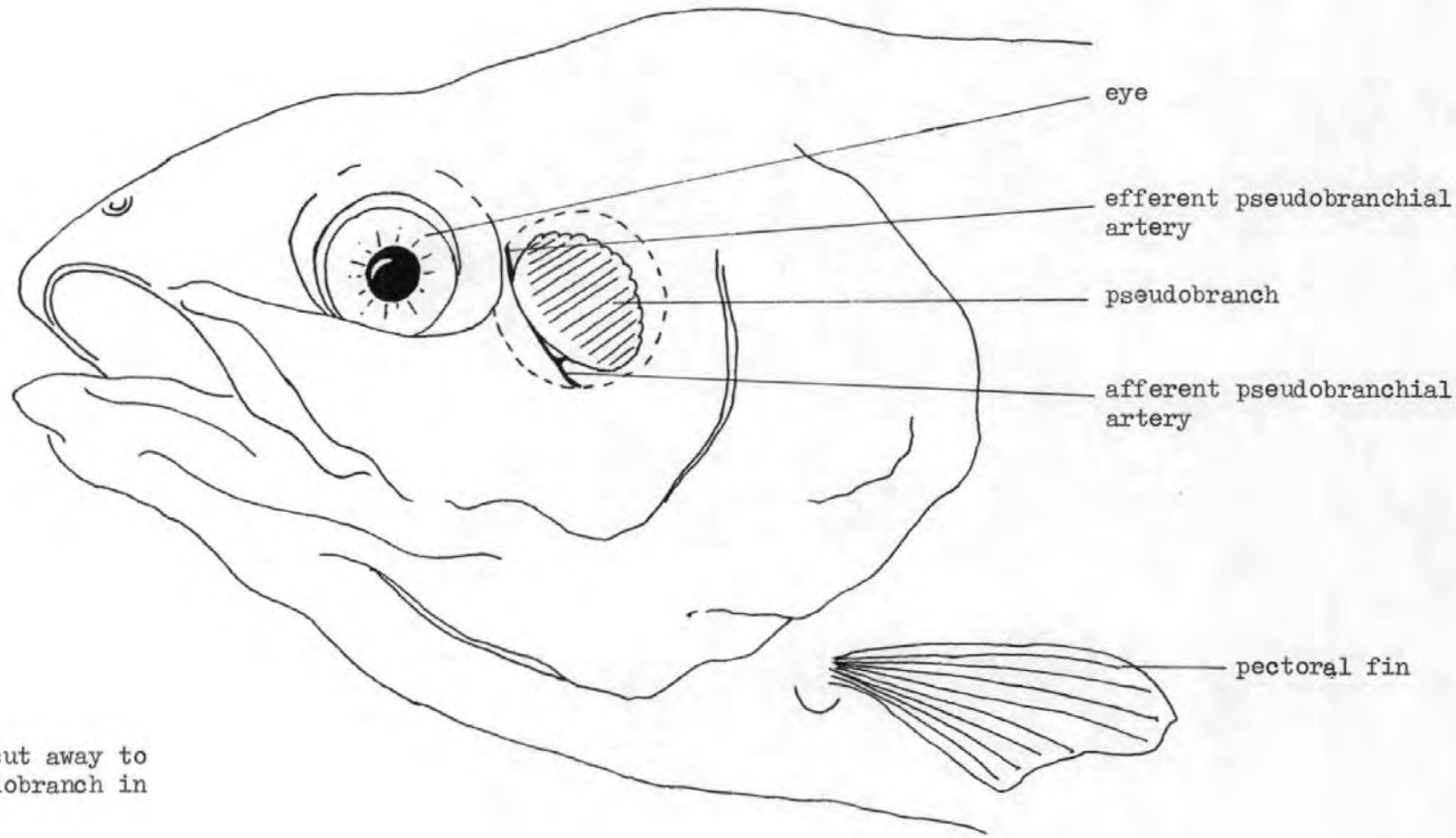


Fig 10

Section of operculum cut away to show position of pseudobranch in Salmo gairdneri

Morphology

General Structure

The pseudobranch structure is basically the same pattern as that of the gill. It consists of a pseudobranch arch bearing a single row of filaments with secondary lamellae arranged alternately on either side of the filament axis. Each secondary lamella consists of a flat capillary system delimited by pillar cells and bordered by either 'chloride type' or 'pseudobranch type' cells. Although the general structure is essentially the same for all pseudobranch types the extent of the epithelial covering and degree of lamellar fusion varies according to the species and forms the basis for the pseudobranch classification used in this study. A general description of the main pseudobranch types is given followed by a more detailed account of the pseudobranch morphology.

Pseudobranch Classification

On the basis of their epithelial covering the pseudobranchs examined were classified into 4 main types (Table 2). This is essentially the same classification as that used by Granel (1927) (see Review), although the terms 'free', 'semi-free', 'covered' and 'buried' are used in the present study to describe the 4 types.

The scanning electron microscope (S.E.M.) was useful for identification of pseudobranch types since it provided quick and accurate information on the extent of the epithelium and any fusion of the secondary lamellae. More detailed information was obtained from serial sections for light and electron microscopy. Using these methods diagrammatic reconstructions of the pseudobranch types were made (Figs 11 to 14).

Table 2 Classification of Pseudobranch Types

Species	Habitat	Pseudobranch type	Number of filaments	Number of lamellae/filament		Av. number of lamellae/mm	
				Longest	Shortest	Tip	Base
Dicentrarchus labrax (Bass)	Seawater	Free	33 - 35	140	43	20	17
Scomber scombrus (Mackerel)	Seawater	Free	N.E. *	N.E.	N.E.	N.E.	N.E.
Mugil capito (Grey mullet)	Seawater/ Brackish water	Semi-free	19 - 21	125	35	28.5	26
Osmerus eperlanus (Smelt)	Seawater/ Brackish water	Semi-free	N.E.	N.E.	N.E.	N.E.	N.E.
Salmo gairdneri (Rainbow trout)	Freshwater	Covered	19 - 20	130	50	29	26
Rutilus rutilus (Roach)	Freshwater	Covered	N.E.	N.E.	N.E.	N.E.	N.E.
Perca fluviatilis (Perch)	Freshwater	Covered	N.E.	N.E.	N.E.	N.E.	N.E.
Belone belone (Garfish	Seawater	Covered	N.E.	N.E.	N.E	N.E	N.E.
Cyprinus carpio (Carp)	Freshwater	Buried	14 - 15	N.E.	N.E.	28	27
Pollachius pollachius (Pollack)	Seawater	Buried	N.E.	N.E.	N.E.	N.E.	N.E.

* N.E. Not examined

Figs 11 - 13 Diagrammatic sections of a 'free', 'semi-free' and 'covered' pseudobranch.

Fig 11 Free pseudobranch (Bass)

Fig 12 Semi-free pseudobranch (Mullet)

Fig 13 Covered pseudobranch (Trout)

paa primary afferent artery

pea primary efferent artery

afa afferent filament artery

efa efferent filament artery

ad adipose tissue of operculum

c chloride cell region

ca cartilage

cvs central venous sinus

f filament (buccal edge)

ps pseudobranch cell region

sl secondary lamellae

v pseudobranch vein.

Arrows indicate the direction of water flow

Fig 11

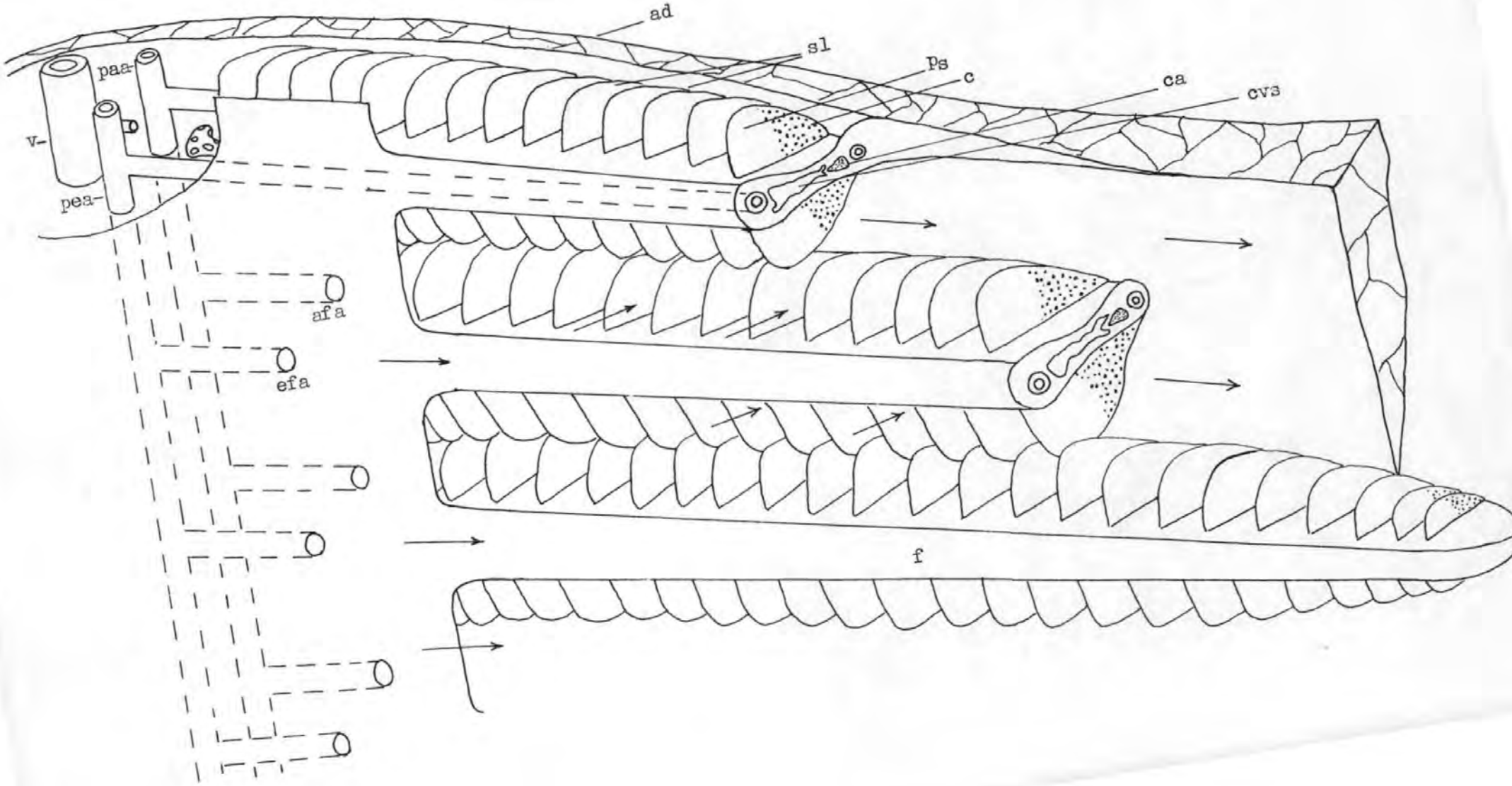


Fig 12

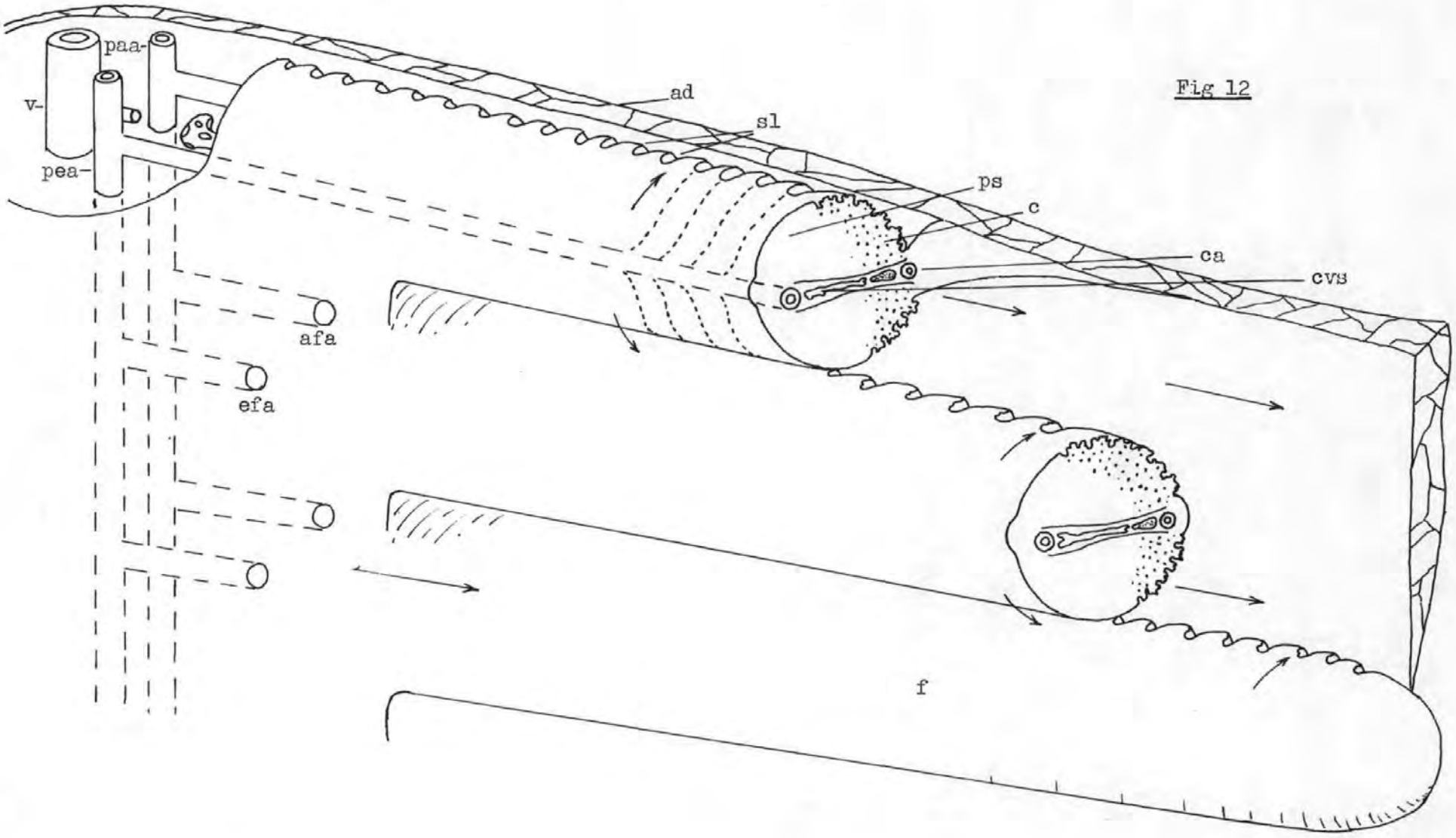


Fig 13

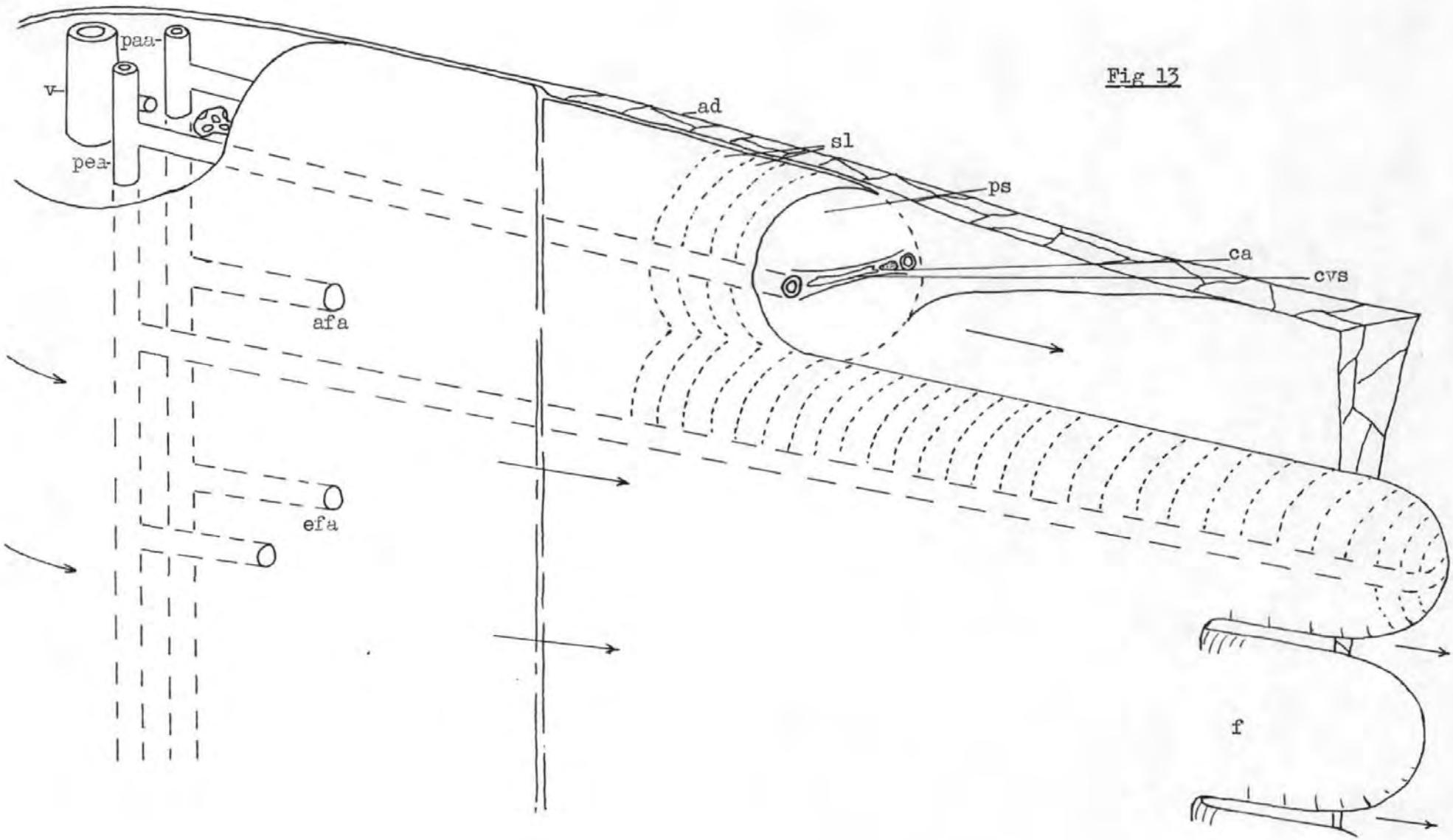
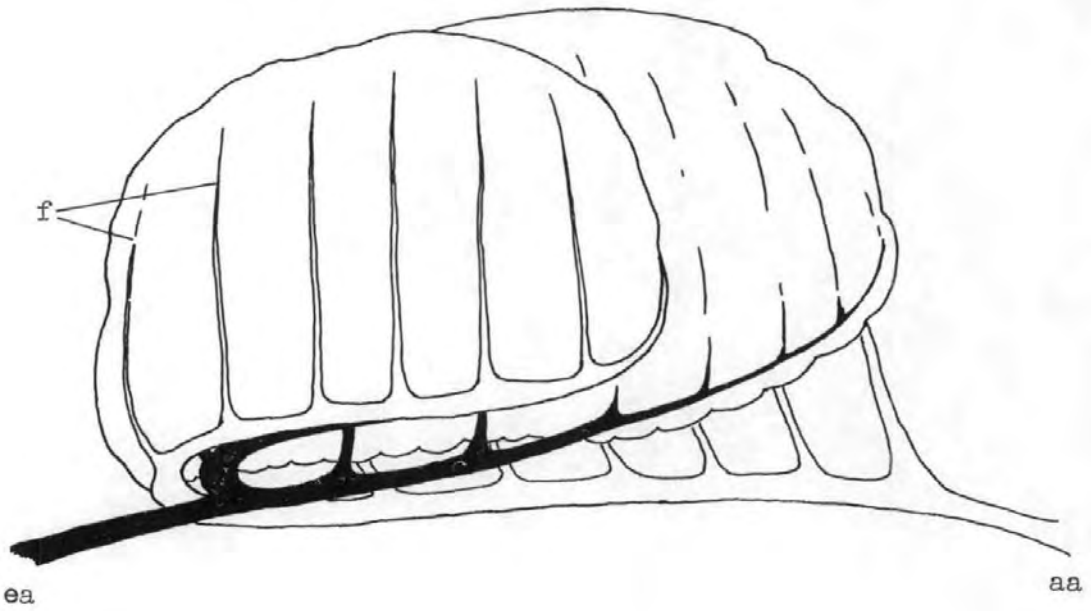


Fig 14 Right pseudobranch of Cyprinus carpio



aa Afferent pseudobranchial artery

ea Efferent pseudobranchial artery

f filaments

Note folded nature of pseudobranch

Pseudobranch Types

(1) Free pseudobranch

The 'free' pseudobranchs of the marine teleosts Dicentrarchus labrax and Scomber scombrus are typically gill-like structures with triangular shaped secondary lamellae arranged alternately on either side of the supporting filaments at an angle of approximately 60° to the filament surface (Plates 4-2, 5-1, 5-2, Fig 11). The apex of the triangular lamellae is offset and nearest the side of the efferent filament artery and the site of water entry. The single row of parallel filaments are attached to the opercular epithelium at the base of the organ (the pseudobranch arch) but are 'free' for much of their length, with the tips projecting into the buccal cavity. Although the secondary lamellae are usually separate, areas of fusion between some lamellae are seen on their leading (buccal) edge overlying the region of the 'pseudobranch type' cells (Plates 5-1, 5-2). The degree of fusion varied from filament to filament and in some cases occured only on one side (Plate 5-1). The secondary lamellae are always free along their trailing (opercular) edge in the region of the 'chloride type' cells.

(2) 'Semi-free' pseudobranch

The 'semi-free' pseudobranchs of the marine teleosts Mugil capito and Osmerus eperlanus still retain the single row of parallel filaments but the secondary lamellae are no longer so apparent. Instead, they are fused and covered with a thin layer of epithelium except for the opercular edge where 'chloride type' cells are situated (Plates 3-2, 6-1, 6-2, 7-2). The secondary lamellae in this region still retain a 'free' aspect (Plates 6-1, 6-2, 7-2), but the epithelial cells are greatly folded and possess highly convoluted microridges and microvilli which give the surface a serrated appearance (Plate 6-2).

The filaments are attached to the operculum for most of their length but are free at the tips which project into the buccal cavity. (Plate 7-1, Fig 12).

(3) Covered pseudobranch

Covered pseudobranchs were found in 3 freshwater fish; Salmo gairdneri, Rutilus rutilus, Perca fluviatilis and one marine fish, Belone belone. The pseudobranchs are still visible on the underside of the operculum but they have lost their gill-like appearance and have become compressed (Plates 2-1, 3-3, 8-1). The whole organ is covered by an epithelium which is thicker over the pseudobranch arch and the filament region proximal to it (Plate 2-3, Fig 13). The filaments are attached to the operculum for most of their length and are only free at the tips (Plate 8-3). The secondary lamellae are no longer apparent since they have become completely fused and covered by connective tissue and epithelium (Plate 8-2). Apart from Perca the secondary lamellae are completely isolated from the water and do not contain any 'chloride type' cells. Perca does contain some 'chloride type' cells on secondary lamellae near the tips of the filaments.

The pseudobranch of Belone belone should be classified as an intermediate stage between the 'covered' and 'buried' pseudobranch since it possessed the folded, lobulated appearance of a 'buried' pseudobranch but was still covered only by a relatively thin epithelium.

(4) Buried pseudobranch

Buried pseudobranchs were found in one freshwater fish Cyprinus carpio and one marine fish, Pollachius pollachius. The pseudobranchs are no longer visible on the underside of the operculum since they are buried within connective tissue beneath the buccal epithelium (Plates 9-1, 9-2).

The buried pseudobranch is folded back on itself (Fig 14) so that the filaments and secondary lamellae present an irregular, lobulated appearance in microscopical studies (Plates 9-1, 9-2). The secondary lamellae are completely fused and contain only 'pseudobranch type' cells which are completely isolated from thewater. The filaments and secondary lamellae are not directly covered by epithelium since the whole organ is invested in loose connective tissue made up of areolar tissue surrounding numerous fat cells. The areolar tissue contains many spaces filled with tissue fluid and ground substance (Plate 11-3), which are bounded by cytoplasmic strands of fibroblasts and fibrocytes. A few fibres are present. Numerous mast cells are seen as well as occasional wandering leucocytes.

The filaments of buried pseudobranchs are very irregularly arranged in groups so it is difficult to ascertain the number of filaments without careful reconstruction of each organ using serial sections.

Microscopic Structure

Pseudobranch Arch

The pseudobranch arch contains a primary afferent and efferent artery, a large vein with several, irregular branching vessels, a network of small nutritional vessels, nerve fibres and extensions of the filament cartilage (Plate 10). There is no cartilaginous axis, comparable to that of the gill arch, supporting the cartilaginous cores of the pseudobranch filaments. All the structures of the arch are embedded in connective tissue consisting mainly of areolar tissue surrounding many fat cells (Plate 10). The fat cells appear as pale lobular masses surrounded by a meshwork of fine collagenous and reticular fibres as well as by a rich capillary network. Numerous large and small nerve bundles can be seen running within the connective tissue between the fat cells (Plate 11-1). The nerve fibres are branches of the glossopharyngeal and/or facial nerves depending on the species (see Innervation). The mature fat cells consist of a large fat droplet with the cell cytoplasm forming a thin pellicle around it and the nucleus displaced to a corner of the cell with a small amount of cytoplasm (Plate 11-4).

The epithelium covering the pseudobranch arch is several layers thick and contains large numbers of mucous cells (Plate 10). No taste buds were found in the epithelium of the pseudobranch arch although small numbers were found in the epithelium covering the main efferent artery after its exit from the arch (Plate 11-2). The epithelium covering the arch extends over the filaments to a greater or lesser degree according to the species (see Pseudobranch Classification). The surface of the epithelial cells consists of numerous microridges, usually arranged in concentric whorls (Plate 12-1).

Pseudobranch Filaments

The pseudobranch arch bears a single row of parallel filaments. Each filament contains a supporting cartilaginous ray which extends into the arch where it is invested by connective tissue. An afferent filament artery runs along the inner border (opercular edge) of the filament and an efferent filament artery along the outer border (buccal edge) (Plate 13-1). The filament arteries unite at the tip of the filament and connect with the primary afferent and efferent arteries in the pseudobranch arch (Figs 11 to 13). The filament arteries are lined with endothelial cells containing flattened nuclei, mitochondria, vesicles, ribosomes and specific endothelial granules (Weibel & Palade 1964) (Plate 13-2). The endothelial layer is surrounded by a thin elastic coat, a thick muscle layer and an adventitia of elastic and collagen fibres (Plate 13-3).

The filament vessels and cartilaginous ray lie within a connective tissue compartment which also contains the central venous sinus (C.V.S.), unmyelinated nerve fibres, collagen fibres and fibroblasts. The collagen fibres are continuous with those lying beneath the basal lamina (Plate 14-2), and with the collagen layer beneath the proximal pillar cells at the base of the secondary lamellae.

The main channel of the central venous sinus gives rise to a network of smaller channels and spaces, the extent of which varies between species. The C.V.S. and its channels are lined by a very thin endothelium 0.1 - 2.0 μm thick, containing flattened, elongate nuclei, a few mitochondria, vesicles and occasionally specific endothelial granules (Plate 14-1). The partial occlusion of the central compartment by connective tissue imparts a very irregular form to the C.V.S. and its channels (Plate 14-1).

Red and white blood cells are occasionally seen within the spaces of the C.V.S. (Plate 13-1). The C.V.S. drains into the large vein of the

pseudobranch arch. Small nutrient arteries and veins of the arterio-venous system are also found in the connective tissue of the central compartment (see Pseudobranch Circulation).

Small bundles of unmyelinated nerve fibres are found within the connective tissue, often close to small arteries (Plates 14-2, 3). A large bundle of unmyelinated fibres also runs the length of the filament just beneath the efferent filament artery.

The pseudobranch filaments are not always covered directly by an epithelium since connective tissue is sometimes interposed between the pseudobranch and its epithelial covering. This is the case in the buried pseudobranch where the filaments are covered with loose connective tissue beneath the epithelium. In the covered pseudobranch the filaments proximal to the pseudobranch arch are covered by an intermediate layer of connective tissue which is only lost near the filament tips where they become directly covered with epithelium (Plates 2-3, 8-3). Only in pseudobranchs where the filaments are free does the epithelium cover the outer (buccal) and inner (opercular) edges of the filament. Where the filaments are attached to the operculum the inner, attached border is invested by connective tissue (Plates 3-1, 2, 3). The filament epithelium consists of several layers of unspecialised cells with a few chloride cells and numerous mucous cells near the epithelial surface (Plates 15-1, 2). The epithelial covering of buried and covered pseudobranchs also contains numerous mucous cells but no 'chloride type' cells.

In the 'free' pseudobranch of Dicentrarchus labrax and the 'semi-free' pseudobranch of Mugil capito scanning electron microscopy revealed that mucous cell openings were more numerous on the opercular edge than the buccal edge of the filament (c.f. Plates 5-1, 5-2). Droplets of mucus were often seen within the pores (Plate 15-2). Mucous pores were usually

found at the junction of epithelial cells (Plate 12-2) and were larger in Dicentrarchus labrax (3-6 µm diam.) than Mugil capito (1.5-3 µm). In Mugil capito dome-like protrusions probably representing closed mucous cells were found on the buccal surface of the filament (Plate 7-1). Using the T.E.M. the surface of the epithelial cells appears to have numerous microvilli-like projections covered with a filamentous coat (Plate 16-1). However studies of Dicentrarchus labrax and Mugil capito pseudobranchs using the scanning electron microscope (S.E.M.) revealed that most of the projections do in fact belong to a complicated pattern of microridges although occasional microvilli are present (Plates 12-2). The epithelial cells are usually pentagonal or hexagonal in shape and individual cells can often be seen since they are bordered by long straight microridges at the cell junctions, where they form a double ridge with an adjacent cell (Plates 12-2, 16-1). This double ridge is often 1.5 - 2x higher than the other microridges. In Dicentrarchus labrax the ridges of adjacent cells are often some distance apart and the cell boundaries are not always clearly defined. The junctions between epithelial cells are notable for the numerous desmosomes along their borders and tight junctions near the surface (Plate 16-1).

The filament epithelial cells usually possess long convoluted microridges up to 8 µm long, and often arranged in a concentric whorl (Plate 12-2). However cells with microvilli-like ridges are also present, especially near the tip of filament in Mugil capito. In Dicentrarchus labrax the height of the microridges as measured from transmission electron micrographs varied between 0.19 - 0.88 µm with a mean of 0.29 µm on the buccal edge of the filament (Table 3).

The cytoplasm and nuclei of the epithelial cells are fairly densely staining especially just beneath the microridges. Numerous randomly arranged fibres and free ribosomes are present as well as a few mitochondria, granular endoplasmic reticulum and an extensive supra-nuclear Golgi

apparatus. A large number of dense vesicles (0.15 μm av. diam.) are found throughout the cell and especially near the Golgi complex (Plate 16-1).

Secondary Lamellae

The secondary lamellae are supplied with blood from the afferent filament artery via the afferent lamellar arterioles, two or more of which may have a common origin (Plate 13-1). Blood leaves the secondary lamellae via the efferent lamellar arterioles. Like the filament arteries the lamellar arterioles are lined with endothelial cells containing numerous electron dense endothelial granules. These cells also contain a flattened nucleus, mitochondria, granular endoplasmic reticulum, ribosomes, vesicles and a prominent Golgi complex. Vesicles of various sizes containing a fairly electron dense material appear to arise from the Golgi apparatus, and may be developing endothelial granules (Plate 17-1). The cytoplasm of some endothelial cells contained bundles of thin filaments. These measure about 60 - 80 Å in diameter and do not show any cross striation, nor contact with other cytoplasmic components. They are usually oriented parallel to each other and to the cell surface, and sometimes are found in close contact with the basal lamina (Plate 17-2). An occasional foot-like process emerges from the base of endothelial cells and penetrates the basal lamina (Plate 17-3). It may establish membranous contacts (gap junctions) with smooth muscle to form myoendothelial junctions. The endothelial cells are surrounded by a continuous layer of muscle cells which is thicker at the junction of the lamellar arteriole and its filament artery (Plate 18-1). Nerve bundles of unmyelinated fibres are often found in this region (Plate 18-2).

The afferent lamellar arterioles supply blood to a network of interconnecting blood spaces delimited by pillar cells and the flanges which entend from them. These are surrounded by a basement membrane which

separates them from the 'chloride type' or 'pseudobranch type' cells (Plate 18-3). The outer two layers of the basement membrane constitute a basal lamina (Fawcett 1966) beneath which there is a collagenous layer. Collagen fibres run from the basement membrane into a series of columns around the periphery of the pillar cells (Plates 18-3, 19-1). These columns are extra cellular lying in deep foldings of the cell membrane of the pillar cells. They vary in number between 6 and 10 per pillar cell. The collagen fibres of these columns are oriented along the length of the columns changing direction at their extremities to become randomly arranged in the plane of the basement membrane.

The blood channels are never in direct contact with the basement membrane since the channels are lined by the flanges of pillar cells or what appears to be a thin fenestrated endothelium (Plate 25-2), except at the outermost part of the marginal channel which is lined with true endothelial cells (Plate 19-2). The junctions between the flanges of adjacent pillar cells are often overlapping or abutting and are united by tight junctions and desmosomes. Each pillar cell has a large central nucleus, and contains numerous mitochondria, free ribosomes and vesicles. Fine cytoplasmic fibrils are often seen adjacent to the columns of the pillar cells.

The pillar cells which lie proximal to the filament lie adjacent to one another and contain columns on the lamellar side only. A continuous thick layer of collagen fibres is present on the filament side of this row of pillar cells.

The dimensions of the intralamellar vascular channels delimited by the pillar cells are approximately the same as the average diameter of the erythrocytes (4 - 8 μm). The marginal channel is wider (12.5 - 17.5 μm) than the blood spaces between adjacent pillar cells and runs from

afferent to efferent secondary lamellar capillaries around the margin of the secondary lamella. The outer border of the marginal channel is lined by typical endothelial cells containing mitochondria, vesicles, electron dense endothelial granules and occasionally bundles of cytoplasmic filaments (Plate 19-2). They do not surround columns. The endothelial cells form simple abutting or overlapping junctions with adjoining pillar cell flanges.

Secondary Lamellae Epithelium

The blood channels of the secondary lamellae are bordered by a single layer of specialised cell types (either 'chloride' or 'pseudobranch type' cells). In 'free' and 'semi-free' pseudobranchs these cells are covered in 'free' areas of the lamellae by a thin layer of unspecialised, flattened epithelial cells. This layer may also contain specialised cells such as mucous and rodlet cells. This outer epithelial layer completely isolates the 'pseudobranch' cells from the external environment but the 'chloride' cells make contact with the water by means of apical pits in the overlapping epithelium (Plates 21-1, 2).

The external surface of the outer epithelial layer is provided with microridges similar to those described for the filament epithelium. Although the length of the microridges does vary there does appear to be a general pattern depending on the position of epithelial cells in the secondary lamellae. In Dicentrarchus labrax cells at the marginal base of the secondary lamellae close to the filament usually have short microridges or microvilli, as do those along the edges of the lamellae. (Plate 20-1). The epithelial cells covering the 'pseudobranch type' cells have flattened, highly convoluted microridges (Plate 20-2) whereas those covering the chloride cells are narrower and further apart (Plate 21-1). The average height of epithelial microridges, as measured from transmission

electron micrographs, is usually greater (although not significantly so) in the region of the 'chloride' cells on secondary lamellae or areas of high mucous cell density on the filament (Table 3).

Table 3

Area	Height of microridges (μm)	
Filament (buccal edge)	0.19 - 0.88	(0.29)*
Filament (opercular edge)	0.19 - 0.81	(0.32)
Secondary lamellae (pseudobranch cell region)	0.12 - 0.42	(0.23)
Secondary lamellae (chloride cell region)	0.14 - 0.65	(0.28)
Secondary lamellae (margin)	0.10 - 0.54	(0.21)

Correlation of microridge height with distribution (Bass pseudobranch)

* Mean of 25 readings.

As in the filament epithelium the microridges are covered by a thick fuzzy glycocalyx (Plate 16-3). A double ridge is often formed at cell junctions and adjacent cells are bordered by numerous desmosomes as well as tight junctions.

The cytoplasm of epithelial cells immediately below the microridges appears very dense and contains few organelles apart from a small number of mitochondria, a little granular endoplasmic reticulum and a number of small dense vesicles (0.05 - 0.16 μm diam.).

The 'pseudobranch type' cells of 'semi-free', 'covered' and 'buried' pseudobranchs are not covered by an epithelial layer since the lamellae are joined together by a layer of interstitial tissue located between the antivascular poles of 'pseudobranch' cells from opposing lamellae (Plate 22-1, 2). This interstitial tissue is formed by cells with long

fine extensions joined to each other by desmosomes. They also make contact with the 'pseudobranch type' cells by means of desmosomes (Plate 22-2, 3). The cytoplasm of interstitial cells contains numerous free ribosomes, extensive bundles of fibres (80 - 100 Å diam.), mitochondria, vesicles and occasional profiles of granular endoplasmic reticulum (Plates 22-1, 2, 3).

The Water-Blood Barrier

The water-blood barrier is considerably thinner in 'free' pseudobranchs and the 'free' areas of 'semi-free' pseudobranchs than in 'covered' and 'buried' pseudobranchs. In 'free' pseudobranchs the main parts of the barrier separating water and blood are the surface epithelial cells, 'chloride' or 'pseudobranch type' cells, basement membrane and pillar cell flanges or endothelium. In 'semi-free', 'covered' and 'buried' pseudobranchs the lamellae are joined together in the region of the 'pseudobranch type' cells which are covered further by connective tissue and layers of epithelial cells. The water-blood barrier is therefore greatly increased. The thinnest part of the barrier in this case covers the blood channels on the efferent side of the lamellae, but this is still much thicker than the water-blood barrier of 'free' pseudobranchs.

The overall distance of the barrier was measured from (a) the tips of the epithelial microridges to the plasma membrane of the pillar cell flange or endothelial cell and (b) from the base of the microridges. As the concavities between the microridges may be filled with mucus the latter case is not necessarily the minimum distance. The mucus layer frequently extends over the surface of the microridges and should perhaps be included as part of the overall barrier. The results are summarised in Table 4 where the maximum, minimum and mean values of about 50 measurements are given. The mean values for the pseudobranch region of Mugil capito, Salmo gairdneri and Cyprinus carpio are not given because there are such

large differences between the shortest (efferent side and tip of filament) and longest routes (afferent side and base of filament) of the water-blood pathway.

Table 4

Species	Width of water-blood barrier /um	
	'Chloride type' cell region	'Pseudobranch type' cell region
Dicentrarchus labrax	9.92 - 17.36 (13.64)*	8.9 - 15.26 (12.65)*
Mugil capito	4.83 - 13.41 (8.12)*	12.8 - 127
Salmo gairdneri		23.78 - 1120
Cyprinus carpio		845 - 2365

The water-blood barrier in the four main representative types of pseudobranch.
* Mean of 50 measurements.

Specialized Cell Types

Four types of specialized cell were found in the secondary lamellae of pseudobranchs examined: (1) 'chloride type' cells (2) 'pseudobranch type cells' (3) mucous cells and (4) rodlet cells. The occurence and distribution of these cells varies according to the species (Table 5), although they show only slight variations in morphology between species.

Chloride Type Cells

The 'chloride type' cells of the pseudobranch were so named because of their similarity to the 'chloride' cells of the teleost gill. Although the term 'chloride' cell is now well established it may be a misnomer since

Table 5

Species	Habitat	'Pseudobranch cell'	'Chloride cell	Mucous cell	Rodlet cell
Dicentrarchus labrax	Seawater	++	++	++	++
Scomber scombrus	"	++	++	++	-
Mugil capito	"	++	++	++	-
Osmerus eperlanus	"	++	++	++	-
Belone belone	"	++	-	++	-
Pollachius pollachius	"	++	-	++	-
Salmo gairdneri	Freshwater	++	-	++	-
Rutilus rutilus	"	++	-	++	+
Perca fluviatilis	"	++	+	++	-
Cyprinus carpio	"	++	-	++	-

Distribution of cell types according to species

++ Large numbers present

\+ Small numbers present

\- Absent

it has been suggested that they transport other ions beside chloride (see Review and Discussion). Nonetheless the conventional term 'chloride type' cell is used in this study to describe similar cells in the pseudobranch.

Apart from small numbers in the freshwater Perca fluviatilis, 'chloride type' cells were only found in the pseudobranch of marine fish, although not necessarily in all species (e.g. they were absent in Pollachius pollachius and Belone belone).

When stained with haematoxylin and eosin or Mallory's triple stain for light microscopy, the contents of 'chloride type' cells appear finely granular and are a pale red or purple colour from base to apex, indicating that they are acidophilic. Transmission electron microscopy revealed the cells to be filled with numerous mitochondria which are only absent near the basement membrane and in the apical region of the cell. (Plate 23-2). The cells are fairly large and are usually cuboidal or columnar (up to 20 µm long and 12 µm wide, although usually about 14 µm x 9 µm). They are covered by a layer of flattened epithelial cells apart from an open region in contact with the water known as an apical pit (Plates 23-1, 2). As in the gill there is only one apical pit per cell although more than one cell sometimes opens into the same pit. In the pseudobranch of Dicentrarchus labrax scanning electron microscopy revealed the pits to be irregularly shaped openings in the epithelial surface. They are usually found at poorly defined epithelial cell junctions and are between 2 - 4 µm wide (Plate 21-1). In Mugil capito the openings are approximately circular with a diameter of 1.5 - 3.0 µm and are present in flattened regions of the surface epithelium where microridges are sparse or absent (Plate 21-2).

The 'chloride type' cells are always found bordering the capillary system on the afferent side of the secondary lamellae and on the filament in the afferent interlamellar region. Small numbers are also found in the epithelium of the afferent (opercular) edge of the filament tips, which are free from the operculum epithelium. The secondary lamellae are always free or semi-free in the region of 'chloride type' cells, allowing them free access to the water via their apical pits.

The number of chloride cells per secondary lamella is far higher in the pseudobranch than in the gill. In Dicentrarchus labrax and Mugil capito the 'chloride type' cells of the pseudobranch cover a third to a half the area of the secondary lamellae at a density of 90 - 125 cells/ 0.1 mm^2. The chloride cells of the gill are mainly restricted to the base of the secondary lamellae proximal to the filament, and the interlamellar region of the filament (Plates 24-1, 2). A few are also found in the epithelium of the afferent and efferent edges of the filament. 'Chloride' cells were found in the respiratory epithelium of the secondary lamellae of Salmo gairdneri, but not in Dicentrarchus labrax.

Ultrastructure

The 'chloride type' cell consists of three distinct regions; a vascular pole, a central region and an apical pole (Plate 25-1).

(1) The vascular pole

The cell membrane is separated from the blood channel by a basement membrane and the flanges of pillar cells or what often appears to be a very thin fenestrated endothelium (Plate 25-2). It is uncertain if this represents a true endothelial layer or if in fact it belongs to the pillar cell flanges which have become modified in the form of a fenestrated endothelium.

The basal and lateral membranes of the 'chloride type' cell are invaginated to give rise to a branching system of tubules (500Å diam.) which form a three dimensional reticulum extending throughout the cytoplasm. In the basal region of the cell these tubules connect with bundles of narrow, parallel saccules (120 - 150 Å diam. and 180 Å apart) which appear as a series of folded loops (Plate 25-2). The saccules are up to 3 /um long and are enlarged at each end where they are connected to the rectilinear tubules formed from invaginations of the plasma membrane. In some sections the tubules show a lattice-like arrangement where they are connected to the saccules. (Plate 26-1). The presence of these saccules was not shown in this or previous studies on the gill chloride cell, although similar lattice networks of tubules have been observed in the chloride cells of rainbow trout (Abel 1973) and river lamprey (Pickering 1976).

(2) Central region

The central region of the 'chloride type' cell is rich in mitochondria which are surrounded by a highly branching network of intracellular tubules which connect with the narrow saccules near the base of the cell. The mitochondria usually appear round or rod-like and are up to 4.5 /um long (although usually about 3.0 /um) with a diameter of 0.5 - 0.8 /um. They contain well developed membranous cristae which may run transversely or longitudinally. The matrix of the mitochondria is slightly electron opaque and contains small numbers of dense mitochondrial granules. In some areas the outer membrane of the mitochondria appeared to be continuous with membranes from the tubular system (Plate 27-1). The surrounding tubular network contains material slightly more electron dense than the cytoplasmic matrix. This is particularly noticeable in material fixed in glutaraldehyde and cacodylate buffer.

The nucleus is basally or laterally placed and is often associated with profiles of granular endoplasmic reticulum and Golgi apparatus. Clusters of free ribosomes are scattered throughout the cell. Some chloride cells were darker than others and contained a more extensive G.E.R. which occupied the central region of the cell (Plate 26-3).

Apical Pole

The mitochondria are absent from the apical region of the cell where the tubular network gives rise to large numbers of membrane bound vesicles (500 - 2000 Å diam.) of similar electron density to the tubules. This was more noticeable in Dicentrarchus labrax than Mugil capito where the tubules sometimes extend virtually to the surface membrane (c.f. Plates 26-2 and 27-1). The outer surface of the apical plasma membrane is covered with a fine filamentous coat whilst the cytoplasmic side of the membrane appears slightly denser than the rest of the cytoplasm. Vesicles coated with a fine filamentous material as well as smooth vesicles were occasionally seen within the apical pit. The cell surface is relatively smooth although occasional, small cytoplasmic projections can be seen using the scanning electron microscope (Plate 21-1). In comparison the surface of the gill 'chloride' cell of both freshwater and seawater fish, is thrown into numerous microvillous-like, cytoplasmic projections. (Plate 28-1). In the seawater fish, Dicentrarchus labrax, large numbers of coated vesicles are found within the apical pit (Plate 28-1). No apical pits were found in the gill 'chloride' cells of the freshwater Salmo gairdneri. Instead the apical membrane was level with, or protruded beyond the surface epithelial cells.

In Mugil capito pseudobranch, apical pits were often not so apparent since the 'chloride' cell surface is flattened and almost level with the surface epithelial cells. A thick layer of amorphous granular material is often found adhering to the surface of the cell. (Plate 27-1).

Invaginations of the apical plasma membrane are due to cytoplasmic extensions of smaller mitochondria-rich cells adjacent to the 'chloride type' cells (Plate 26-4). They are much smaller and narrower (3 µm x 5 µm) than the 'chloride type' cells and often contain a more extensive G.E.R. and a less prominent tubular system. These cells were first noted by Dunel and Laurent (1973) who named them accessory cells. Although they have not been previously reported in the gill the present study showed the presence of these cells in association with the 'chloride' cells of the gill of Dicentrarchus labrax* (Plate 28-2). However they were not found in the gills of Salmo gairdneri or Cyprinus carpio.

The external plasma membranes of the accessory cells and chloride cells interdigitate at the level of the apical pit where they are separated by shallow junctions (Plate 27-2). The tubular network of the 'chloride type' cells opens into the extracellular spaces between neighbouring 'chloride type' cells or between 'chloride type' and accessory cells. The shallow junctions at the level of the pit are not true tight junctions so the extracellular spaces may communicate with the water at these points. Occasionally clear cytoplasmic processes containing clear vesicles extrude from the accessory cell cytoplasm into the apical pit (Plate 26-2).

Desmosomes were often seen along the adjoining membranes of chloride and accessory cells in the vicinity of the apical pit (Plate 27-2). Both types of cell made similar contact with the neighbouring epithelial cells in this region but no junctional complexes were found on their lateral and basal surfaces.

*They have since been reported in recent studies by Hootman and Philpott (1979), and Sardet, Pisam & Maetz (1979), in the gills of euryhaline and seawater fish (see Discussion).

'Pseudobranch Type' Cells

The 'pseudobranch type' cells were so named because they were specific to the pseudobranch and were found in all the pseudobranchs examined. In those pseudobranchs possessing 'chloride type' cells, the 'pseudobranch type' cells were always found bordering the capillary system on the efferent side of the secondary lamellae, but were not found in the filament epithelium. In all freshwater teleosts examined (except Perca) and the seawater Pollachius pollachius and Belone belone, the pseudobranch type cells covered the whole area of the secondary lamellae.

The 'pseudobranch type' cells do not possess apical pits and are always completely isolated from the external environment. In 'free' pseudobranchs the 'pseudobranch type' cells are covered by a layer of flattened epithelial cells, which in some regions are joined together between apposing secondary lamellae (Plates 28-1, 2). These areas of fusion only occur in the region of the 'pseudobranch type' cells and sometimes only on one side of the filament (Plate 5-1). In all other pseudobranchs the secondary lamellae are completely fused in the region of the 'pseudobranch type' cells by means of narrow, fibrous interstitial cells, intercalated between the antivascular poles of the 'pseudobranch type' cells (Plates 22-1,2).

When stained with H & E, Mallory's triple stain or methylene blue for light microscopy the 'pseudobranch type' cells are characterised by a clear apical zone and a denser, finely granular central region and vascular pole (Plates 29-1, 2; 30-1, 2). Transmission electron microscopy showed the apical region to be virtually free of mitochondria and other organelles whilst the central and vascular regions of the cell are packed with numerous mitochondria and tubules, arranged in a very characteristic pattern (Plates 31 - 1, 2).

The 'pseudobranch type' cells are quite large (normally between 16 - 20 µm long and 8 - 12 µm wide) and are usually oriented with their long axis parallel to the basement membrane (c.f. 'chloride type' cells).

Ultrastructure

As in the 'chloride type' cell the 'pseudobranch type' cell can be divided into three regions; a vascular pole, a central region and an apical pole.

Vascular pole

The plasma membrane rests on a distinct basement membrane surrounding the pillar cells and fenestrated endothelium of the blood channels. The basal plasma membrane is deeply invaginated and gives rise to a network of intracellular tubules (500 - 700 Å diam.). The continuity of the endothelial cells beneath the basement membrane is also highly interrupted indicating the possible communication between the lumina of the blood vessels and that of the tubules (Plate 35-3). The tubular invaginations of the basal membrane are fairly straight and unbranched for 0.25 - 0.35 µm, before giving rise to a highly branched region of anastomosing tubules which extends for 0.5 - 1.0 µm beneath the mitochondrial region. Unlike the 'chloride type' cells there are no narrow sacculès and the tubular system shows no connections with the lateral membranes of the 'pseudobranch type' cells. In the branched region of the tubular system, bundles of cytoplasmic filaments run parallel to the basement membrane and communicate with desmosomes situated at the lateral membranes (Plate 31-2).

Central Region

In the central region of the cell the tubular system becomes arranged into an orderly pattern of long parallel tubules which surround numerous closely packed mitochondria. The tubules have a relatively constant diameter (400 - 500 Å) and do not appear to be branched in this region.

Obliquely cut sections often revealed the tubules to be spirally coiled (Plate 32-1). The tubular network forms a closed loop system in the 'pseudobranch type' cells, since the tubules incline around the mitochondria and return towards the basal plasma membrane.

The mitochondria are usually long and rod-like (up to 6 /um long with a diameter of 0.3 - 0.5 /um), but in some cells they show a variety of irregular shapes including V shaped and circular mitochondria (Plate 32-2). They possess well developed membranous cristae arranged transversely or longitudinally, and the matrix appears darker than in the mitochondria of the 'chloride type' cells. Also, fairly large numbers of dense mitochondrial granules are found in the matrix (Plate 31-2). The outer mitochondrial membranes and those of the tubules are closely associated and continuities between the two are sometimes seen (Plate 32-3).

No other organelles are usually visible in the central region of the cell apart from clusters of ribosomes. The large pale nucleus is usually placed towards the apical pole of the cell. Occasionally a pair of centrioles (diplosome) is seen nearby (Plate 32-4).

In Cyprinus carpio a previously undescribed organelle was often seen in close association with the nucleus, sometimes as an extension of the nuclear envelope (Plates 33-1, 2). It appeared to be a form of modified granular endoplasmic reticulum and was very regular, consisting of four or five parallel cisternae bordered on either side by a row of dense granules, possibly ribosomes. The cytoplasmic extensions (600 - 700 Å wide) between the cisternae contained a narrow membranous plate (75 Å wide and 0.5 - 0.6 /um long) sandwiched between the rows of ribosomes. The organelle showed no continuity with other cell organelles apart from the perinuclear cisterna of the nuclear envelope. Only one of these organelles was found per cell. It was found in all carp pseudobranchs examined but was absent from all other species.

Apical Pole

The apical pole is free of mitochondria and tubules and contains a clear cytoplasm with profiles of granular endoplasmic reticulum and clusters of free ribosomes. Long bundles of cytoplasmic filaments (tonofilaments) which often communicate with desmosomes are common in this region. Desmosomal contact, often in the form of long chains of desmosomes, is frequently found between adjoining cells (Plate 33-3).

The apical pole also contains many small, irregularly shaped vacuoles (0.1 - 1.0 /um diam), and sometimes a single large vacuole, up to 10 /um long and 6 /um wide, which fills the apical region of the cell. (Plate 34-1). These vacuoles are surrounded by bundles of cytofilaments and a band of dense material just below the surface of the membrane (Plates 34-2, 3). Small numbers of vesicles, which appear to be 'pinched off' from the surrounding membrane (Plate 34-3), are found within the vacuoles. These vacuoles are mostly found in the 'pseudobranch type' cells of freshwater fish although they also appear in the 'pseudobranch type' cells of seawater bass when osmotically stressed.

In any one section 'light' and 'dark' 'pseudobranch type' cells were seen, probably representing different stages of activity. Three stages could be recognised:

(1) The cell contains a clear cytoplasm and possesses relatively few mitochondria which are loosely packed in the basal and central regions of the cell. The tubular system is poorly organised at this stage and is not intimately associated with the mitochondria. The nucleus is large with a pale nucleoplasm (Plates 30-3, 35-1).

(2) The mitochondria increase in number and become closely packed in the basal and central regions of the cell. The tubular system becomes well organised and surrounds the mitochondria in a characteristic pattern of parallel tubules (Plates 31-1, 35-2).

(3) The cell cytoplasm becomes much darker and contains large numbers of ribosomes and glycogen particles. The whole cell appears full of dark, closely packed mitochondria leaving room only for a small, dark nucleus and profiles of granular endoplasmic reticulum (Plates 35-1, 2, 3). In the bass pseudobranch these dark cells are found in the central region of the secondary lamellae (Plate 29-2) and are the first cells to receive blood that has passed from the 'chloride' cell region.

Mucous Cells

Large numbers of mucous cells were found in the epithelial covering of 'covered' and 'buried' pseudobranchs. In 'free' and 'semi-free' pseudobranchs, mucous cells were found mainly in the filament epithelium, with smaller numbers in the lamellar epithelium. Where the filaments hang freely, they were more numerous on the opercular (trailing) edge than the buccal (leading) edge of the filament. In the lamellae they were more common in the epithelium around the marginal channel, and proximal to the filament.

Ultrastructure

Each mucous cell contains a basally situated, irregularly shaped, dark nucleus. Most of the cell volume is made up of tightly packed mucous droplets. The contents varied in staining intensity from lightly staining globules (Plate 36-1) to dense osmiophilic granules (Plates 15-1, 36-2), and may reflect different stages in activity or different forms of mucous cell. The mucous cells in the filament epithelium usually possess denser globules than those in the secondary lamellae. Using the scanning electron microscope, droplets of mucus were often seen within the mucous cell pores (Plate 15-2). The remainder of the cytoplasm is occupied by granular endoplasmic reticulum, a few mitochondria, small vesicles and an extensive Golgi complex. The latter usually occupies a supranuclear

position towards the base of the cell. The mucous cells lie in close contact with adjacent epithelial cells and make contact with the external environment by means of a pore (1.5 - 6.0 µm diam). These pores are usually found at the junctions of filament epithelial cells. In Mugil capito, dome-like protrusions, probably representing closed mucous cells were often found on the buccal surface of the filament (Plate 7-1).

Rodlet Cells

Rodlet cells have not been previously reported in the teleost pseudobranch although they have been found in a wide variety of fish tissues including the gill epithelium (see Morrison & Odense 1978). This particular cell is also sometimes referred to as Rhabdospora thelohani (Laguesse 1906) since some authors believe it to be a parasite, (e.g. Bannister 1966).

Distribution of Rodlet Cells

Large numbers of rodlet cells were found in the gill and pseudobranch epithelium of Dicentrarchus labrax whilst small numbers were found in the pseudobranchs of Rutilus rutilus and the gills of Salmo gairdneri.

In the gill, rodlet cells were found at the base of the secondary lamellae, on the filament between adjacent lamellae and along the edges of the filaments. They were often in close association with 'chloride' and mucous cells and were usually separated from the blood space by flattened epithelial or suprabasement cells and the basement membrane. No rodlet cells were found in the respiratory epithelium of the secondary lamellae.

Mature rodlet cells in the pseudobranch were found as a single layer of cells just below the surface of the epithelium covering the area of the secondary lamellae (Plate 37-1). They were always in close association

with mucous and 'chloride' or 'pseudobranch' cells (Plate 37-2). As in the gill they were also found on the edges of the filament and between adjacent lamellae. In the secondary lamellae they were separated from the blood space by a thin layer of flattened epithelial cells, an underlying layer of either 'chloride' or 'pseudobranch' cells, and the basement membrane.

In both gill and pseudobranch, rodlet cells were more numerous in the region of the afferent filament artery than the efferent artery. They were present in all the gills and pseudobranchs of Dicentrarchus labrax examined (10 fish), and all the gills of Salmo gairdneri (3 fish) and pseudobranchs of Rutilus rutilus (3 fish). It was difficult to make an accurate assessment of the numbers of rodlet cells present in a gill arch or a pseudobranch since there were large variations between individual filaments and also between individual fish. In a random sample of 10 pseudobranch filaments from each of 5 Dicentrarchus labrax, the density of rodlet cells ranged from 122 - 7575 per sq. mm with a mean of 2974 per sq. mm.

Development of Rodlet Cells

Many of the rodlet cells appeared to be in different stages of development. The mature stage (Plate 39-2) and stage preceding it were easily recognisable being unlike any other cells in the epithelium. Various characteristics of these two stages were used to identify the developing and immature phases of rodlet cell maturation.

The earliest development of rodlet cells was traced back to a cell (Type I) containing a network of granular endoplasmic reticulum and free ribosomes at one end and a Golgi complex of numerous saccules and vesicles at the other (Plate 38-1). There are a number of electron dense inclusions in the region of the Golgi complex. The nucleus has a light coloured

nucleoplasm with darker clumps of heterochromatin especially around the periphery, and a few mitochondria are scattered throughout the cytoplasm. These cells are often associated with maturing rodlet cells in the sub-surface epithelial tissues.

The next stage appears to be an intermediate cell type (Type II) which contains characteristics of both mature and immature rodlet cells (Plate 38-2). The cell contains a network of granular endoplasmic reticulum and free ribosomes at one end similar to cell type I. At the opposite end of the cell is a prominent Golgi complex with flattened saccules, large irregularly shaped vacuoles, and numerous smooth vesicles. Cell type II shows the earliest development of a fibrous border, consisting of a layer of widely spread microfibrils beneath the plasma membrane, and surrounding the vesicles and a few rodlet sacs. Some of the sacs contain electron dense cores. A few apparently isolated dense cores are present which may represent the tips of rodlet sacs where they are surrounded by little or no sac material. The microfibrils have a diameter of 50 Å (thin fibrils) to 150 Å (thick fibrils). The cell is close to the surface of the secondary lamellae and is covered only be a thin layer of flattened epithelial cells.

As the cell matures it becomes surrounded by a thin fibrous border (0.1 - 0.2 µm wide) beneath the plasma membrane (Cell TypeIII, Plate 39-1). The cisternae of the granular endoplasmic reticulum become distended and contain a light flocculent material. The granular endoplasmic reticulum is continuous with a large Golgi complex and large irregularly shaped areas containing a similar flocculent material. Numerous free ribosomes are present throughout the cytoplasm and the mitochondria aggregate at the opposite end of the cell to the nucleus which becomes darker and more regular in shape.

In the mature rodlet cell (Type IV) the fibrous border becomes much wider usually about 0.5 /um but up to 1.5 /um wide (Plates 39-2, 40-1). It is bounded on the outside by a plasma membrane 100 - 125 Å thick. The fibrous border contains numerous microfibrils (300 - 400 Å apart), arranged in parallel around the circumference of the cell. Beneath the plasma membrane are regularly spaced dense bands (750 - 1000 Å wide) which run parallel to the microfibrils.

The club shaped rodlet sacs take up a large part of the cytoplasm of the mature cell. Each sac is bounded by a membrane and contains a granular matrix which is less concentrated and sometimes almost absent from the peripheral region of the sac (although this may be a fixation artefact). The sacs usually contain a single electron dense core which may extend the whole length of the sac. The cores are circular in cross section and vary in diameter from 1000 - 1600 Å. The sacs are orientated with the tapered end towards the apex of the cell and bulbous end anterior to the basal nucleus.

A closely packed aggregation of mitochondria is found laterally near the apex of the cell. The fibrous border is absent in the apical region which contains large numbers of smooth surfaced vesicles (400 - 500 Å diam.) and is bounded only by the plasma membrane (Plates 39-2, 40-2).

The remainder of the cytoplasm contains many clear vesicles, a few multi-vescular lysosome-like bodies and a network of membranous vacuoles containing a lightly staining material. The Golgi complex still occupies a supranuclear position and contains numerous saccules and vesicles. Ribosomes are present throughout the cytoplasm apart from the apical region of the cell.

The apex of mature cells opens between adjacent epithelial cells. The cytoplasm protruding through the opening is bounded by a limiting membrane

which is covered with a filamentous glycocalyx. Near the apex desmosomal contact between rodlet cells and surrounding epithelial cells is sometimes found (Plate 40-2).

Rodlet cells containing only membranous vacuoles or no cytoplasmic contents at all were observed. These may have secreted their contents and are dead or dying cells. A few rodlet cells with very dark borders have been observed (Plate 41-1) and some cells contained hyperchromatic nuclei (Plate 41-2). No cells were observed in the process of secreting their contents.

In a S.E.M. study of the bass pseudobranch, a number of conical, circular openings (1 - 2 μm diam.) were found in the interlamellar filament epithelium and at the base of the secondary lamellae (Plate 41-3). The epithelium is raised in a continuous circular ridge and cytoplasmic projections can often be seen within the opening. This type of opening is thought to belong to that of the rodlet cell.

Vascular System

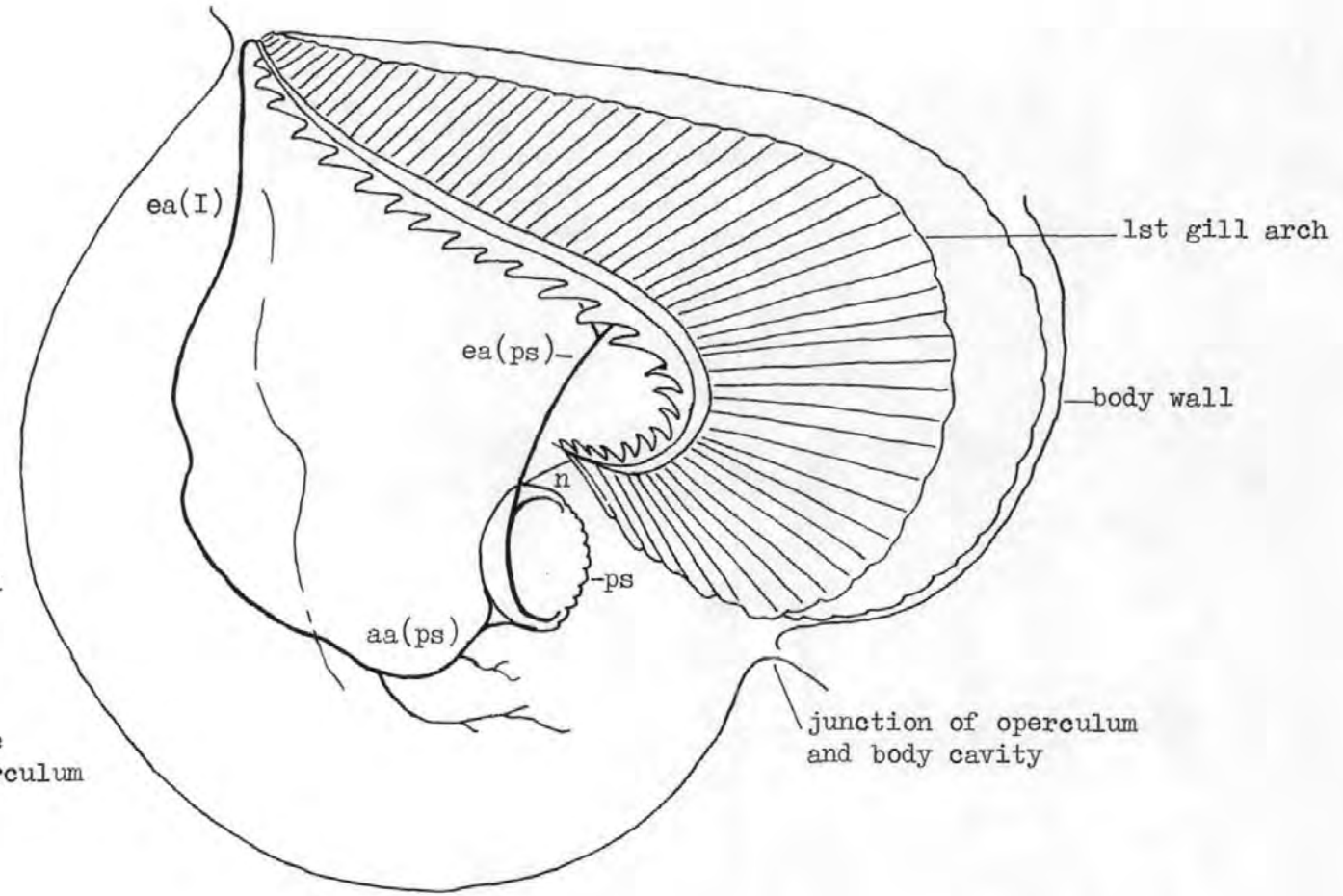
A general study was made of the vasculature in all pseudobranch types, with more detailed examinations of those of freshwater Salmo gairdneri and seawater Dicentrarchus labrax. Microfil casts of the pseudobranch vascular system were compared with gill casts from Salmo gairdneri, Dicentrachus labrax and Perca fluviatilis.

General Organisation

In all pseudobranchs studied the organisation of the vascular system was similar to that of the gills, but it varies in certain details according to the type of pseudobranch.

The primary afferent artery was always supplied ventrally by the mandibular artery which is connected to the efferent branchial arch of the first gill arch. Whilst this is the sole blood supply in Salmo gairdneri and Cyprinus carpio, an additional dorsal supply from the cephalic circle is found in Perca fluviatilis and Dicentrarchus labrax. (c.f. Figs 2c and 2d). The mandibular artery is always visible running close to the outer (free) edge of the operculum, beneath the opercular cartilage (Fig 15). In Cyprinus carpio the artery disappears beneath a thick layer of connective tissue covering the pseudobranch whilst in Salmo gairdneri it is covered only by a thin epithelium before entering the pseudobranch. When the blood supply is of mixed origin the mandibular artery forms an anastomosis with the dorsal vessel from the cephalic circle before entering the pseudobranch. Close to the pseudobranch the artery divides into two branches supplying opposite ends of the pseudobranch (Fig 15). Smaller afferent filament arteries arise from the primary afferent artery at regular intervals and these in turn supply blood to the secondary lamellae capillaries via the afferent lamellar arterioles (Plates 42-2, 43-1). Blood leaves the secondary lamellae via the efferent lamellar arterioles

Fig 15 Arterial pseudobranch circulation of Salmo gairdneri



Key

aa(ps)	afferent pseudobranchial artery
ea(ps)	efferent pseudobranchial artery
ea (I)	efferent branchial artery (lst gill arch)
ps	pseudobranch
n	pseudobranch nerve
	operculum

and passes to the efferent filament arteries which supply the primary efferent artery (Plate 42-1). The primary efferent artery supplies blood to the ophthalmic artery and the circulation of the eye. In all pseudobranchs examined a venous/lymphatic system was also found connecting with a large vein in the pseudobranch arch, which supplies the jugular system returning to the heart. A venous system has not been described previously for the pseudobranch but it is clear from serial sections and microfil injections that one does exist (see Arterio-venous System).

Arterio-arterial System

In all species studied the arterio-arterial vascular system is similar, with minor differences. The primary afferent and efferent arteries are always located on opposite sides of the pseudobranch arch although the exact disposition and morphology of these arteries varies according to the species. In Dicentrarchus labrax and Perca fluviatilis the arteries run close together and parallel to each other whilst in Salmo gairdneri the arteries are further apart and situated at different levels (Plates 44-1, 2; 46-1). In Cyprinus carpio the arteries curve around to accommodate the pseudobranch folding back on itself (Fig 14). The primary afferent artery is always situated close to the cartilaginous extensions of the pseudobranch filaments on the inner face of the organ (Plates 44 - 1, 2).

The wall of the afferent artery is very thick in most species with well developed conjunctive and muscular layers (Plates 44-1, 2; 45-1, 4; 46 - 1). The muscular layer is particularly noticeable in the trout and bass (up to 20μ thick) whilst in the perch the afferent arterial wall is thinner and less muscular.

The secondary lamellae are supplied by the afferent filament arteries which depart at regular intervals from the primary afferent artery. The

trout pseudobranch is unusual since the primary afferent artery also provides a direct supply to secondary lamellae at the base of the organ via short arterial segments.

The afferent filament arteries run very close to and are sometimes contiguous with the cartilaginous rod of the filaments. In the trout, two or more afferent filament arteries are sometimes interconnected by means of short cross vessels between them (Plate 42-2). In this species the filament arteries at the distal end of the filaments converge towards the centre of the organ where they form a complicated network of interconnecting vessels (Plate 42-2). In the bass, mullet and perch the filament arteries show no tendency to converge and do not possess transverse vessels.

From the afferent filament arteries emanate short lamellar arterioles through which blood passes into the secondary lamellae. They often branch to communicate with one to three lamellae (e.g. trout, Plate 42-2).

The vascular system of the secondary lamellae is a network of interconnecting blood spaces delimited by pillar cells and their flanges. There is no discernible difference in the vascular system of the secondary lamellae in the species examined. Each secondary lamella is bordered by a marginal vessel (Plate 19-2) which is derived from the afferent lamellar artery. In microfil casts of bass pseudobranch only the marginal vessels were filled and not the secondary lamellae network (Plates 47-1, 2). This provides evidence for the marginal channel as a direct blood pathway from the afferent to the efferent arterioles, bypassing the vascular network of the secondary lamellae. However in trout pseudobranch the marginal vessels were never filled alone and the secondary lamellae were always partially or completely filled. Channels connect the marginal vessel and the capillary network of the secondary lamellae but the individuality of the marginal vessel is always distinct in the cast

preparations. In the case of incomplete Microfil perfusion of the secondary lamellae, only the parts closest to the filament axis contained casting material. The marginal channels and secondary lamellae on the distal parts of the filament were empty or incompletely filled.

In preparations of trout pseudobranch perfused via the primary efferent artery the secondary lamellae were particularly well filled at the base of the organ where there are direct connections between the primary efferent artery and the secondary lamellae by means of short arterial segments (Plate 48-1). Secondary lamellae in the distal regions of the filaments were often poorly filled.

The efferent lamellar arteries of the secondary lamellae, communicate with the efferent filament artery via one to three branches (Plate 48-1). The efferent filament arteries run along the outer edge of the pseudobranch filaments and finally connect independently to the primary efferent artery (Plate 42-1).

The wall of the primary efferent artery is usually thinner and less muscular than that of the primary afferent artery. This is particularly noticeable in the trout and bass pseudobranch although in the perch they are of similar thickness (Plates 44-1, 2; 45-3; 46-1).

Arterio-venous System

The venous circulation has not been previously described in the teleost pseudobranch. In fact it has long been considered that the circulation is purely arterio-arterial (Laurent 1974). However in this study the use of microfil injections and serial sections clearly shows the existence of an arterio-venous pathway.

In all species studied a large central vein was found in the pseudobranch arch. It was especially noticeable in seawater fish which possess a 'free' pseudobranch (e.g. Dicentrarchus labrax (Plates 43-1, 2; 46-1, 2).

Also the central venous sinus/lymphatic system was particularly well developed in 'free' pseudobranchs. Arterio-venous anastomoses were found between the efferent filament artery and the central venous sinus of Dicentrarchus labrax although these structures could not be found in the covered and buried pseudobranchs of Salmo gairdneri and Cyprinus carpio. It is possible that they do exist in trout and carp but they may be very rare and widely spaced.

In the Pseudobranch Arch

When the pseudobranch of Dicentrarchus labrax was perfused via the ventral aorta the primary afferent and efferent arteries were well filled as was a large vessel running between and slightly above them (Plates 43-1,2). Transverse sections of wax embedded pseudobranch revealed this vessel to be a large thin walled vein or lymph vessel situated between the primary afferent and efferent arteries (Plate 46-1). The distinction between the venous and lymphatic systems in teleosts appears less clear than in higher vertebrates and will be discussed later (see Discussion). In line with currently held views of the gill vasculature (Vogel, Vogel & Kremers 1973; Laurent & Dunel 1976; Girard & Payan 1976, 1977; Dunel & Laurent 1977) the vessel described here will be referred to as the pseudobranchial vein.

The lumen of the pseudobranchial vein is not uniform along its length. Usually it is widest in the central region of the arch and narrower at its extremities where the single vessel becomes replaced by a series of interconnecting venous channels. The vein leaves the pseudobranch close to the entry of the primary afferent artery and connects with a large venous sinus in the opercular tissues behind the pseudobranch. (Plates 43-1, 44-2).

One surprising feature of the microfil casts of bass pseudobranch was the filling of the pseudobranchial vein without filling of the filament central venous sinus. This provided evidence for an arterio-venous pathway between either the primary afferent artery or the primary efferent artery and the pseudobranchial vein. Confirmation of such a pathway between the primary afferent artery and the pseudobranchial vein was made from serial sections of wax embedded material.

In the bass, perch, trout and carp the primary afferent artery has direct connections to numerous capillaries which are often packed around the main vessel (Plates 45-1, 2). The primary afferent artery of the perch is surrounded by a particularly rich capillary network and frequent connections to the artery by means of short sphincter-like vessels are found (Plate 45-2).

In addition to the capillary network the primary afferent artery also gives off numerous small arteries which often run parallel to the primary afferent artery. This system of small arteries and capillaries constitutes an afferent blood pathway of nutritive vessels to the tissues of the pseudobranch arch and the surrounding tissues of the pseudobranch. The blood from this system is eventually collected in veins which drain into the pseudobranchial vein.

The primary efferent artery is also surrounded by a vascular network of capillaries and small arteries but no connection could be found between these and the primary efferent artery (Plate 45-3). It seems likely that these small vessels arise from the primary afferent system.

In the Filament

The arterio-venous system of the filament arises from both the afferent and efferent circulation. One pathway is provided by the primary afferent artery to the nutritive vessels of the filament and the

other by direct connections between the efferent filament artery and the central venous sinus. The second pathway was only found in the bass pseudobranch.

The first pathway consists of a rich vascular network of nutritive vessels in the outer and central filament regions. This system of arterioles, venules and capillaries is derived from the primary afferent artery at the base of each filament. The vessels are arranged mainly parallel to the longitudinal filament axis with some transverse or oblique connections at both sides of the filament cartilage. On the outer edge of the filament in trout and perch, two small arteries run along each side of the afferent filament artery (Plate 47-3). They are often interconnected with those from neighbouring filaments (Plate 47-3). A similar, but less regular system of small arteries is also seen on the afferent side of the bass pseudobranch (Plate 47-2).

In the central region of the filament small vessels of identical origin run close to the cartilage and the afferent filament artery. (Plate 49-1).

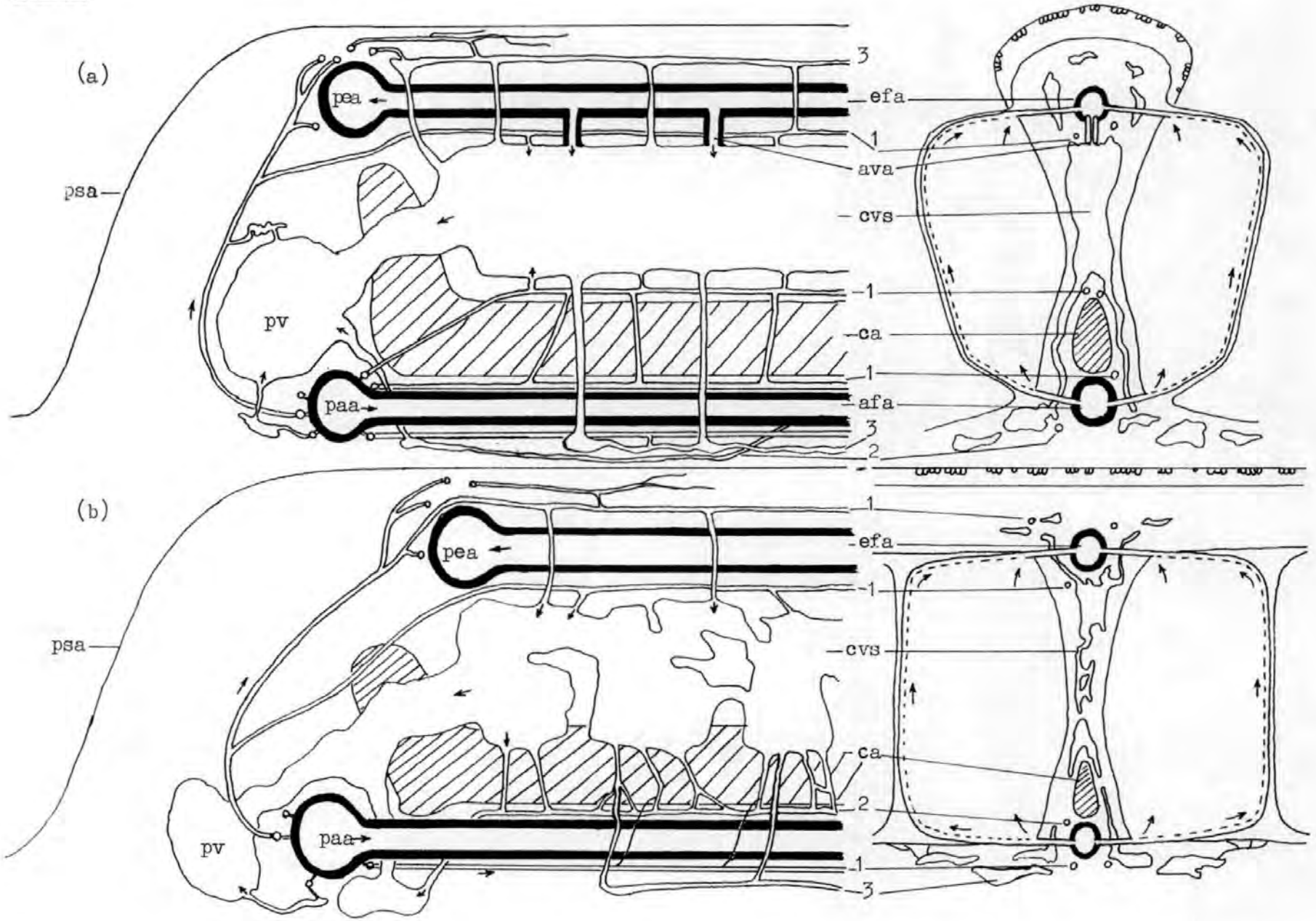
The nutritive vessels eventually connect to the central venous sinus or its extensions which finally drain into the pseudobranchial vein.

The central venous sinus of the filament appears as an irregularly shaped vascular channel, extending almost from the tip of the filament to its base where it drains directly into the venous system of the arch. The exact location of the pseudobranchial vein and its connections to the C.V.S. vary from species to species, as does the morphology of the C.V.S. itself (c.f. Figs 16-1 & 16-2). In many sections of histological material the C.V.S. remained tightly closed. However, when open the C.V.S. of the seawater fish, bass and mullet, is usually larger than that of the freshwater trout, perch and carp. Whilst the C.V.S. of the bass

Fig 16 Circulation diagrams of the pseudobranch arch, filament and secondary lamellae of (a) bass and (b) rainbow trout. Cross sections of the filaments and their secondary lamellae are shown to the right. Arrows indicate the direction of blood flow.

psa	pseudobranch arch
paa	primary afferent artery communicating with collateral arteries
pea	primary efferent artery
afa	afferent filament artery
efa	efferent filament artery
pv	pseudobranchial vein
ava	arterio-venous anastomose
ca	cartilage
cvs	central venous sinus
f	filament
sl	secondary lamella
1	small artery of the arterio-venous system
2	small vein of the arterio-venous system
3	extensions of the c.v.s.

Fig 16


(a)
psa
pea
pv
paa
3
efa
1
ava
cvs
1
ca
1
afa
3
2
(b)
psa
pea
pv
paa
1
efa
1
cvs
ca
2
1
3

pseudobranch takes the form of a large open channel, that of the trout appears as a branching system of interconnecting vessels with the size of the sinus compartments near the cartilage and efferent filament artery often greater than those nearer the afferent artery, particularly at the base of the filament.

The wall of the central sinus is extremely thin, consisting of a basement membrane and a thin endothelial layer (Plates 14-1, 2). No muscle cells and only a few adventitial cells are visible. The central sinus vessels often reach close to the afferent filament artery wall (Plates 46-2, 3), the afferent lamellar vessels and sometimes also to the basement membrane separating the lamellar blood spaces from the central core of the filament (Plate 49-1). However, no direct connections between the central sinus, or its branches, and the lamellar blood spaces were seen. In cases where the filaments are attached to the opercular wall the C.V.S. gives rise to sinus-like vessels outside the core of the filament and running in the connective tissue between the afferent filament arteries (Plates 49-1, 2). On the efferent side of the filament, shorter extensions form a system of venous channels around the efferent filament artery (Plate 50-1). In the buried carp pseudobranch, extensions of the C.V.S. open into particularly large sinus-like channels outside the core of the filament (Plate 44-3). The folded nature of this pseudobranch allows a number of filamental sinuses to connect with each channel (Plate 49-3) which may or may not be filled with blood in histological sections.

The second arterio-venous pathway between the efferent filament artery and the C.V.S. was only found in the bass pseudobranch, although it is possible that these connections may occur less frequently in other species.

The connections are provided by special arterio-venous anastomoses (A.V.A's) which occur in great numbers and at fairly regular intervals along the efferent filament artery ($A.V.A._{eff.}$). While most of them lead directly to the C.V.S. some open into nutritive vessels connected with the C.V.S. at numerous points along the filament. No A.V.A.s were found between the afferent filament artery and the central sinus.

The number of anastomoses varies from filament to filament (Table 6). They are more common on the central filaments of the pseudobranch with fewer numbers in the tip and distal regions than in the basal region. The distribution of anastomoses and of lamellae counted on one side of each filament of bass pseudobranch is shown in Table 6.

Table 6

Pseudobranch region		Distance covered (mm) per filament	No of filaments examined	Av. No of lamellae per mm *	Av. No. of anasto-moses per mm*	Av. No. of anasto-moses per lamella
Outer filaments	Distal region	0.60	8	21.0	12	0,571
	Basal region	0.40	8	18.0	25	1.388
Central filaments	Distal region	0.75	7	20.5	18.5	0.902
	Basal region	0.50	7	16.5	33	2.000

* The mean number of lamellae and anastomoses per mm were calculated to the nearest 0.5 of a whole number.

The foregoing table shows that anastomoses in the basal region of any one filament are more than twice as frequent as those in the distal region. They are almost four times as common in the basal region of the central filaments as in the distal region of the outer filaments.

The A.V.As have a characteristic morphology, and are easily identified in paraffin sections (Plate 50-2). The vascular channel from the efferent filament artery to the C.V.S. is tightly occluded by conspicuously large endothelial cells, with a relatively dense cytoplasm and big, often lobated nuclei. The endothelial cells are found inside a layer of smooth muscle cells, and often protrude into the lumen of the efferent artery. Vesicles are sometimes discernible at the protruding cell surface in resin sections stained with methylene blue (Plate 50-1).

Ulstrastructure of the A.V.A.

The A.V.A. endothelial cells are highly specialised with a number of characteristic features. Two types of cell were identified although intermediate forms were also observed.

Type I Endothelial Cells

These cells are located on the arterial side of the A.V.A. where they frequently bulge into the arterial lumen. Their surface is usually rounded although microvilli or short rounded protrusions sometimes extend into the lumen (Plate 51; 53-1). These cells are elongated and often two or three times as large as type II endothelial cells of the same A.V.A. They make contact with normal arterial endothelia by means of tight junctions or desmosome-like junctions.

The nucleus is large with a pale nucleoplasm. The cytoplasm of the cell is highly filamentous with numerous intracellular filaments (Plate 52-1) often arranged in concentric whorls. The filaments are found throughout the cytoplasm except around the periphery of the cell. Numerous

micropinocytic vesicles are found in this region close to the cell membrane (Plate 52-2). In addition to these the cells contain larger membrane bound vesicles with an electron dense core. These are similar to the Weibel-Palade type organelles in normal endothelia but are generally smaller and less numerous. Small numbers of mitochondria are present in the cell and an occasional supra-nuclear Golgi complex is seen. Clusters of dark granular material resembling glycogen are situated amongst the filaments and occasional free ribosomes are found in the peripheral cytoplasm.

Type II Endothelial Cells

These cells are located on the venous side of the anastomoses (Plate 52-3). They are usually cuboidal in shape and smaller than the type I cells. The nucleus takes up a large part of the cell. There are few filaments but large numbers of vesicles are present. There are a few mitochondria and some granules, probably glycogen, are scattered throughout the cytoplasm.

The opening of the A.V.A. into the C.V.S. is characterized by protruding type II endothelial cells which have occasional microvilli-like extensions on their surface (Plate 52-3).

Endothelial cells intermediate in morphology between type I and type II A.V.A. endothelial cells occur approximately in the middle of the anastomoses (Plate 51; 52-2).

In general, therefore, there is a distinct morphological polarity in each anastomosis with distinct endothelial cell types at either end, and a progressive change in morphology from one end of the anastomosis to the other. Exceptionally, however, type I cells are also seen close to the C.V.S. All A.V.A. endothelial cells also contain numerous micropinocytic

vesicles. These are most prominent in type II cells, and are more numerous than in normal endothelia of the afferent or efferent arteries.

The anastomoses do not always take a direct course from the efferent filament artery to the C.V.S. Sometimes the A.V.As run parallel to the efferent artery for some way and then perpendicular to it before they open into the artery or the C.V.S.

The lumen of the A.V.A. was always narrowed to a small slit or even to a labyrinth by the endothelial cells and their processes (Plate 51). The endothelial cells were joined by means of tight junctions.

Cover Cells

Each anastomosis is ensheathed by a layer of cells with a pale cytoplasm, numerous mitochondria and micropinocytic vesicles (Plates 51; 52-3). These cover cells, which are usually flatter than the A.V.A. endothelial cells, surround most of the anastomosis apart from the region near the C.V.S. The space between endothelial and cover cells is usually occupied by a basal lamina which often disappears in regions of close contact between the cell types (Plate 51). Near the C.V.S. it usually disappears altogether and the endothelial cells make direct contact with the interstitial space (Plate 52-3).

Nerves

Perpendicular to the general direction of the A.V.As run bundles of nerve fibres containing mainly unmyelinated axons. The two bundles either side of the anastomosis are branches of a single large bundle normally running parallel to the efferent filament artery. Partly or completely naked axons are often in close contact with A.V.A. cover cells and also occasionally with endothelial cell processes (Plates 53-1, 2). However no specialized synaptic contacts have been observed. Although nerve endings

containing synaptic vesicles have not been positively identified so far, in relation to any specific A.V.A. structure, axonal varicosities are sometimes found. These varicosities contain numerous clear vesicles and occasional mitochondria while neurotubules and neurofilaments are rare. However the latter two elements are common along most of the axon length.

Comparison with Gill Vasculature

Microfil casts were made of gills from trout, perch and bass. The general organisation of the fish gill is well known (see Review by Hughes & Morgan 1973). Recently more detailed studies have been made on the vasculature using conventional histological techniques and microfil perfusion (Laurent & Dunel 1976).

The Arterio-arterial System

The arterio-arterial vascular system of the gill is similar to that of the pseudobranch. The afferent branchial artery which runs from the ventral to the dorsal side of the operculum corresponds to the primary afferent artery of the pseudobranch, since it supplies blood to the filament arteries. However in the gill this artery supplies two rows of filaments (hemibranchs) (Plates 54-1, 2) as opposed to one in the pseudobranch. The afferent filament arteries depart at regular intervals from the afferent branchial artery and run along the inner edge of the filaments. In trout and perch the afferent filament artery has an enlargement at the bifurcation of the two hemibranchs (Plates 55-1, 2), in the most distal part of the septal area, roughly one third of the distance from base to tip of the filament. These enlargements of the afferent filament vessels were first noted in rainbow trout by Fromm (1974) who called them 'blebs'. In the septal region up to these swellings the opposite filaments are joined together by adductor muscles which possess a rich vascularisation. Distal to the 'blebs' the filaments separate and remain free to their tips. There was no evidence of 'bleb-like' swellings on the afferent filament vessels of the pseudobranchs examined.

As in the pseudobranch the afferent filament arteries supply the secondary lamellae by means of short lamellar arteries which may divide

into two or three branches, each supplying a secondary lamella. The vascular system of the gill secondary lamella is similar to that of the pseudobranch, consisting of a network of vessels surrounding pillar cells and a distinct marginal vessel bordering the lamella. The marginal channel arises from the afferent lamellar vessel and connects with the capillary network by means of channels whilst still retaining its own individuality. In the gill, no casting of the marginal vessel alone was ever observed.

The secondary lamellae are drained by efferent lamellar arteries which are always shorter and of smaller diameter than the afferent lamellar vessels. Usually a single lamella is drained by one vessel only. The efferent lamellar arteries communicate separately with the efferent filament artery which runs on the external edge of the filament. Finally all the efferent filament arteries independently connect to the efferent branchial artery.

Arterio-venous System

In the gill arch

The arterio-venous system of the gill is somewhat different to that of the pseudobranch. In the gill this system arises solely from the efferent circulation whilst that of the pseudobranch arises from both the afferent and efferent systems. In the gill, it is the efferent branchial artery which gives off numerous small arteries supplying the tissues of the gill arch and filaments. This is in contrast to the pseudobranch where the primary afferent artery performs this function. Also, in the gill, the efferent branchial artery has direct connections to numerous capillaries which surround the vessel, similar to those surrounding the primary afferent artery of the pseudobranch. Similarly, blood from this capillary network is collected in veins which drain into

the branchial vein. In the gill another capillary system is located in the proximal part of the efferent filament arteries near their connection to the efferent branchial artery (Plates 54-3; 55-3; 56-2).

In the Filament

Two types of vessels supply blood to the arterio-venous system of the filament; (1) small arteries emanating from the efferent branchial artery or the proximal part of the efferent filament artery and (2) direct connections between the efferent filament arteries and the central venous sinus.

In the gills of trout, perch and bass the base of the filament is surrounded by a highly branched network of small arteries which arise from the efferent branchial artery. Two arteries branch from the trunk on the filament and run along each side of the efferent filament artery (Plate 54-3). Near the base of the filament these arteries often interconnect with neighbouring filaments. In the trout, small arteries of identical origin also run parallel with the afferent filament artery.

The second arterio-venous pathway is made up of direct connections between the efferent filament artery and the central venous sinus. Although not visible in microfil castings these connections are clearly visible in histological sections as sphincter-like structures similar to the arterio-venous anastomoses (A.V.A's) found between the efferent filament artery and the C.V.S. of the bass pseudobranch. They were identified in the gills of trout, perch and bass where they appeared frequently but at irregular intervals along the wall of the efferent filament artery. Whilst most of the A.V.A's lead directly to the central sinus some were also seen to originate from small nutritive vessels which are connected with the central sinus at numerous points along the filament. No A.V.A's were found between the afferent filament artery and the C.V.S.

As in the pseudobranch the central venous sinus is an irregularly shaped channel occupying the central core of the filament for most of its length. It lies mainly between the cartilage and the efferent artery with branches extending around the cartilaginous process and the afferent filament arteries. Shorter prolongations also form a network around the efferent filament arteries.

The morphology of the sinus is not uniform from species to species. In the bass the C.V.S. is a simple sac-like structure (Plate 56-4) whilst in trout and perch the morphology is more complicated. In these species the C.V.S. has a network of branches which extend towards the inside part of each filament. These prolongations extend between the base of the secondary lamellae and partially surround the cartilage and afferent filament artery. In the trout the C.V.S. is compact near the efferent filament artery but becomes divided around the cartilage rod and finger shaped around the afferent filament artery. On the efferent side of the filament, shorter extensions form a vascular network around the efferent filament artery and between the base of the secondary lamellae, particularly in the more distal region of the filament (Plate 56-3).

In trout and perch the junctions between the C.V.S. and the branchial vein are made by veins running parallel to the afferent filament arteries of each filament (Plate 55-2). The branchial venous vessels have been described by J. Muller (1839) who found that they leave the gill arch in two directions and drain the blood back to the heart.

Choroid Rete Mirabile

In all cases the efferent pseudobranchial artery gives rise to the ophthalmic artery supplying the choroid rete mirabile at the back of the eye. The ophthalmic artery has no communication with neighbouring vessels so all the blood to the choroid rete has first to pass through the

pseudobranch. The existence of the choroid rete mirabile is closely dependent on the presence of a pseudobranch (Wittenberg & Haedrich 1974) so there may be some functional relationship between the two. The structure of the choroid rete may give a clue to this relationship.

When the pseudobranch was injected with microfil via the ventral aorta the choroid rete was also filled. The eyes were removed from microfil injected trout and the choroid rete was dissected out for examination.

The choroid rete mirabile is a large horseshoe-shaped capillary network which lies in the choroidal layer of the eyeball (Plate 57-1). It lies exterior to the retina and is separated from the sclera by an intense black membrane. There is no strong attachment of the rete to structures other than blood vessels in the interior of the eye.

The ophthalmic artery pierces the sclera behind and dorsal to the optic nerve and passes into the lumen of the wide ophthalmic venous sinus that lies along the inner border of the choroid rete (Plate 57-1). Therein it divides into two branches which run along the inner border of each limb of the rete, giving off parallel twigs into it so that clusters of large capillaries arise almost directly from the thick-walled artery. These capillaries radiate in almost parallel columns occasionally giving off collateral branches towards the periphery of the rete from which they pass into the choriocapillaris network (Plate 57-2). The rete mirabile proper is only a thin sheet consisting of many thousands of closely arranged and parallel arterial and venous capillaries which cap an enormous tangled array of coarse, intermediate vessels filling much of the back of the eye.

Blood is returned from the choriocapillaris vessels by small vessels to the venous capillaries of the rete mirabile proper. These empty directly into the ophthalmic venous sinus which drains into the large ophthalmic vein.

INNERVATION

The studies on the pseudobranch innervation were carried out primarily on the trout pseudobranch which is richly innervated and has a readily accessible nerve supply suitable for denervation and electrophysiological studies. Structural studies were also made on the pseudobranchs of carp, bass, mullet and perch although none of these were as densely innervated as the trout pseudobranch.

Depending on the species the pseudobranch is supplied by one or several branches of the glossopharyngeal (IX) and/or facial (VII) nerves. The exact position of the nerves varies between species with slight variations even between individuals (Laurent & Dunel 1966). Trout and carp pseudobranchs are supplied solely by the glossopharyngeal nerve (Fig 17-1) whilst perch and bass are supplied by the glossopharyngeal and a branch of the facial nerve which joins with the glossopharyngeal before entering the pseudobranch (Fig 17-2). The nerve supply to the pseudobranchs of species representing the main families is covered in an extensive review by Stork (1932).

In the trout the glossopharyngeal nerve runs ventro-medially in a groove formed at the anterior margin of the opercular opening and the body wall (Fig 15, Plate 2-1). The nerve supply to the pseudobranch is provided by the pretrematic branch of the IX nerve which leaves peripheral to the ganglion (Fig 18). Near the pseudobranch it runs close to the efferent blood vessel leaving the structure (Fig 18). Just before innervating the pseudobranch the nerve divides into three main branches, two of which run ventrally away from the pseudobranch. The organ is innervated by a horizontal branch of the nerve and little branches which stem from the trunk at several points. The branches entering the pseudobranch are surrounded by adipose tissue and small nutrient blood vessels (Plate 58-1, 2).

Fig 17 Arterial circulation and innervation in the pseudobranchs of (a) carp and (b) bass

aa(ps) afferent pseudobranchial artery

ea(ps) efferent pseudobranchial artery

aab(1) afferent branchial artery

eab(1) efferent branchial artery

da dorsal aorta

va ventral aorta

ma mandibular artery

op.a ophthalmic artery

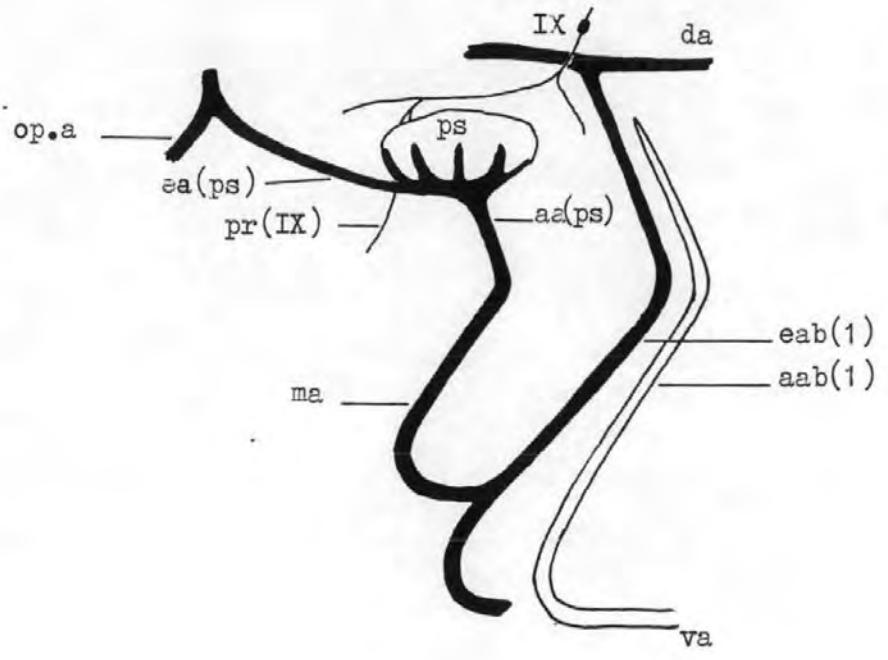
ps pseudobranch

VII ganglion of nerve VII (facial)

IX ganglion of nerve IX (glossopharyngeal)

pr(IX) pretrematic branch of the glossopharyngeal nerve

Fig 17


IX
da
op.a
ps
ea(ps)
pr(IX)
aa(ps)
eab(1)
aab(1)
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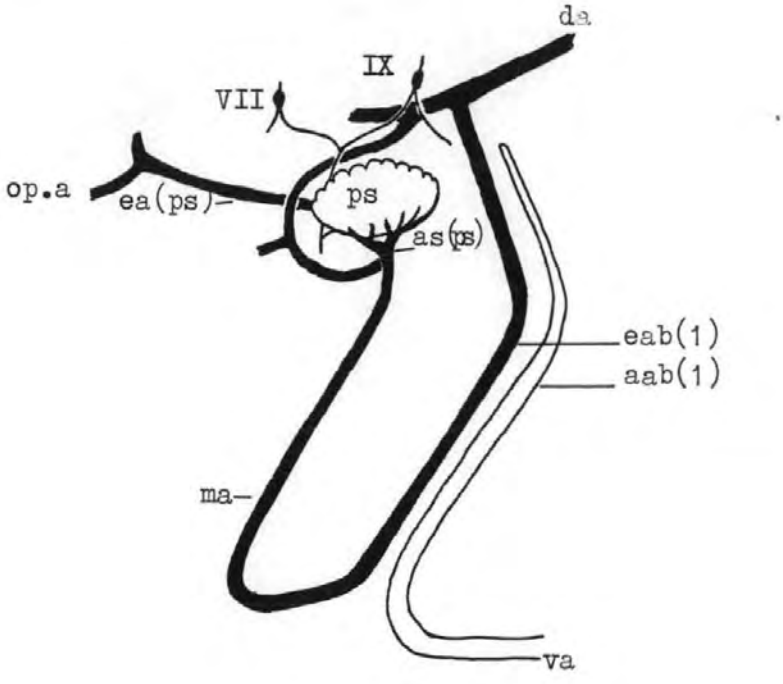

da
IX
VII
op.a
ea(ps)
ps
as(ps)
eab(1)
aab(1)
ma
va

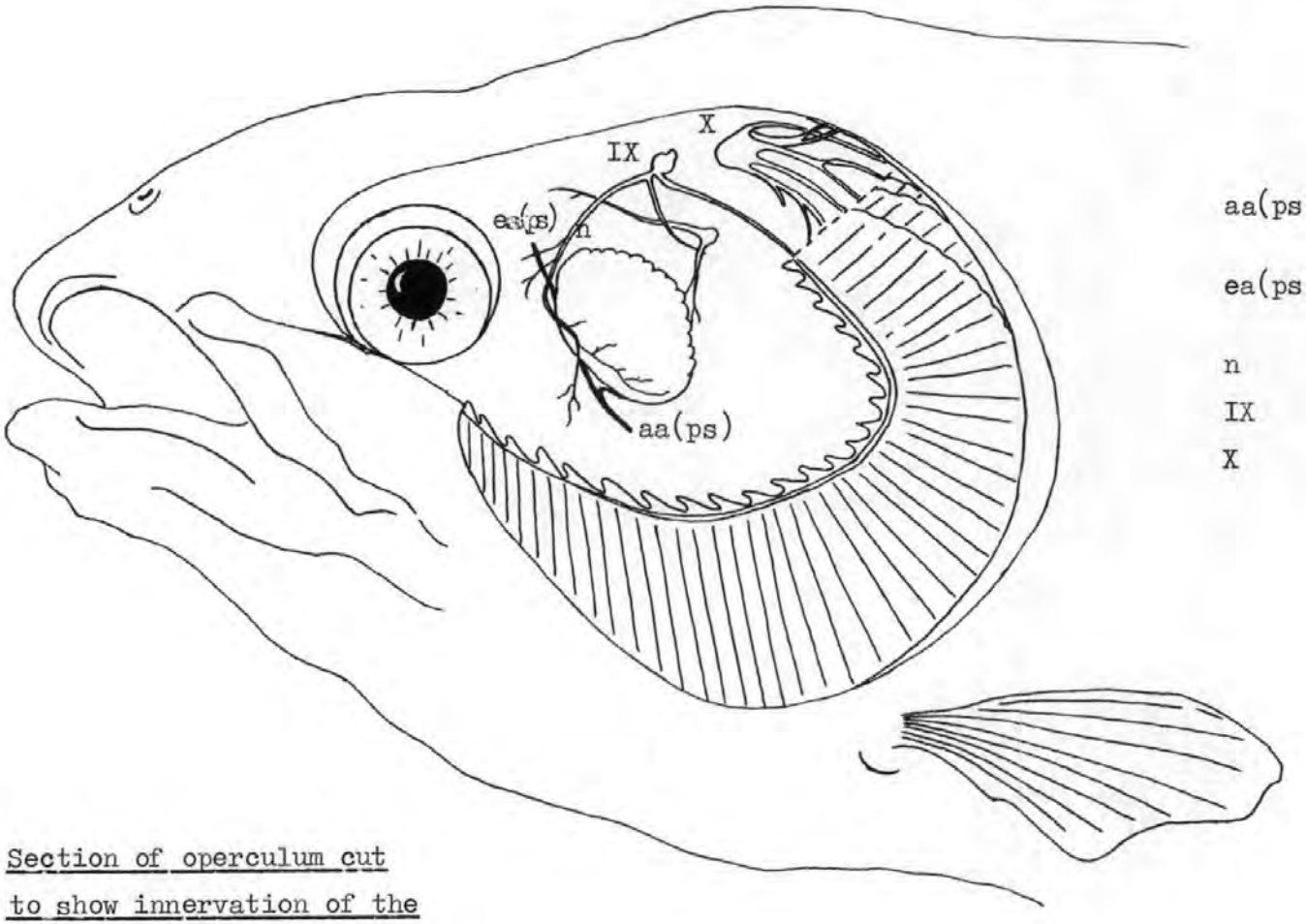
The main trunk of the pseudobranch nerve supply contains both large and small myelinated fibres as well as bundles of unmyelinated fibres. For degeneration studies, the normal intact nerve of one pseudobranch was compared with the sectioned nerve from the contralateral pseudobranch of the same fish.

Myelinated Fibres

The diameter and fibre populations of intact nerves from five trout (250 - 300 g) were measured. Sections of whole nerve were cut near its entry to the pseudobranch, parallel with the primary efferent artery (Fig 18). The average diameter of the nerve was between 140 and 165 /um with a mean of 1219 (1014 - 1403) myelinated fibres. The diameters of the fibres were measured and the percentage of the total fibre population at each diameter was calculated and plotted on a histogram. Most fibres (85.2%) were less than 4 /umin diameter with the majority between 1 and 2 /um. The largest fibres were up to 14 /umin diameter (Plate 59-1, 2, 3; Fig 19).

The entire nerve is divided up into a number of loosely connected bundles containing fibres of various diameters. Each fibre is surrounded by a thin layer of Schwann cell cytoplasm and an outer layer of collagenous fibres (endoneurium) oriented longitudinally parallel to the nerves (Plates 60-1, 2). Schwann cell nuclei are occasionally seen in the cytoplasm surrounding the fibres. The axoplasm of each nerve fibre contains microtubules and neurofilaments with small numbers of mitochondria (Plate 61-2). The whole nerve is ensheathed by loose connective tissue (epineurium) and surrounded by large fat cells.

As the nerve is followed distally towards the pseudobranch there is a gradual separation of the initially heterogenous population of fibres into bundles within which the majority of fibres are of a similar diameter. (Plate 59-3)



aa(ps) afferent pseudobranchial artery

ea(ps) efferent pseudobranchial artery

n pseudobranch nerve

IX glossopharyngeal nerve

X vagus nerve

Fig 18 Section of operculum cut to show innervation of the pseudobranch and gill arches in Salmo gairdneri

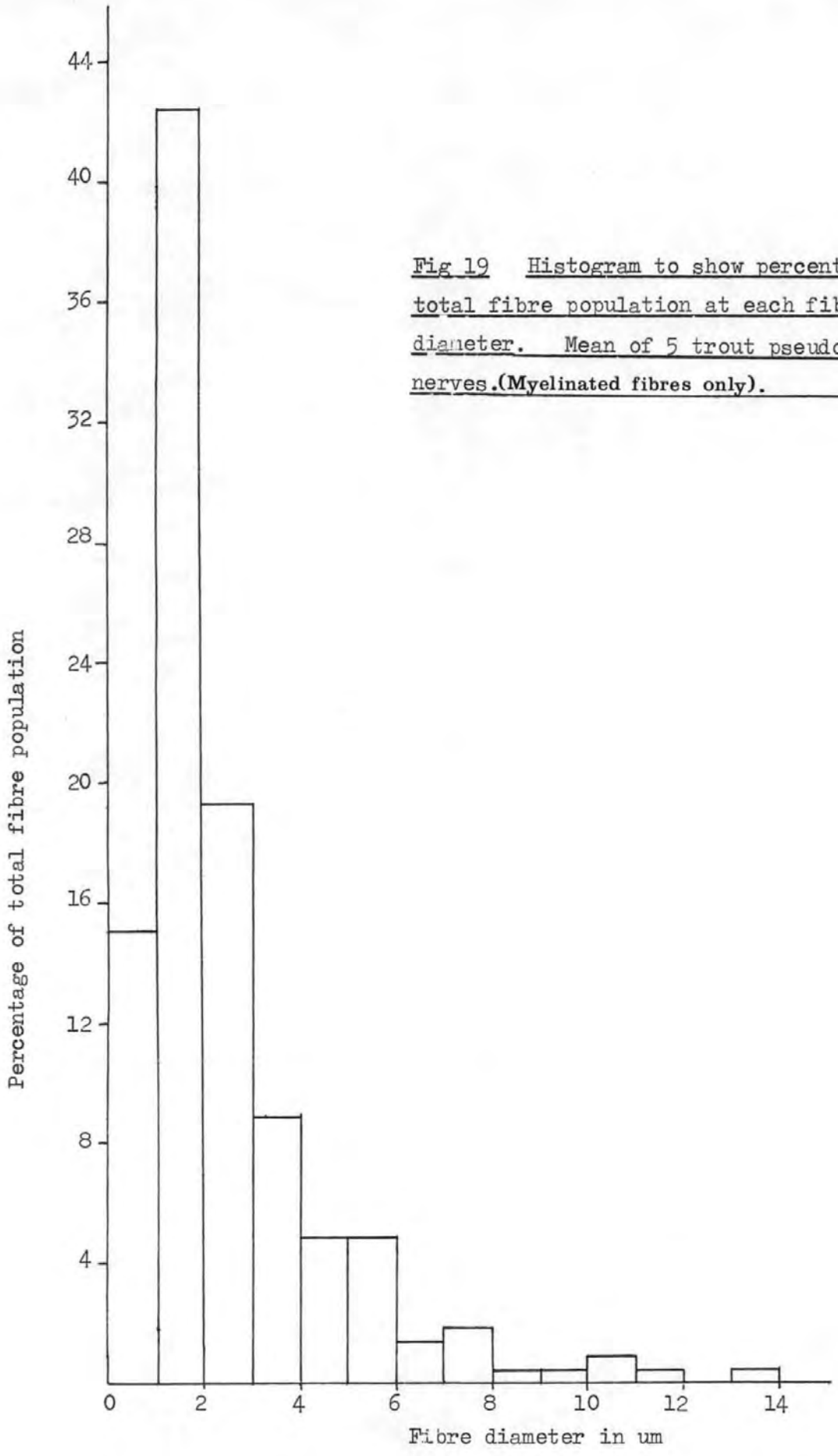


Fig 19 Histogram to show percentage of total fibre population at each fibre diameter. Mean of 5 trout pseudobranch nerves.(Myelinated fibres only).

Some bundles contain predominantly medium and large myelinated fibres (4 - 14 µm) whilst others contain mainly small myelinated fibres and unmyelinated fibres. At the base of the pseudobranch these bundles become segregated and provide branches to different regions of the pseudobranch and the surrounding tissues.

Unmyelinated Fibres

Although the main part of the nerve consists of myelinated axons there are also small bundles of unmyelinated fibres which make up a relatively small part of the nerve. These bundles are situated between the myelinated fibres and contain 5 - 20 unmyelinated axons 0.1 - 1.0 µm in diameter (Plates 60-1, 2; 61-1). Each bundle is ensheathed by a thin sheet of Schwann cell cytoplasm and separated from the myelinated fibres by the collagen fibres of the endoneurium. The unmyelinated fibres are rich in neurotubules with occasional neurofilaments and small mitochondria (Plate 61-3).

Primary Plexus

Close to the primary efferent artery at the base of the organ, the pseudobranch nerve and its branches give rise to a large plexus (primary plexus) containing a complex network of myelinated and unmyelinated fibres and large numbers of nerve cell bodies (Plate 62-3, 4). Serial sections of pseudobranchs impregnated by Cajal's method revealed an extensive, dense innervation (Plate 62-1, 2) which supplies the primary blood vessels and the secondary plexus in the axis of each filament.

Myelinated Fibres

There are great numbers of small myelinated fibres, usually 1 - 2 µ in diameter and rarely greater than 4 µ. They are usually found in bundles accompanied by groups of unmyelinated fibres (Plate 63-1). A small bundle containing 5 - 10 myelinated fibres may contain ten times as

many unmyelinated fibres. Bundles containing only one or two myelinated fibres together with a number of unmyelinated fibres form an extensive branching system between the fat cells of the adipose tissue. Occasional myelinated fibres or axons losing their myelin sheath are often seen close to the nerve cell bodies. Myelinated axons are rich in microtubules and neurofilaments, and occasional clusters of elongated mitochondria. Vesicular or tubular profiles of agranular endoplasmic reticulum are also seen.

Unmyelinated Fibres

A considerable number of unmyelinated fibres ranging in diameter from less than 0.1 μm to 3.0 μm are found in the primary plexus. The smaller fibres (0.1 - 1.0 μm) are usually arranged in bundles containing up to 150 unmyelinated fibres invested by extensive Schwann cell cytoplasm. Unmyelinated fibres associated with myelinated axons are usually larger (0.2 - 1.5 μm) and surrounded by less Schwann cell cytoplasm. The axoplasm of the smallest fibres contains mainly microtubules with occasional mitochondria and a few neurofilaments. The largest fibres often have a pale axoplasm with fewer neurotubules but larger numbers of mitochondria and a more extensive agranular endoplasmic reticulum (Plate 63-2).

Medium to large unmyelinated fibres (0.5 - 2 μm) are usually found in small bundles of 10 - 20 fibres whilst the largest fibres are often found singly, sometimes without any apparent Schwann cell cytoplasm. In the regions of the plexus near the neuron cell bodies the unmyelinated fibres show a considerable variation in size and morphology. There is often little or no Schwann cell cytoplasm between individual axons with the consequent formation of small groups of naked axons engulfed as a single unit by a surrounding Schwann cell sheath (Plate 64-3, 4).

The smaller axon processes (0.05 - 0.5 /um) are rich in microtubules (220 - 250 Å diam.) with occasional neurofilaments (75 - 100 Å thick). The larger processes (> 0.5 /um) contain few or no microtubules and neurofilaments but may possess clusters of clear vesicles (usually about 400 Å in diameter, but up to 1000 Å) and occasional dense core vesicles (700 - 1000 Å). Mitochondria and clusters of glycogen particles (≃300 Å diam.) are often seen in these processes. Some are closely apposed to the cell membrane of the neuron perikaryon where they form axo-somatic synapses (Plate 64-2).

Synapses

The axo-somatic synapses are characterised by the formation of an axon bouton and its specialised membranous contact with the perikaryon of a neuron. The boutons contain mitochondria and clear vesicles (≃ 500 Å diam.) which accumulate at the pre-synaptic membrane of the axon. (Plate 64-2) Both the axon and perikaryon cell membranes show thickening with an accumulation of osmiophilic material which is denser at the cytoplasmic aspect of the perikaryon post-synaptic membrane. The thickened zone may be 0-3 - 0.4 /um long with an intermembrane synaptic cleft of 220 - 250 Å . Serial sections showed the presence of several synaptic regions on the same neuron.

Neurons

The primary plexus contains a large number of neuron cell bodies (perikarya) of variable morphology. They vary in size but are usually quite large (7 - 30 /um in diameter) and may be unipolar, bipolar or multipolar with numerous dendritic processes (Plate 62-3, 4). The greatest numbers are found at the base of each filament where they may form large aggregates containing up to 50 cell bodies (Plate 62-3).

The cell bodies are surrounded by numerous dendritic and axonal processes and their satellite and Schwann cell sheaths. They are embedded in connective tissue which consists of thick layers of collagen fibres and fibroblasts, especially around small groups of isolated neurons (Plates 63-3; 64-1). In large groups the individual neurons and their processes are surrounded by only a thin layer of collagen.

The cytoplasm of the neuronal perikarya contains characteristic stacks of granular endoplasmic reticulum (Nissl substance) which may be arranged in parallel cisterns as well as loosely scattered throughout the cytoplasm (Plates 63-3; 64-1). Clusters of free ribosomes are scattered abundantly throughout the cytoplasm as polysomal rosettes. Profiles of Golgi apparatus are commonly seen as flattened membranous sacs and cisternae with associated small vesicles. The cell body also contains a large number of small, spherical or elongated mitochondria ($\simeq 0.2\,\mu$m diam.). In addition less obvious neurofilaments form a fine meshwork throughout the cytoplasm and occasional lysosomes are seen. The nucleus is large and round with evenly dispersed chromatin and a round compact nucleolus.

Innervation of Primary Blood Vessels

(a) Primary afferent artery

The adventitia of the primary afferent artery is densely innervated by a highly branching plexus of nerve fibres and their arborescent endings (Plates 62-2; 65-1). The fibres are grouped in small bundles containing unmyelinated axons 0.3 - 1.5 μm in diameter and frequently accompanied by one or two myelinated fibres (Plate 66-1). The fibres do not always contain microtubules and neurofilaments but may contain mitochondria, clear and dense core vesicles and agranular endoplasmic reticulum (Plate 66-3). Some of the larger fibres contain a very extensive

agranular endoplasmic reticulum with irregularly branched tubular and vesicular components (Plate 66-2). The bundles are surrounded by collagen fibres and fibroblasts.

Towards their endings the fibres lose their Schwann cell sheath and become naked axons separated from each other only by a narrow intercellular space. These naked axons are 0.2 - 0.8 μm in diameter and contain few microtubules and neurofilaments. Some contain clear and dense core vesicles, mitochondria, profiles of agranular endoplasmic reticulum and small clusters of glycogen particles. They may be found isolated in the adventitia surrounded by collagen fibres (Plate 66-3) or situated near the smooth muscle cells of the artery wall (Plate 67-1).

(b) Primary Efferent Artery

Bundles of unmyelinated and naked axons similar to those in the wall of the primary afferent artery, are found in the adventitia of the primary efferent artery (Plate 67-2). Naked axons rich in vesicles and mitochondria are often found amongst the collagen fibres or in close proximity to the smooth muscle cells of the vessel. Occasionally a neuron cell body is seen adjacent to the adventitia of the vessel (Plate 67-3).

Using Cajal's silver impregnation method a region containing short, thick nerve processes was found around the primary efferent artery of the trout pseudobranch (Plate 65-2). These processes (10 - 15 μm long and 3 - 5 μm wide) are polarised towards the lumen of the vessel. Some give rise to a number of thinner extensions. They are found in a region close to the exit of the vessel from the pseudobranch but it has not been possible to locate it for electron microscope studies.

Innervation of Nutritional Vessels

Small bundles of fibres are often seen close to the nutritional arteries within 0.2 µmof the smooth muscle cell layer (Plate 67-4). The bundles usually contain 2 - 7 unmyelinated fibres 0.2 - 0.9 µmin diameter. They are ensheathed by little or no Schwann cell cytoplasm and may contain microtubules and neurofilaments, clear and dense core vesicles and agranular endoplasmic reticulum.

Secondary Plexus

The secondary plexus originates from the primary plexus and develops in the axis of each filament parallel to the internal side of the efferent filament artery. It extends from the base to the tip of the filament as a complex network of nerve fibres and nerve cell bodies (Plate 68-1).

Fibres

The nerve fibres of the secondary plexus are mainly unmyelinated although a few myelinated fibres are found at the base of the filament. They are usually found amongst bundles of unmyelinated fibres running between the secondary and primary plexes. However they soon disappear and are absent for most of the filament length. The unmyelinated fibres vary in diameter from 0.1 - 1.0 µmwhilst the few, small myelinated fibres are rarely more than 1.0 µm.

The arrangement of the fibres varies according to the filament region.

Running the length of the filament, between the efferent filament artery and the central venous sinus is a single large nerve bundle (up to 15 µmacross) containing unmyelinated fibres and Schwann cells rich in granular endoplasmic reticulum, Golgi membranes, mitochondria and free ribosomes (Plate 69-1, 2). Although the Schwann cells may be found at the centre of the bundle the individual unmyelinated axons are generally

separated by little or no Schwann cell cytoplasm, and are found as groups of naked axons with closely apposed cell membranes. The axons may contain neurotubules and neurofilaments or may be devoid of these elements and contain mitochondria and agranular endoplasmic reticulum. In the region of the arterio-venous anastomoses in the bass, the bundle splits into two branches running either side of the specialized endothelial cells of the anastomosis. Axonal varicosities containing clear vesicles may run close to these cells (see Vascular System).

Around the outside of the efferent filament artery but still within the central compartment are found small isolated groups of 1 - 4 unmyelinated fibres. Some of these are quite large (2 - 3 μm in diameter), and contain a clear cytoplasm with varying amounts of mitochondria, clear and dense core vesicles, and tubular elements (Plate 69 -3, 4).

In the connective tissue of the central compartment between the basement membrane and the central venous sinus there is a dense system of unmyelinated nerve fibres, 0.1 - 1.0 μm in diameter, found in bundles which vary in composition from a few naked axons to large bundles 5 - 10 μm across (Plate 68-2, 3, 4). Fibres in the larger bundles are usually ensheathed by Schwann cells and their extensions whilst the smaller bundles have little or no Schwann cell cytoplasm and contain closely apposed naked axons (Plate 68-4). These naked axons may contain clear vesicles (350 - 500 Å diam.), dense core vesicles ($\simeq$ 1000 Å), small mitochondria, and tubular and vesicular profiles of agranular endoplasmic reticulum. Neurofilaments and microtubules are sparse or absent. The fibres in the larger bundles, however, contain mainly microtubules and neurofilaments, with occasional mitochondria and a little agranular endoplasmic reticulum.

At the base of each secondary lamella bundles of unmyelinated fibres supply the terminal plexus of the lamellae (Plate 70-1). These bundles

are up to 5 - 12 μmacross and contain fibres 0.1 - 1.0 μmin diameter which are rich in microtubules, neurofilaments and a few mitochondria (Plate 70-2, 3). One or two fibres may contain principally agranular endoplasmic reticulum. The individual fibres (80 - 100 in a single bundle) are unsheathed by a single Schwann cell and its cytoplasmic processes.

The most complex arrangement of fibres of the secondary plexus occurs in association with the nerve cell bodies in the filament axis and the base of the secondary lamellae. Most of the fibres are unmyelinated although a small myelinated fibre is occasionally seen amongst them. They are ensheathed by varying amounts of Schwann cell cytoplasm, which often disappears close to a neuron. The fibres are usually 0.1 - 1.0 μm in diameter and contain neurotubules and neurofilaments. However a number of large dilated regions are found (1 - 3 μmacross) which possess many mitochondria, clear and dense core vesicles (500 - 1000 Å) and agranular endoplasmic reticulum, in addition to microtubules and neurofilaments (Plate 71-1). Also, large processes (up to 4 μmacross) containing mitochondria, Golgi apparatus, ribosomes and small lysosomes, but lacking microtubules and neurofilaments, are found adjacent to some nerve cell bodies. These may represent dendritic expansions of the latter (Plate 71-3).

Neurons

Two distinct types and possibly a third are recognisable.

(a) Large neurons

These may be multipolar or bipolar and measure 10 - 20 μmin diameter. They are usually spherical and similar to those found in the primary plexus at the base of the organ. The cytoplasm is rich in Nissl substance, clusters of ribosomes, Golgi membranes and mitochondria. They give rise

to a highly branched dendritic system which forms part of the population of unmyelinated fibres in this plexus.

(b) Small neurons

There is a large population of small neurons, measuring 4 - 8 μm in diameter, located principally at the base of the secondary lamellae on the efferent side of the circulation. Some penetrate the secondary lamellae where they are surrounded by fibrous, interstitial tissue. They may be divided into two possible types:

(1) Regular, ovoid cell bodies with large rounded nuclei. The cytoplasm is rich in Nissl substance, occasional Golgi profiles, mitochondria and a few lysosomes. Occasionally small groups of smooth surfaced vesicles (600-800 Å in diam.) with homogenous electron dense cores are seen in these neurons. Some of the dense cores appear to be in tubules of smooth endoplasmic reticulum rather than in vesicles (Plate 72-1).

Specialized regions of contact are sometimes seen between neighbouring neurons. These regions (0.3 - 0.5 μm long) appear to be gap junctions with an accumulation of dense osmiophilic material on the cytoplasmic side of each of the closely apposed membranes (Plate 72-2).

(2) Irregularly shaped cell bodies with an elongated or irregularly shaped nucleus (Plate 71-2, 3). The cytoplasm is pale and contains a less organised Nissl substance than the type I neurons, but has a well developed Golgi apparatus with numerous tubular and vesicular elements. There may be large numbers of lysosomes of various sizes (0.1 - 1.0 μm in diam.) and mitochondria 0.4 - 0.5 μm in diameter. Some of the larger lysosomes contain whorls or stacks of membrane-like material. This type of neuron is often surrounded by large, pale cytoplasmic processes which may be devoid of organelles or contain mitochondria (≃ 0.3 μm diam.), free ribosomes, small lysosomes and sometimes Golgi apparatus (Plate 72-3).

These processes do not contain microtubules or neurofilaments, but groups of unmyelinated fibres (0.1 - 1.0 μm diam.) containing both these elements as well as clear vesicles and mitochondria may be seen in close proximity (Plate 71-3).

Synapses

Axo-somatic synapses similar to those described in the primary plexus are occasionally seen on the large neurons, but could not be identified on the small neurons. No axo-dendritic or axo-axonic synapses could be found in the secondary plexus.

Innervation of filament blood vessels

Both the afferent and efferent filament arteries are densely innervated by small unmyelinated fibres (0.1 - 0.7 μm in diam.). They are found in small bundles in the adventitia of the vessels surrounded by collagen fibres or close to the smooth muscle cells (Plate 73-3). The individual axons are surrounded by little or no Schwann cell cytoplasm and contain varying amounts of agranular endoplasmic reticulum, microtubules and neurofilaments (Plate 73-2). Groups of naked axons rich in clear vesicles, mitochondria and glycogen particles are found near the smooth muscle cells of the vessel wall (Plates 74-1, 2).

Bundles of naked axons are also found close to the walls of the afferent lamellar arteries near their origin from the afferent filament artery. These axons contain mainly microtubules and neurofilaments although some contain clear vesicles ($\simeq$ 500 Å diam.) and mitochondria.

In the axis of the filament, small nutritional arteries are often accompanied by bundles of naked axons containing microtubules, neurofilaments, clear and dense core vesicles, and mitochondria. (Plate 74-3). They are found close to the smooth muscle cell layer, often separated only by an intercellular space 0.05 - 0.2 µm wide.

Terminal plexus

The terminal plexus is made up of those cell bodies, fibres and their endings within the secondary lamellae of the pseudobranch (Plates 75-1, 2, 3). This interlamellar plexus is located at the antivascular pole of the pseudobranch cells and is common to two contiguous lamellar in the case of buried, covered and semi-free pseudobranchs. It is located in the fibrous interstitial issue which separates two neighbouring layers of 'pseudobranch' cells. In the free pseudobranch the plexus is situated between the 'pseudobranch' or 'chloride' cells and the epithelial cells. The lower epithelial cells correspond to the interstitial tissue in this case. The secondary lamellae of free pseudobranchs (e.g. in bass) are fairly sparsely innervated and no definite endings could be found.

The interlamellar plexus is made up of numerous unmyelinated fibres and dendritic expansions of the small neurons at the base of the lamellae and in the filament axis. The nerve processes may be found as bundles of naked axons or isolated fibres devoid of all Schwannian elements.

Fibres

All the fibres of the interlamellar plexus are unmyelinated although these may vary considerably in size and morphology. The majority of fibres are between 0.1 and 1.0 µm in diameter and contain varying amounts of neurotubules, neurofilaments, mitochondria, agranular endoplasmic reticulum and clear and dense core vesicles. Larger processes (up to 3 µm in diam.) are also found particularly at the base of the secondary lamellae

and near nerve cell bodies. These large processes usually lack neurotubules and neurofilaments and may contain a clear cytoplasm or a complicated agranular endoplasmic reticulum with tubular and vesicular profiles, large and small vacuoles and lamellar whorls. They may also contain mitochondria or free ribosomes. Serial sections showed many of these processes to be expansions of smaller fibres containing neurotubules, mitochondria and vesicles.

At the base of the secondary lamellae the unmyelinated fibres are grouped in bundles which penetrate the interstitial tissue between the 'pseudobranch type' cells (Plate 75-3; 76-1). These fibres lose their Schwann cell sheath as they penetrate the secondary lamellae and become arranged in small bundles of naked fibres. These gradually branch and separate into small groups or individual fibres. Some fibres have a thick and thin appearance due to expansions found along their length (Plate 76-1, 2). These thick portions (1 - 2 μm long) appear as varicosities measuring 1 μm or more in diameter and filled with small clear vesicles (350 - 500 Å diam.), occasional dense core vesicles (500 - 1000 Å) and mitochondria. Some expansions may be filled with small, dense or whorled mitochondria whilst others contain mainly agranular endoplasmic reticulum and whorled membranous structures (Plate 77-1). The thin inter-varicose parts can be as thin as 0.1 μm in diameter and mainly contain microtubules and neurofilaments (Plate 76-2).

The fibres run very close to the 'pseudobranch type' cells and may be separated only by an intercellular space or a little interstitial tissue.

Although they contain microtubules for much of their length, in some regions there are large accumulations of fibre expansions and possible endings. These fibre expansions are often found closely apposed to the

'pseudobranch type' cells and may contain a variety of elements:

a. Clear vesicles (350 - 500 Å) and mitochondria (Plates 76-2; 78-1, 3).

b. Clear vesicles, a few dense core vesicles (500 - 1000 Å) and mitochondria (Plate 78-2).

c. An accumulation of small dense mitochondria (0.15 - 0.2 µm in diam.), clear vesicles and glycogen particles (Plates 77-2; 78-4).

d. An accumulation of mitochondria and whorled membranous structures (Plates 77-1; 79-1, 3).

e. A highly branched network of agranular endoplasmic reticulum, vacuoles, vesicles, whorled membranous structures and mitochondria (Plate 79-2, 4).

f. A variety of large vesicular and tubular elements (500 - 3000 Å diam.) of varying electron density, agranular endoplasmic reticulum and mitochondria (Plate 76-3).

Large accumulations of glycogen particles are also often associated with endings containing mainly clear vesicles and mitochondria (Plates 77-2; 78-1).

All these nerve processes may be found in close contact with the apical membrane of the 'pseudobranch type' cells, or surrounded by the fibrous cytoplasm of the interstitial cells. In the perch these processes may also be found isolated in small extracellular spaces between the 'pseudobranch type' cells.

In the 'free' pseudobranch of the bass, small numbers of isolated fibres containing microtubules, clear vesicles and mitochondria are found close to the 'pseudobranch type' and 'chloride type' cells but they are generally small (< 1.0 µm in diam.) and no large accumulations of fibre expansions or endings could be found.

Synapses

Although there were large numbers of endings and varicosities per section, regions of specialised membrane contact with 'pseudobranch type' cells were found only occasionally. They were always of one type, characterised by an accumulation of clear, spherical vesicles (350 - 500 Å diam.) at the thickened membrane of the nerve ending. One or two mitochondria and a few dense core vesicles ($\simeq$ 700 Å diam.) were also sometimes present. The membrane of the nerve ending always bore a number of small dense areas whilst that of the 'pseudobranch type' cell showed a slight uniform thickening (Plates 80-1, 2). Occasionally, opening of a vesicle into the synaptic cleft was seen (Plate 80-3).

Very rarely nerve endings were found to make possible synaptic contacts with another nerve or nerve ending. An ending containing principally clear vesicles and a few dense cored vesicles is shown in close contact with a small ending containing mitochondria in Plate 73-1. Although there appears to be a dense region at the point of contact it is difficult to establish as a synapse since there is poor definition between the apposing membranes.

Degenerative and Regenerative Changes following Nerve Section

Pseudobranch Nerve

Nerves examined 24 hours and 72 hours after section showed no apparent degenerative changes but after 7 days obvious signs of damage were present in many of the myelinated fibres. At this stage many axons contained accumulations of large and small membrane bound vacuoles within the axoplasm (Plate 81-2). In some cases the axoplasm appeared to be disintegrating leaving large spaces containing amorphous or granular material. Some distortion and fragmentation of the myelin sheath was evident although the extent of this varied from fibre to fibre (Plate 81-1, 2). The laminar

disintegration of the myelin seemed to commence at random without any preferred site of initiation. Fragmentation was evident near the surface adjacent to the Schwann cell cytoplasm or deep and close to the axon remnant. The lamellae tended to separate from each other somewhat haphazzardly, creating spaces, often of considerable size, and commonly filled with a dispersed amorphous material. Isolated globular masses of degenerating myelin were evident in the axoplasm of some axons (Plate 81-3).

The unmyelinated axons did not show any convincing structural damage which could be said to be distinct from possible fixation artefacts. Some of the larger axons contained occasional vacuolated mitochondria but these were often found in fibres from normal intact nerve.

By the third and fourth weeks after section most of the myelin sheaths had disintegrated and disappeared. Only occasional profiles of disintegrating myelin were observed (Plate 82-1). However clear regenerative processes were well underway by this time. Cords of Schwann cells were observed in various stages of innervation with the beginning of myelination in some regenerating axons. As well as proliferation of new Schwann cells, many cells appeared to have been previously associated with myelinated axons, as suggested by the debris in the cytoplasm. Although reinnervation was not observed in all the Schwann cells, many were reinnervated by multiple axons which varied in diameter from 0.05 - 1.0 μm The proliferating Schwann cell cords possessed highly folded basement membranes and the axons become embedded in gutters with the consequent formation of mesaxons (Plates 82-2, 3). In most Schwann cell cords at this stage it was difficult to distinguish genuine unmyelinated fibres from axon sprouts awaiting myelination. Each Schwann cell destined to envelop a myelinated fibre may be initially reinnervated by numerous axon sprouts, although only one of these will become myelinated. Another difficulty arises in trying to distinguish regenerating axons from proliferating Schwann

cell processes and their overlapping profiles.

Because of the large number of regenerating axon sprouts per Schwann cell the total nerve fibre population at this stage is far greater than in the normal nerve, although most of the axons are below 1 μm in diameter. However because of the difficulties of distinguishing unmyelinated fibres from potential myelinated fibres it is difficult to estimate the different fibre populations.

A common observation at this time was the occurence of small electron dense droplets (500 - 5000 Å) in the axons or the surrounding Schwann cell cytoplasm (Plates 82-2, 3). Although initially resembling staining artefacts they were found in identical positions in serial sections and were absent from other areas of the section without Schwann cells and their axons.

Besides the increase in Schwann cells and axons there was also an increase in the amount of collagen fibres which formed uniform, dense bands around the Schwann cells (Plates 82-1, 2).

Remyelination of some axons was fairly advanced in nerves examined after 8 and 10 weeks although the numbers undergoing myelination was small compared with the total axon population (Plates 83-1, 2). Occasional fragments or globules of degenerating myelin were still observed in some Schwann cells.

There was a general increase in the diameter of the axons, with the largest up to 3 μm across. Only the larger fibres (1 - 3 μm) were undergoing myelination and some of the smaller axons appeared to be breaking down (Plates 83-1, 2).

The morphology of nerve examined 15 weeks after section was nearly normal and the regeneration process was essentially complete in most fibres (Plates 84-1, 2). However some Schwann cells still contained degenerate

myelin debris and showed no evidence of undergoing reinnervation (Plate 84-3). The number of myelinated fibres was significantly less than in normal nerve (about 20% of the normal value) and none were found above 4 μm diameter. There was an apparent increase in the numbers of unmyelinated fibres although some of these may still undergo myelination or resorption.

Neurons

Changes were noted in the perikarya of all neurons 3 - 7 days after nerve section. In some large neurons the granular endoplasmic reticulum was disorganised with a marked fragmentation and swelling of the cisternae (Plate 85-1). The cytoplasm appeared darker and there was a marked increase in the number of free ribosomal clusters. Many of the mitochondria were ruptured or vacuolated and there was an increase in the number of electron dense, lysosyme-like bodies. In some cells the nucleus had lost its rounded appearance and showed deep infoldings (Plate 86-1). Similar changes were noted in small type I neurons, as well as dilation and disintegration of the nuclear envelope.

In some cells the changes were not as obvious since there was no swelling of the granular endoplasmic reticulum, although it was often dispersed into haphazzardly arranged, short segments. Also, the mitochondria were usually swollen and vacuolated and there appeared to be an increase in the number of free polysomes (Plate 85-2).

In the small type 2 neurons the small amount of granular endoplasmic reticulum was only slightly dilated but there was a large increase in the number of dense, lysosome-like bodies. The Golgi zone appeared very active, giving rise to numerous vesicles and small, dense bodies (Plate 86-2).

After 4 weeks some of the large neurons still showed fragmentation and dilation of the granular endoplasmic reticulum, whilst others showed a complete loss of ribosomes even though the endoplasmic reticulum cisternae were arranged in an orderly, parallel manner (Plate 86-3). In the latter

case the cytoplasm is filled with a 'powdery', flocculent material which is densest between the cisterns of the endoplasmic reticulum. The Golgi apparatus occupies a central or supranuclear position and is highly vesiculated. Small dense vesicles and lysosome-like bodies are associated with it. There are a few dilated cisternae, some of which contain dense vesicles. The mitochondria are swollen and vacuolated, and the dense nucleus is irregularly shaped. Neurons showing these characteristics could not be found in tissues examined 8 weeks (and beyond) after section. Most neurons observed between 8 and 15 weeks after section still showed fragmentation and swelling of the granular endoplasmic reticulum, and vacuolation of the mitochondria. In some cells the ribosomes appeared to be dissociated from the E.R. membranes and were distributed free or in clusters throughout the cytoplasm. There was also an increase in the electron density of the cytoplasm which was particularly noticeable 15 weeks after section. These cells have a much denser cytoplasm than normal cells as well as a much larger population of free polyribosomes (Plate 87-1). In some cells there was evidence of organisation of the G.E.R. cisternae into parallel arrays (Plate 87-2). The nucleus appeared uniformly electron dense at this stage and showed no signs of distortion (Plate 87-1).

The type 2 neurons examined 15 weeks after section appeared atrophied with almost total disappearance of the granular endoplasmic reticulum and a marked reduction in other cellular organelles such as mitochondria, Golgi apparatus and dense bodies (Plate 88-1). The nucleus was electron dense with a darker peripheral region and the nuclear envelope was distended in some cells. The cytoplasm was pale and filled with single ribosomes, small vesicular and tubular profiles containing material of varying electron density, multivesicular bodies and lysosome-like, dense bodies containing either a homogenous granular matrix or stacks of membrane-like material. Occasional neurotubules and neurofilaments were also seen.

Nerve fibres and endings

The earliest changes seen in fibres within the pseudobranch were found in myelinated fibres associated with the large type I neurons. Seven days after section the axoplasm of many was vacuolated and the myelin sheath showed signs of folding and disintegration (Plate 85-1). Similar changes were seen in the myelinated fibres of mixed nerve bundles in the secondary plexus (Plate 88-2). Definite degenerative changes were difficult to identify in the secondary and terminal plexus although some fibres did show vacuolation of mitochondria and the formation of irregular membranous profiles (Plates 89-1, 2, 3, 4). Clumping of vesicles was observed in some fibres (Plates 89-2, 4). In others, electron dense lysosomal bodies appeared to arise from tubules of smooth endoplasmic reticulum, or sometimes from degenerating mitochondria which showed an increased electron density (Plate 90-1). It was not usually possible to positively identify nerve endings since they were not characterised by a synapse with another cell. Also, varicosities containing mitochondria and vesicles were found at regular intervals along most fibres and not just at the terminal. Ultrastructural changes were seen along the length of unmyelinated fibres but initially these were most obvious in the varicosities containing mitochondria and vesicles. Changes were seen in fibres associated with 'pseudobranch type' cells as well as fibres close to the pseudobranch vasculature.

No obvious ultrastructural changes were noted in 'pseudobranch type' cells 7 days after denervation. This was true for the majority of 'pseudobranch type' cells over a period of 15 weeks, but in a few

cases degeneration of nerve fibres was accompanied by breakdown of the plasma membrane of the 'pseudobranch type' cell. This was particularly noticeable 4 weeks after denervation and usually occured in regions where the 'pseudobranch type' cell was in close apposition with a degenerating nerve fibre or terminal which also showed breakdown of its plasma membrane (Plate 90-2). No breakdown of other cell membranes was observed and such phenomena was never observed in normal pseudobranch material. It therefore seems unlikely to be an artefact of fixation or tangential sectioning and is probably a true breakdown of the membranes in question (or, some localised change in them that prevented visualisation in the electron microscope). Furthermore a similar effect was produced within 4 hours of an intraperitoneal injection of the false transmitter 5-Hydroxydopamine (5-OHDA) (see later).

Apart from breakdown of the plasma membrane the nerve fibre or terminal also showed degeneration of mitochondria and the formation of 'myelin' figures which may arise from autolysed mitochondria (Plate 90-3). Occasionally all the organelles aggregated into a dense mass to form large osmiophilic lamellar structures. Myelin figures were also sometimes seen in the associated 'pseudobranch type' cell (Plate 90-4).

Further degenerative processes observed between 4 and 15 weeks after denervation included fragmentation and disorganisation of neurotubules and neurofilaments (Plate 91-1, 2), vacuolation of the fibre cytoplasm (Plates 91-2; 92-1; 93-1), disintegration of the plasma membrane and complete dissolution of fibres (Plate 93-1). Some large unmyelinated fibres appeared to lose most of their organelles and appeared as large pale structures with only an occasional dense body or mitochondrion and a few

scattered neurotubules (Plate 93-2). The ultrastructural changes were extremely variable between fibres. Some fibres were apparently unaffected and remained intact throughout the experimental period. Unaffected fibres were usually under 1.0 μm in diameter and belonged to a population of fibres which made close contact with the 'pseudobranch type' cell and often contained large numbers of clear vesicles (Plate 91-3). A synapse between this type of fibre and a 'pseudobranch type' cell was observed 15 weeks after denervation (Plate 92-3). Fibres above 1.0 μm in diameter invariably showed some degree of degeneration or ultrastructural change. This was particularly true of fibres containing large numbers of mitochondria or a network of smooth endoplasmic reticulum (Plate 92-2).

CYTOCHEMISTRY

The pseudobranchs of freshwater and seawater fish were compared in their reactions to various staining procedures and histochemical tests at the light and electron microscope level. Seawater bass and freshwater trout were used for most tests, although seawater mullet and freshwater carp were sometimes used when available. The reactions of the four principal cell types at the light microscope level are summarised in Table 7.

1) Light Microscopy

(i) 'Pseudobranch type' cells

The 'pseudobranch type' cells of freshwater and seawater fish were unstained by P.A.S. and alcian blue indicating the absence of carbohydrate and acidic mucosubstances (Plate 94-1). The negative result to Schiff's reagent without previous oxidation indicates the absence of free aldehyde. Lipid is present as shown by the moderate staining of the central and basal region of the cell by Sudan black B. There is also a very strong succinic dehydrogenase and weak alkaline phosphatase activity in this region (Plate 94-3). No significant amounts of chloride reaction product were seen in these cells although small amounts were occasionally seen adhering to the apical membrane.

(ii) 'Chloride type' cells

The 'chloride type' cells in the pseudobranchs of seawater fish contain some carbohydrate material since they are stained lightly by the P.A.S. test (Plate 94-1). The weak staining reaction of the apical region to alcian blue at pH 0.5, 1.0 and 2.5 indicates the presence of acidic mucosubstances containing both sulphated and carboxylated material. The extinction of staining at a critical electrolyte concentration of 0.2 M $MgCl_2$ in alcian blue, pH 5.7, indicates the presence of polycarboxylates

and weakly oxidised sulphomucins, but the absence of most sulphated mucosubstances. The absence of free aldehyde is shown by the negative result to Schiff's reagent without previous oxidation. The central and basal regions of the cell are stained moderately by Sudan Black showing the presence of lipids. Strong succinic dehydrogenase activity was present throughout the cell but no alkaline phosphatase activity could be found (Plate 94-3). Significant amounts of chloride reaction product were localized in the apical region of the cell as a dark brown or black precipitate outlining the apical pit (Plate 100-1). Precipitate was rarely seen on the free surface of the filament or the lamellar epithelium.

(iii) Mucous cells

The mucous cells at the epithelial surface were stained strongly by P.A.S. in both seawater and freshwater fish (Plate 94-1). The acidic nature of the mucopolysaccharide material was confirmed by the strong staining reaction of alcian blue at pH 2.5. The mucous cells of bass showed a higher degree of sulphation than those of trout. Bass cells were stained by alcian blue at pH 1.0 and in the range 0 - 0.4 M Mg Cl_2 in the alcian blue C.E.C. series. Trout mucous cells were only weakly stained in alcian blue at pH 1.0, and only stained from 0 - 0.1 M Mg Cl_2 in the C.E.C. series, indicating the presence of polycarboxylates but absence of most sulphomucins.

(iv) Rodlet cells

The rodlet cells of the bass pseudobranch gave a strong positive reaction to the P.A.S. test, with both the rodlets and the wall staining (Plate 94-2). However, they do not contain acidic mucosubstances since they failed to stain with alcian blue at any pH or critical electrolyte concentration. They were unstained by the Sudan black method for lipids and showed no alkaline phosphatase activity. They did show some succinic dehydrogenase activity near the apex of the cell.

Table 7

Test	'Pseudobranch type' cell	'Chloride type' cell	Mucous cell	Rodlet cell
P.A.S.	(Bass) -	(Bass) + +	(Bass) + + +	(Bass) + + +
	(Trout) -		(Trout) + + +	
Alcian blue pH 0.5	(Bass) -	(Bass) +	(Bass) +	(Bass) -
	(Trout) -		(Trout) +	
pH 1.0	(Bass) -	(Bass) +	(Bass) + +	(Bass) -
	(Trout) -		(Trout) +	
pH 2.5	(Bass) -	(Bass) +	(Bass) + + +	(Bass) -
	(Trout) -		(Trout) + + +	
Alcian blue C.E.C.				
0.00 M $MgCl_2$	(Bass) -	(Bass) +	(Bass) + + +	(Bass) -
	(Trout) -		(Trout) + + +	
0.05 M	(Bass) -	(Bass) +	(Bass) + + +	(Bass) -
	(Trout) -		(Trout) + + +	
0.10 M	(Bass) -	(Bass) +	(Bass) + + +	(Bass) -
	(Trout) -		(Trout) + + +	
0.20 M	(Bass) -	(Bass) -	(Bass) + + +	(Bass) -
	(Trout) -		(Trout) -	
0.30 M	(Bass) -	(Bass) -	(Bass) + + +	(Bass) -
	(Trout) -		(Trout) -	
0.40 M	(Bass) -	(Bass) -	(Bass) + +	(Bass) -
	(Trout) -		(Trout) -	
Sudan Black	(Bass) + +	(Bass) + +	(Bass) -	(Bass) -
	(Trout) + +		(Trout) -	
Schiff's Reagent without oxidation with periodic acid	(Bass) -	(Bass) -	(Bass) -	(Bass) -
	(Trout) -		(Trout) -	
Chloride	(Bass) -	(Bass) + + + apical region	(Bass) -	(Bass) -
	(Trout) -		(Trout) -	
Alkaline phosphatase	(Bass) +	(Bass) -	(Bass) -	(Bass) -
Succinic dehydrogenase	(Bass) + + +	(Bass) + +	(Bass) -	(Bass) + apical region
	(Trout) + + +		(Trout) -	

Key: - No reaction + Weak reaction + + Moderate reaction + + + Strong reaction

2) Electron Microscopy

(i) 'Pseudobranch type' cells

(a) Localisation of Chloride ions

Occasional clumps of precipitate were seen adhering to the inner or outer surface of the cell membrane at the apical pole of the cell (Plate 95-1). Small amounts of precipitate were also found around small nerve fibres abutting against the apical membrane of the cell. These fibres often contained clear vesicles (Plate 95-1). Little or no precipitate was seen in the main body of the cell, apart from a small number of random dense particles.

The overall appearance of pseudobranch tissues fixed in silver-acetate osmium tetroxide solution was comparable to, but not of the same high quality as material fixed in glutaraldehyde-osmium tetroxide. There were certain obvious signs of cell damage. The cytoplasm was partially extracted so the ground substance appeared less dense than it does after conventional glutaraldehyde fixation. The Golgi cisternae and to a lesser extent the intracellular tubules occasionally showed varying degrees of swelling although the extent of this varied from cell to cell. Extraction of the nucleoplasm and fragmentation of nuclear material indicated some nuclear damage.

(b) Localisation of Sodium ions

Tissue fixed in pyroantinomate-osmium tetroxide solution was of poorer quality than that fixed in glutaraldehyde and osmium tetroxide. The cells showed signs of damage and extraction of the cytoplasmic ground substance. The mitochondrial cistae appeared disorganised and the cytoplasm was vacuolated in some regions, particularly near the apical pole.

A considerable amount of precipitate was found in the cytoplasm of mullet 'pseudobranch type' cells (Plate 95-2). Small deposits were also

found in the mitochondria, and vacuolated areas of the cytoplasm. A very fine precipitate of small particles was found in the nuclei, particularly around the periphery in the region of the heterochromatin (Plate 95-3). No precipitate was found in the basement membrane or lateral membranes, or in the tubular network surrounding the mitochondria. In 'dark' pseudobranch type cells a large number of dense particles were associated with the tubular system. These particles are clearly visible in unstained sections (Plate 95-4) but become much denser when stained with lead citrate. Their position corresponds to that of glycogen granules observed in glutaraldehyde-osmium tetroxide fixed tissues.

(c) Localisation of alkaline phosphatase activity

Localisation of reaction product as a fine granular deposit was observed within the tubules of the basotubular membrane system (Plates 96-1,2). The deposits were localised in the centre of the tubules within the border of an intracisternal tubular system inside the main tubules (Plate 96-2). This intracisternal system has not previously been observed after normal preparation and fixation procedures so it is possible that this observation is due to an artefact. It could be due to contraction of the 'inner leaflet' of the unit membrane, although preservation of other membranes (basal and lateral plasma membranes, mitochondrial membranes) generally appeared to be normal.

No other localisation of reaction product was found although a very fine peppering of particles was often present over the cell. Very little precipitate was found in control sections, and there was no localisation of deposits (Plate 96-3).

(d) Localisation of carbonic anhydrase activity

In the trout pseudobranch, reaction product was associated mainly with the tubular system near the basement membrane (Plates 97-1, 2, 3). Preservation of fine detail was rather poor in unstained sections but the

deposits appeared to be associated with the outer membrane of the tubules (Plate 97-3). A fine precipitate of small individual particles was also present over much of the cell. Fine deposits of this nature were also found in the covering epithelial cells but were absent from endothelial cells, pillar cells and blood cells. Small amounts of precipitate were found in control sections but there was no localisation of deposits.

(e) Localisation of adenosine triphosphatase activity

In trout and bass pseudobranch reaction product was located mainly at the antivascular pole of the 'pseudobranch type' cell within the intercellular spaces of the lateral and apical membranes (Plate 98-1). A fine precipitate was also present over most of the cells, being particularly dense in the covering epithelial cells of the bass pseudobranch. Reaction product was not obviously associated with the tubular system or mitochondria of the 'pseudobranch type' cells.

When A.T.P. or magnesium was omitted from the reaction mixture, reaction product did not appear in any cells of the pseudobranch. No appreciable reduction was observed in the amount and distribution of precipitate after omitting NaCl and KCl from the reaction mixture or after addition of ouabain to the mixture. This indicates the insensitivity of the Wachstein-Meisel method for the localisation of ouabain sensitive, Na^+, K^+-activated A.T.P.ase.

(f) Staining with osmium tetroxide

Prolonged immersion of carp pseudobranch tissues in osmium tetroxide selectively stained smooth and granular endoplasmic reticulum, the nuclear envelope and Golgi apparatus of the 'pseudobranch type' cells (Plate 98-2). Deposits were occasionally found in mitochondrial vacuoles and cristae, although this was not common and usually occured in mitochondria containing tubulovesicular cristae (Plate 99-2). Other staining features included

large homogenous droplets containing osmium black enclosed by a unit membrane, or vacuoles outlined by osmium black deposits (Plate 99-1). These possibly arise from swelling of the endoplasmic reticulum or Golgi vesicles. Occasionally some areas enclosed a number of densely stained granules which may be vesicles of multivesicular bodies (Plate 99-4). The intracellular tubules were mainly unstained as were the basal and lateral plasma membranes. Staining amongst the tubular system seemed to be due to scattered elements of the Golgi complex and smooth endoplasmic reticulum, although the heavy deposits prevented clear identification. This staining amongst the tubules was particularly heavy in 'dark' 'pseudobranch type' cells where the deposits appeared to surround the tubules (Plate 99-3).

The unusual organelle associated with the nuclear envelope of carp 'pseudobranch type' cells (see Fine Structure) was also stained by this method, although it was sometimes difficult to recognise because of swelling of the cisternae (Plate 99-4).

Osmium black was not generally found in extracellular or vascular spaces and other cells in the epithelium were mostly unstained.

(ii) 'Chloride type' cells

(a) Localisation of chloride ions

In both stained and unstained sections, reaction product could easily be seen in the cytoplasm bordering the 'chloride' cell apical pit (Plates 100-2, 3, 4). The precipitate is in the form of granules which vary considerably in size, from less than 150 Å to several thousand Angstroms. While individual granules are usually small and spherical, collectively these appear as large irregular shaped clumps (Plates 100-2, 3, 4). In sites where the reaction product was particularly concentrated, holes were seen in adjacent areas of the embedding matrix (Plate 100-2). This may

be due to the dense precipitate absorbing excessive amounts of energy from the electron beam and the consequent heating melted the resin matrix. Because of this and the density of the large clumps it was difficult to evaluate accurately the specific localisation of reaction product in relation to the apical plasma membrane.

Whilst some precipitate was seen adhering to the outer surface of the plasma membrane, most of it was localised on the cytoplasmic side of the membrane and closely followed the contour of the apical pit (Plates 100-2, 3). Occasional small clumps of reaction product were associated with the vesicles and tubules in the apical cytoplasm. In some cells the precipitate was located primarily at the apical junctions between chloride and accessory cells (Plate 100-4). However it was impossible to tell whether the reaction product was actually in the intercellular space or adhering to the membranes.

In stained sections an amorphous material was often seen within the apical pit (Plate 100-4), although it was not apparent in unstained sections. Since the material lacked the required density to be seen in unstained sections it can be concluded that silver ions do not react with it. On the other hand, dense granules and aggregates of reaction product were frequently associated with the vesicle like inclusions found embedded in the amorphous material (Plate 100-4).

In most thin sections examined a random peppering of fine, dense particles could be seen over much of the 'chloride type' cells. These 'background' particles are fairly evenly dispersed over nuclei and cytoplasm and show no tendency to aggregate.

(b) Localisation of sodium ions

Pseudobranch 'chloride type' cells from seawater adapted mullet were compared with gill 'chloride' cells from the same fish, and with 'chloride'

cells from the gills of freshwater carp.

In the mullet pseudobranch a large amount of precipitate was found in the cytoplasm of the 'chloride type' cells, especially near the base of the cell around the network of narrow saccules (Plate 101-1). The electron opaque precipitate consisted of small, individual particles which varied in size from 200 to 700 Å diameter.

Small numbers of particles were found in the matrix of the mitochondria but no precipitate was found in the saccules or the connecting network of intracellular tubules. No precipitate was observed in the basement or lateral membranes, or in the invaginations of the plasma membrane. In the apical region, precipitate was found mainly in the cytoplasm and some of the larger vacuoles (Plate 101-2). A considerable deposit of much smaller, dense particles was observed in the nuclei, especially in the region of the heterochromatin (Plate 101-3).

The pattern of pyroantinomate deposition was similar in the 'chloride' cell of the mullet gill but very little precipitate was found in the 'chloride' cells of carp gill. In the latter case, deposits were found mainly in the nucleus with occasional particles in the mitochondria and vacuoles of the apical region.

(c) Localisation of alkaline phosphatase activity

Small amounts of precipitate were associated with the lateral membranes in the apical region of the 'chloride type' cell of the bass pseudobranch (Plate 101-4). However no localisation of reaction product could be found in the basal membrane, intracellular tubules or cytoplasm of the cell. Occasionally a fine 'background' precipitate was found over most of the 'chloride' cell organelles and cytoplasm, but showed no distinct localisation.

(iii) Mucous cells

The mucous cells of the bass pseudobranch were compared with rodlet cells from the same tissue in their reactions to staining by phosphotungstic acid (P.T.A.).

(a) 15% P.T.A. (pH 2)

The membranes of mucous cell globules were densely stained by treatment with 15% P.T.A. after previous hydrogen peroxide oxidation. The globules showed varying degrees of staining with the central region often paler than the rest of the globule (Plate 103-1). No other cells in the pseudobranch epithelium were stained by this method which is reported to selectively stain membrane, acid mucopolysaccharides (Marinozzi 1967, 1968; Pease 1970).

(b) 1% P.T.A. in chromic acid (pH 0.3)

The membranes of mucous cell globules were densely stained after this treatment. The globules were lightly stained and often contained a densely stained central region (Plate 102-1). This method has been reported as a selective stain for glycoprotein (Tsuchiya & Ogawa 1973) and for sialic acid (Rambourg 1967; Rambourg, Hernandez & Leblond 1969).

(iv) Rodlet cells

(a) 15% P.T.A. (pH 2)

All membranes and organelles of the rodlet cell were unstained (Plate 103-1). This confirms the absence of acid mucopolysaccharide material as deduced from light microscope studies.

(b) 1% P.T.A. in chromic acid (pH 0.3)

Rodlet sacs were stained densely using this method, which indicates the presence of glycoprotein (Plate 102-2). However this material is apparently absent from the central core of the sac since this region remains

unstained. The fibrous wall of the cell was lightly stained with denser staining bands beneath the plasma membrane. The vesicular network of the cytoplasm was poorly stained or unstained.

(c) Alkaline phosphatase activity

No alkaline phosphatase activity was found in these cells.

(d) Carbonic anhydrase activity

Reaction product was localised in the intercellular spaces of the lateral membranes in the apical region, and associated with the mitochondrial membranes (Plate 103-2). In some cells precipitate was also found outlining the membranes of the apical vesicles (Plate 104-1). The rodlet sacs, Golgi, endoplasmic reticulum and fibrous border contained no localised deposits although a very fine background precipitate was present which was also found in controls.

Miscellaneous

The staining reactions of epithelial cells, endothelial cells, pillar cells and blood cells were also noted in the course of examining the principal cell types.

(i) Epithelial cells

Occasional small clumps of silver chloride reaction product were found adhering to the surface membrane of bass epithelial cells but there was no clear localisation of deposits.

In the localisation of sodium, small amounts of precipitate were found in epithelial cells from seawater and freshwater fish. The amount of precipitate varied from cell to cell and was virtually absent from some cells (Plate 104-2). Only in the nucleus was a precipitate of fine particles always found (Plate 104-3). The cytoplasm and mitochondria occassionally contained a denser, globular precipitate (Plate 104-2). In

some cases the surface membrane of the cell was densely stained (Plate 104-2).

The surface membrane and glycocalyx were strongly stained by 15% P.T.A. (pH 2) and 1% P.T.A. (pH 0.3) to show the presence of acidic carbohydrates and glycoproteins. The very strong staining reaction of the glycocalyx to staining with periodic acid-chromic acid-silver methenamine also shows the presence of polysaccharides (Plate 16-3).

No alkaline phosphatase activity could be found in epithelial cells although there was evidence of carbonic anhydrase and adenosine triphosphatase activity in the basal and lateral membranes.

(ii) Endothelial cells

No chloride reaction product was found in these cells but the pyroantinomate stain for sodium revealed a large number of individual dense particles. However these seemed to be associated mainly with the ribosomes which are common in these cells. No acidic carbohydrate material or enzyme activity was found in the endothelial cells.

(iii) Pillar cells

No chloride was found, but like the endothelial cells, pyroantinomate precipitate was scattered throughout the cell in association with ribosomes. A fine deposit of particles also covered the nucleus. There was no evidence of acid carbohydrates or enzyme activity.

(iv) Blood cells

Using the pyroantinomate stain for sodium, the nuclei of red and white blood cells were densely stained especially around the nuclear membrane of red blood cells (Plate 104-4). The rest of the cell was unstained. Blood cells were not stained in any of the other procedures used.

EXPERIMENTAL INVESTIGATIONS

Effect of Salinity Changes on Specific Cell Types

1) Chloride type cells

(a) Exposure to 0.34‰ salinity

When Dicentrarchus labrax was exposed to water of 0.34‰ salinity, without previous acclimation, the 'chloride type' cells of the pseudobranch showed a number of ultrastructural changes. After 5 hours the mitochondria were distended and often vacuolated (Plate 105-1). They usually possessed fewer cristae and were less electron dense than those from full seawater. The number of intracellular tubules increased and they became more extensively branched. In some cells the tubules showed a more orderly arrangement around the mitochondria, although nowhere near the extent or regularity shown in normal 'pseudobranch type' cells. The cisternae of the granular endoplasmic reticulum became very dilated (Plate 106-1). The narrow saccules near the vascular border were less extensive and tended to be arranged parallel to the basement membrane rather than at right angles as in the normal 'chloride type' cell. In some cells the saccules had completely disappeared (Plate 105-2). Large numbers of smooth vesicles appeared at the apex of the cell and the apical pit had disappeared from many cells. The pits were either much smaller and rounded off, or were completely covered with overlapping epithelial cells (Plate 105-1). The accessory cells lose contact with the external milieu and sink beneath the overlapping epithelium. Occasionally, dense lysosome-like bodies were found within the epithelium (Plate 106-2).

There was little further change after 10 hours but after 24 hours spherical dense inclusions had appeared within the cells. The membrane bound inclusions were smaller than the mitochondria with a diameter of 0.4 - 0.8 um, and contained no apparent substructure (Plate 106-3).

Fish showed clear signs of distress after 36 hours, by which time they were very dark and producing copious amounts of mucus on the gills and body surfaces. Many of the 'chloride type' cells appeared to be degenerating after this time and had become much darker with rounded mitochondria and clear vacuoles. The nucleus was densely staining and contracted, and the tubular network was thrown into lamellar whorls in the basal and lateral regions of the cell (Plates 107-1, 2). The cytoplasm and tubules showed a similar electron density. No apical pits were seen in these cells.

Further signs of degeneration were seen in the cells of the last surviving fish examined after 48 hours exposure. The degenerating cells became highly irregular in shape and were pushed to the surface of the epithelium (Plate 107-3). The nucleus became hyperchromatic and the vacuolated cytoplasm was very electron dense. The tubular system disappeared and the cell became full of degenerating mitochondria and vacuoles. Eventually, the degenerate cell becomes elongated and flattened at the epithelial surface (Plate 107-3) before (presumably) being sloughed off.

(b) Gradual acclimation to changing salinity

No significant changes were seen in 'chloride type' cells of Osmerus eperlanus or Dicentrarchus labrax held in water of 24‰ or 16‰ salinity. Cells from fish adapted to 8‰ salinity contained a more highly branched tubular system but were still distinct from 'pseudobranch type' cells. In fish adapted to freshwater the epithelial sheet covering the lamellae became more extensive and frequently covered the 'chloride type' cells completely, so that no apical pits were present. Where there was a gap in the epithelial layer, instead of a pit, the cytoplasm of the cell often protruded beyond the edges of the epithelial cells (Plate 109-1). These

protrusions of the cytoplasm sometimes had an irregular surface and contained elements of the tubular system, along with an amorphous, sometimes granular ground substance.

Fish held for 36 hours or more in freshwater possessed degenerating 'chloride type' cells similar to those described in water in 0.34‰ salinity.

2) 'Pseudobranch type' cells

(a) Exposure to 0.34‰ salinity

'Pseudobranch type' cells of Dicentrarchus labrax showed very little ultrastructural change after an exposure of 5 - 10 hours, apart from a slight increase in the number of glycogen particles. After 24 hours there was a large increase in the number of glycogen particles, and many spherical electron dense inclusions had appeared (Plates 108-1, 2, 3). The inclusions were similar to those found in the 'chloride type' cell but were far more numerous in the 'pseudobranch type' cell. They tended to aggregate at the antivascular pole of the cell and were often vacuolated. These cells were stained by Sudan Black, indicating the presence of lipid. The mitochondria became rounder with irregular cristae and a less electron dense matrix. The tubular system was disorganised and more randomly branched than that of the cells from full seawater. Large, clear vacuoles similar to those in the 'pseudobranch type' cells of freshwater fish appeared in some cells.

(b) Gradual acclimation to changing salinity

(i) Seawater fish

No major ultrastructural changes occured in the 'pseudobranch type' cells of Osmerus eperlanus and Dicentrarchus labrax gradually acclimated to freshwater, apart from the appearance of a few lightly staining, spherical

inclusions. These were less electron dense, and far fewer in number than those found in fish from 0.34‰ salinity.

(ii) Freshwater fish

The 'pseudobranch type' cells of Salmo gairdneri kept in water of 0 to 34‰ salinity showed little or no ultrastructural change. At 34‰, 42‰ and 60‰ salinity the cells still retained their characteristic appearance although some cells appeared to be degenerating with vacuolation of the mitochondria and cytoplasm. In the higher salinities the cells appeared to be contracted and the lateral membranes became widely separated in the region of the desmosomes (Plate 110-1). A few cells showed reorganisation of their cytoplasmic contents. Most noticeable was the more highly branched, random appearance of the tubules (Plate 110-2), (contrasting with the orderly, parallel arrangement characteristic of normal 'pseudobranch type' cells), and the disruption of the plasma membrane at the vascular border with loss of communication to the blood channel (Plate 109-2).

3) Rodlet cells

Exposure to 0.34‰ salinity

Only the rodlet cells of Dicentrarchus labrax exposed to diluted seawater (0.34‰ salinity) were examined.

After 5 hours exposure, rodlet cells in the gills and pseudobranch could not be identified with any degree of certainty in the light microscope. Under the electron microscope only a tentative identification of rodlet cells could be made since no cells containing rodlets were found, but a number of cells with contracted fibrous borders were present (Plates 110-3,4). After 24 hours exposure, no rodlet cell structures could be identified and many of the epithelial cells were disrupted and vacuolated (Plate 110-5).

Effect of salinity changes on denervated trout

The survival of rainbow trout in 50% seawater was unaffected by bilateral denervation of the pseudobranchs. Operated fish were able to survive at this concentration with or without previous acclimation and showed no differences in feeding or behaviour to control, unoperated fish. No ill effects were produced by transfer of operated fish from 50% to 100% seawater or from 50% seawater to freshwater.

Pseudobranchectomy

In all fish bilateral pseudobranchectomy caused complete body darkening and blindness (judged by the failure of the fish to respond to hand movements above the tank) within 2 - 3 hours of the operation, whereas unilateral pseudobranchectomy had no such effect. The body darkening and blindness were permanent and associated with a gradual increase in opacity of the eyes over a period of several months. It was noted that the operculum of pseudobranchectomised fish was held further away from the buccal cavity than in normal fish. This could be due to damage of the opercular musculature incurred during removal of the pseudobranchs. Fish examined one week after the operation had a slightly increased ventilation rate with an average 12% increase in the number of opercular movements per minute compared with normal fish. However this effect appeared to be temporary since the ventilation rate had returned to a normal value in fish examined after 3 weeks. The only other obvious change in the fish was a reduction in activity of the fish, which spent long periods of time in an almost static position. This may be due to a reduction in metabolic activity after the operation, or more likely due to interruption of an important source of external stimuli (sight). The fish were able to feed however and survived long periods of time (up to 12 months) with no other apparent ill-effects.

Acetazolamide injection

General observations

The effect of acetazolamide injection was initially comparable to that of bilateral pseudobranchectomy since it caused body darkening and blindness of the fish. At the highest concentration used (5 mg/kg) this occured within 1 hour of injection and lasted about 72 hours i.e. the effect is reversible. The onset and disappearance of the effects appeared to be dose related since body darkening took 3 - 4 hours to appear in fish injected with 1 mg/kg acetazolamide, and had almost disappeared within 48 hours.

E. M. observations

Some ultrastructural changes were seen in the trout pseudobranch 4 hours after injection of acetazolamide (1 - 5 mg/kg). In some cells the mitochondria had rounded up and become much denser (Plate 111-1). A number of lipid-like droplets and small, round, electron dense inclusions also appeared within the cells. After 24 hours the number of lipid-like droplets (1.0 - 1.5 μm diam.) had increased and appeared similar to those found in osmotically stressed fish (Plate 111-2). The droplets were often surrounded by profiles of tubular reticulum adhering to the interface membrane of the droplet (Plate 112-1). Occasionally they were associated with expanded regions of granular endoplasmic reticulum containing an amorphous, medium electron dense material (Plate 112-2). The tubular system was disorganised in some cells and the clear cytoplasmic region of the cell was sometimes vacuolated and contained large clear areas and broken membranes (Plate 112-3). Similar changes were seen in trout 'pseudobranch type' cells 4 hours after an intraperitoneal injection of 0.5 ml of 10^{-3}N HCl (Plate 112-4). However all these effects were temporary since most cells appeared normal when examined 96 hours after injection.

Injection of 5-hydroxydopamine (5-OHDA)

Ultrastructural changes were noted in 'pseudobranch type' cells and many nerve fibres within 4 hours of administering 5-OHDA. Most ultrastructural changes in the nerve fibres were associated with those fibres adjacent to a 'pseudobranch type' cell. In many cases the contiguous membrane between a nerve fibre and 'pseudobranch type' cell appeared to be breaking down so that the cytoplasm of the nerve fibre and 'pseudobranch type' cell were in direct contact (Plate 113-1). A large pale cytoplasmic region surrounded by a broken membrane often appeared in the apical region of the 'pseudobranch type' cell where it made contact with a nerve fibre (Plates 113-1, 2). Sometimes large pale cytoplasmic expansions appeared to arise from the end of a nerve fibre (Plates 114-1, 2). Serial sections revealed the presence of small dense bodies and myelin figures in these regions (Plates 114-1, 2). There was no obvious increase in the number of dense core vesicles in the nerve endings, although small dense lysosome-like bodies and electron dense lamellar structures appeared more common than in normal fibres.

Ultrastructural changes in the large type I neurons of the pseudobranch were also noted 4 hours after injection of 5-OHDA. These included vacuolation of mitochondria and fragmentation of the granular reticulum (Plate 115-1), as well as some breakdown of membranes between the neurons and surrounding nerve fibres (Plate 115-2). Some of these fibres were vacuolated and contained small dense bodies or myelin figures. After 24 hours the neurons showed further degenerative-type changes with complete vacuolation of the mitochondria and swelling of the G.E.R. (Plate 115-3).

Injection of 6-hydroxydopamine (6-OHDA)

Pseudobranchs examined 24 hours after injection of 6-OHDA showed serious ultrastructural changes. Many terminal fibres appeared to have degenerated

leaving broken membranes and vacuolated areas in the interstitial tissue (Plate 116-1). Numerous lipid-like bodies were observed within the 'pseudobranch-type' cells whose apical region was vacuolated and filled with broken membranes, dense lamellar and myelin figures, and lysosome-like bodies (Plate 116-1, 2). The orderly arrangement of the tubular system was disrupted in many cells. After 48 hours the number of 'lipid' droplets had increased and in some cases were coalescing into larger droplets (Plate 116-3). No observations were made on any neurons after injection of 6-OHDA because of failure to locate them.

ELECTROPHYSIOLOGICAL STUDIES

1) Spontaneous discharge of the pseudobranch nerve

Spontaneous discharge of electrical activity from the pseudobranch nerve occured when the isolated perfused pseudobranch was perfused with standard teleost ringer at a PO_2 of 220 torr at 15°C. The overall afferent electrical activity from the whole nerve could be differentiated into two types, characterised by the amplitude and the shape of their compound action potentials:

Type A: characterised by a diphasic spike-like potential (amplitude 50-250 μV) of less than 2 ms duration, with an impulse conduction velocity $>10\ ms^{-1}$. (Plates 117-1, 3, 4; Plate 118).

Type B: characterised by an irregular complex wave of lower amplitude (50 μV down to noise level) of longer duration (2 - 5 ms), and impulse conduction velocity $2 - 3\ ms^{-1}$. (Plates 117-2, 5, 6).

Without doubt, many of the B impulses were not detected because they were indistinguishable from the background noise. Also, the absolute number of A and B impulses were difficult to estimate since it was not always easy to distinguish the two types of activity. However the appearance of one or other activity in response to different stimuli does provide proof of their separate existence.

The level of spontaneous activity was usually very high and irregular for the first 15 - 20 minutes before becoming more stable, although it remained irregular apart from brief bursts of regular type A activity. Most of the initial activity appeared to be type B with occasional type A spikes (Plate 117-2). The activity was similar to that obtained under conditions of hypoxia (Plate 117-5). Initially the total discharge rate of activity A + B was sometimes as high as 460 impulses/second although it usually varied between 210 - 350 impulses/second. After the initial high

activity the discharge rate dropped to a level between 55 - 130 impulses/second. The amount of activity varied between preparations, and in some cases the activity remained high, in which case the preparation was discarded. Occasionally the type A activity would show rhythmic bursts of activity either as single regular spikes or volleys of spikes (Plate 118). Sometimes the number of spikes/volley gradually increased then decreased in number. There was no experimental stimulus applied at these times but it seems possible that they were caused by brief pulses in perfusion pressure and the recruitment of new units which had until then remained inactive. Similar bursts of activity occured when the perfusion pressure was raised between 15 and 80 torr. The initial dynamic burst of activity during the raising of the pressure was followed by a static phase at steady pressure. Bursts of type A activity could also be induced by stroking with a glass rod, the primary afferent artery and the proximal part of its left and right branches (Plate 117-3). Similar results could not be obtained in other areas of the pseudobranch.

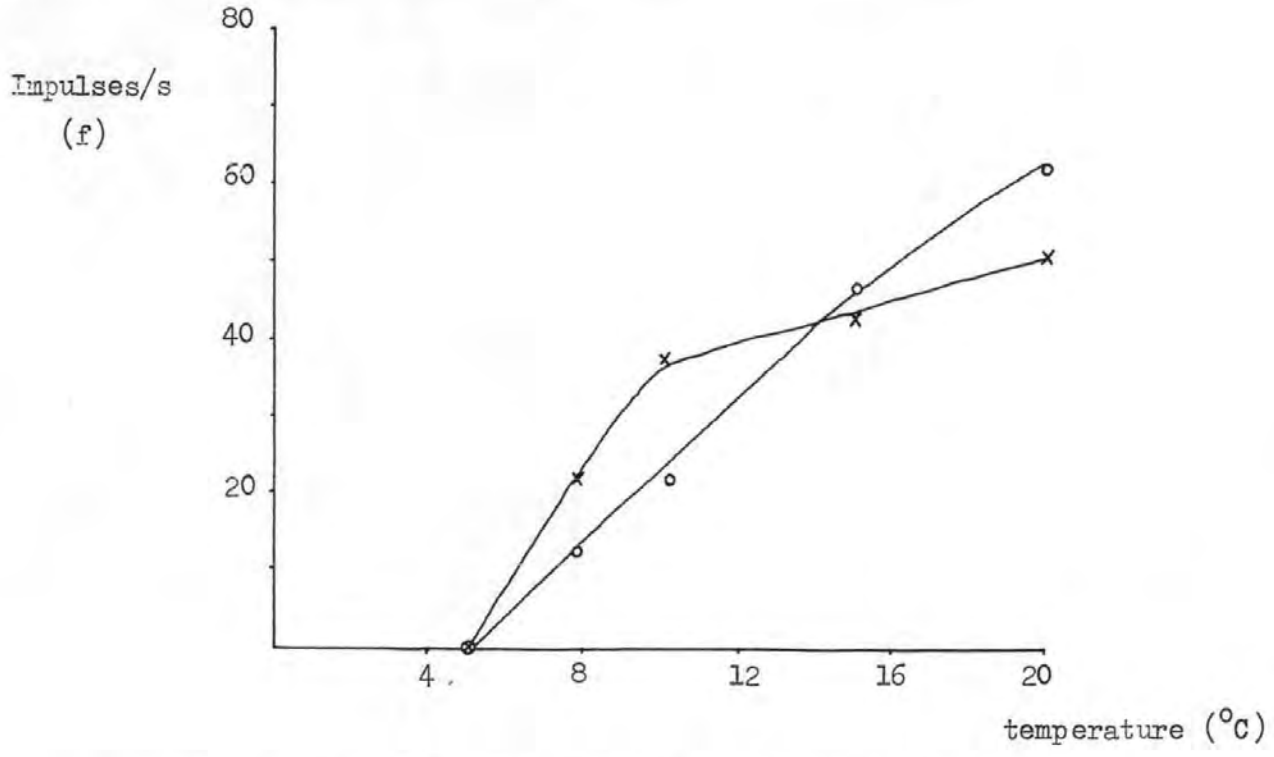
2) Effect of temperature variation

The spontaneous activity of both A and B were affected by temperature (Fig 20a). No activity was found below $5^{o}C$. Above this temperature type A activity increased gradually in an almost linear fashion while type B activity increased rapidly at first before levelling off at about $20^{o}C$.

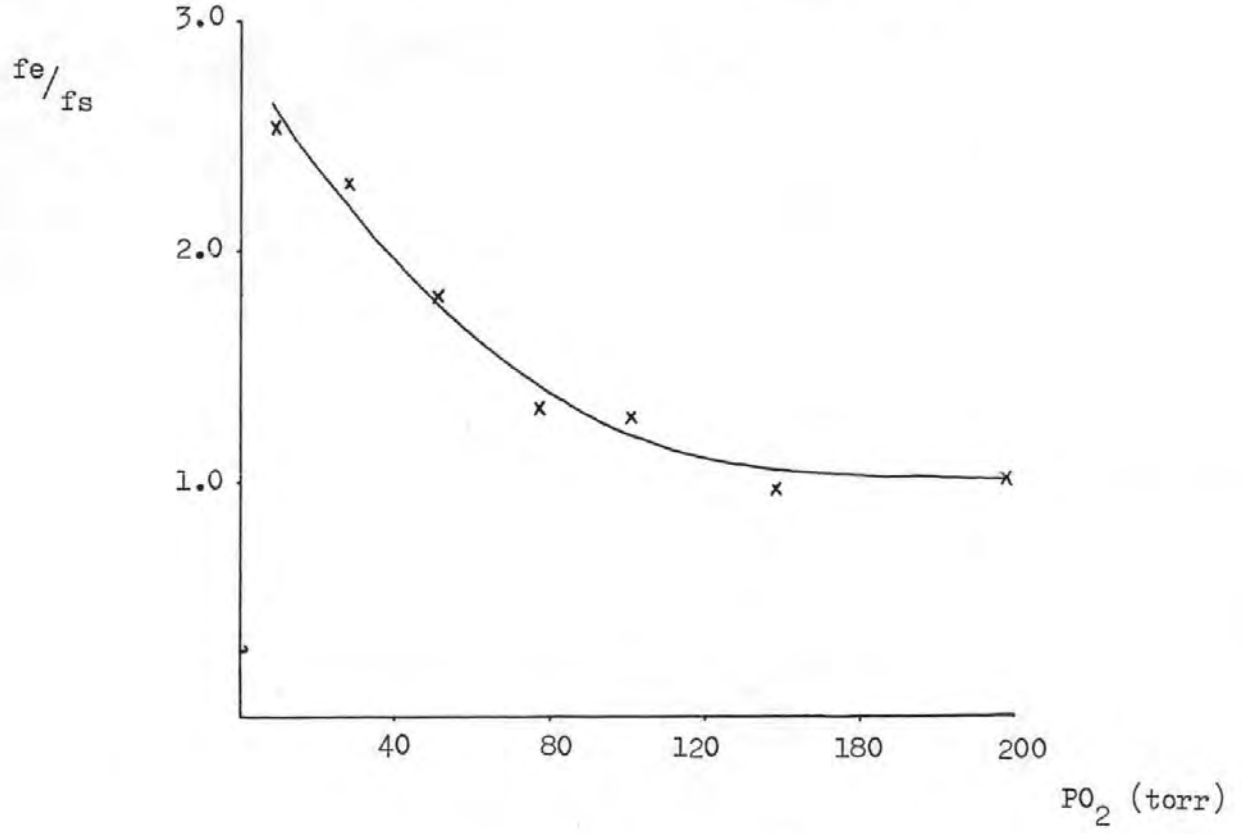
3) Effect of PO_2

When the preparation was perfused with hypoxic solutions (5 - 100 torr), the reduction in PO_2 caused activity B to increase in a hyperbolic way (Fig 20b). Since the absolute value of impulses per second varied between preparations the activity at each PO_2 level was expressed as a function of the impulse frequency under standard conditions for that particular preparation: i.e. $\frac{\text{frequency under experimental conditions}}{\text{frequency under standard conditions}} \quad \frac{fe}{fs}$

Fig 20



a) Effect of temperature on activity A (o) and B (x)



b) Effect of PO_2 on activity B at 15°C.

The mean values of $\frac{fe}{fs}$ from five experiments were calculated together with the standard errors, and plotted against PO_2 (Table 8, Fig 20b).

Table 8

PO_2 (torr)	$\frac{fe}{fs}$	S.E.
200 - 220	1	-
140 - 150	0.96	0.24
90 - 100	1.27	0.11
70 - 80	1.34	0.07
40 - 50	1.80	0.16
20 - 30	2.29	0.13
5 - 10	2.56	0.09

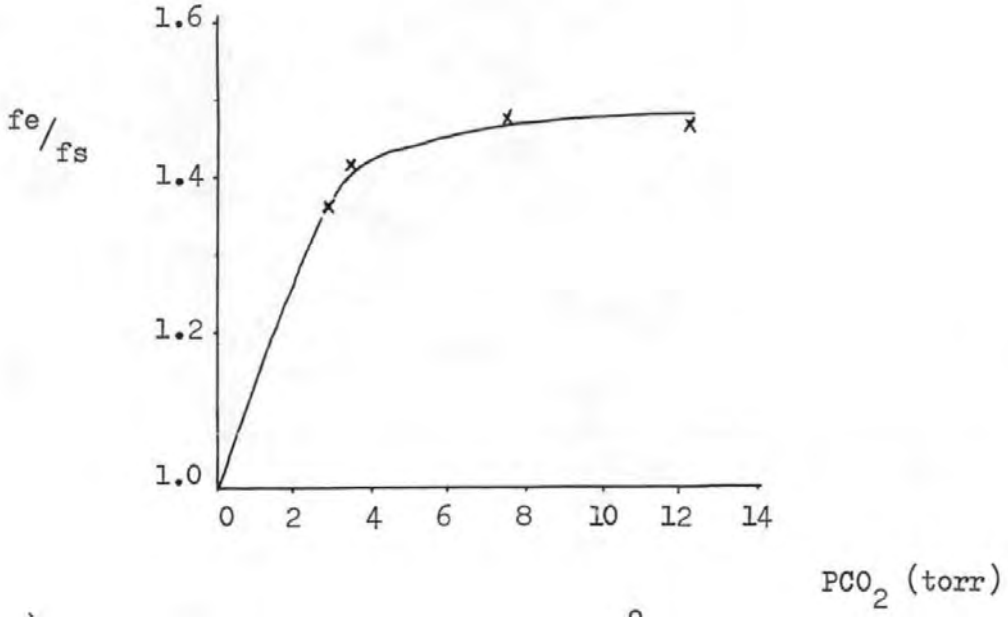
Effect of PO_2 on activity B at 15°C (Mean of five experiments)

Activity A was unaffected by changes in PO_2 whereas activity B appeared within a few seconds of reducing the PO_2. It disappeared as rapidly upon returning to normoxic perfusion.

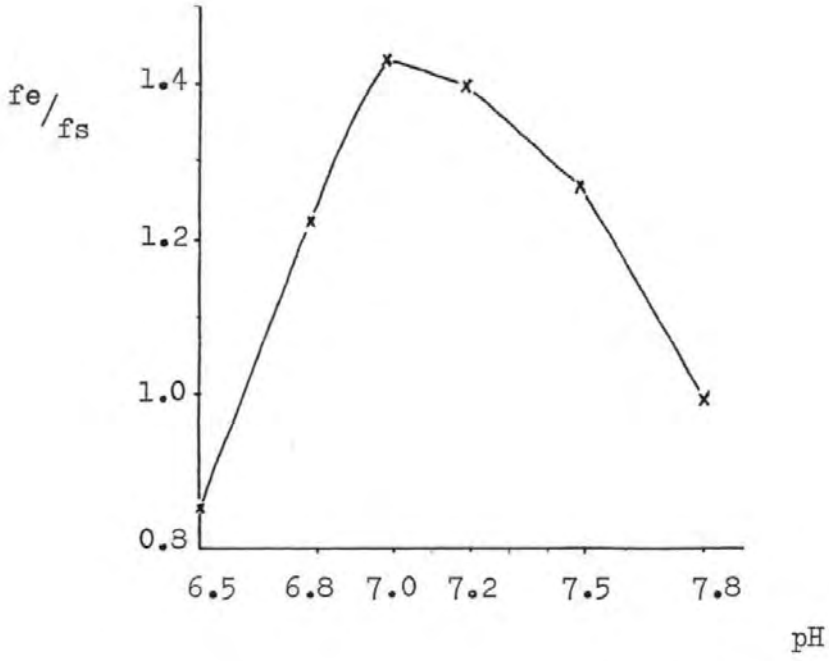
4) Effect of PCO_2

The activity of B in the CO_2 -bicarbonate buffer system at PCO_2 2.5 torr and pH 7.8 was significantly higher ($p = 0.05$) than the same activity in the phosphate buffer system at pH 7.8. Increasing the PCO_2 to 12.3 torr while maintaining the pH at 7.8 caused a small rise in activity although this was not significant. The activity at each PCO_2 level was expressed as a function of the activity using the phosphate buffer system and plotted against PCO_2. (Table 9, Fig 21a).

Fig 21



a) Effect of PCO_2 on activity B at 15°C and pH 7.8



b) Effect of pH on activity B at 15°C

Table 9

PCO_2 (torr)	$\frac{fe}{fs}$	S.E.
0	1	-
2.5	1.36	0.10
3.7	1.41	0.09
7.5	1.47	0.17
12.3	1.46	0.21

Effect of PCO_2 on activity B at 15°C and pH 7.8 (Mean of five experiments)

5) Effect of pH

There was a small but significant rise in activity B when the pH of the standard teleost ringer was dropped from 7.8 - 7.0. Below pH 7.0 the activity began to drop and was greatly reduced at pH 6.5 (Table 10, Fig 21b). Activity A was unaffected.

Table 10

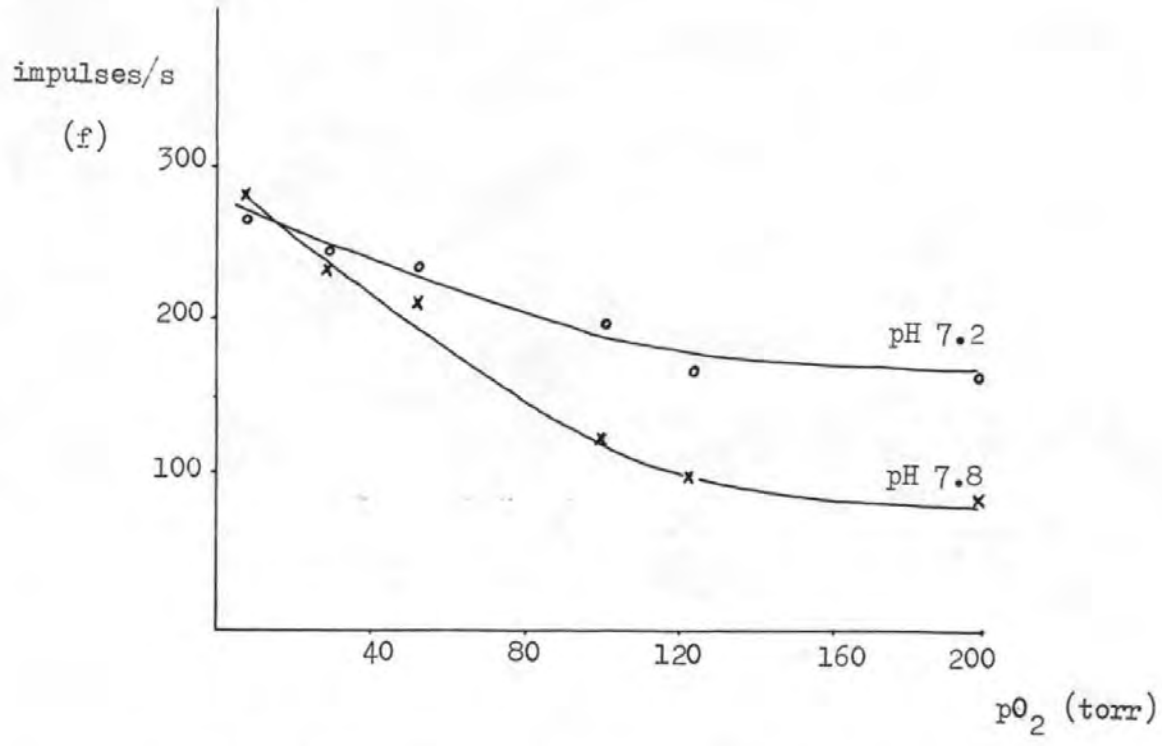
pH	$\frac{fe}{fs}$	S.E.
7.8	1	-
7.5	1.27	0.13
7.2	1.40	0.19
7.0	1.43	0.08
6.8	1.22	0.17
6.5	0.85	0.18

Effect of pH on activity B at 15°C (Mean of five experiments)

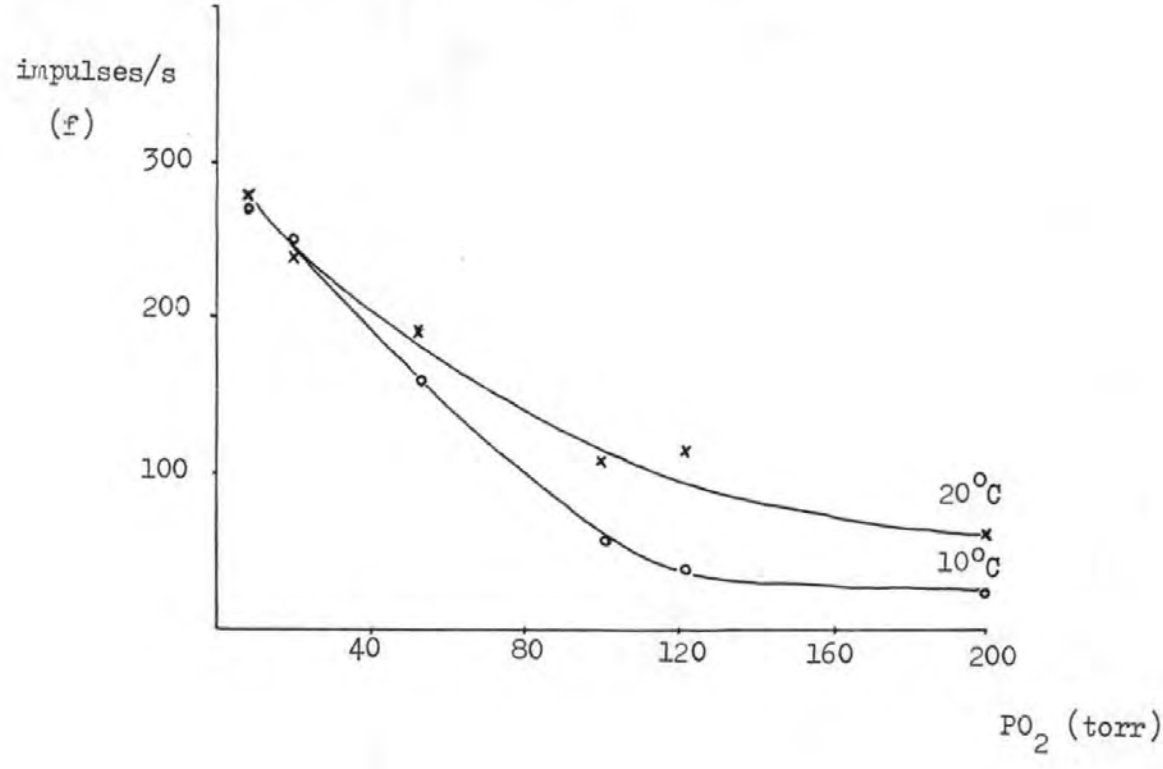
6) Combined effect of PO_2, pH and temperature

At low PO_2 (5 - 10 torr) lowering the pH from 7.8 to 7.2 has little effect on activity B whereas at higher PO_2 the differences in activity at pH 7.8 and 7.2 become more marked (Fig 22a).

Fig 22



a) Effect of pH on the response of activity B to PO_2 at 20°C



b) Effect of temperature on the response of activity B to PO_2 at pH 7.8

Similarly a rise in temperature from 10 to 20°C at pH 7.8 causes only a slight increase in activity B at low PO_2 while a similar rise at high PO_2 causes a larger increase in activity (Fig 22b). The increases in activity are less marked at pH 7.2 (Fig 23a). The results probably indicate an additive effect of PO_2, pH and temperature, particular combinations of which can cause eventual saturation of the receptors.

7) Effect of osmotic pressure and NaCl concentration

Activity A showed a large increase in activity with increasing Na Cl concentration between 62.5 and 250 m M (Plate 117-4, Fig 23b). The activity increases more rapidly above 125 m M . This was not due to an increase in osmotic pressure because a similar increase in osmotic pressure by addition of mannitol did not cause a similar rise in activity. The effect was shown to be due to Na^+ ions alone since replacement of the Na Cl fraction above 125 m M in the hyperosmotic solution by choline chloride suppressed the activity, whereas sodium propionate did not affect the response.

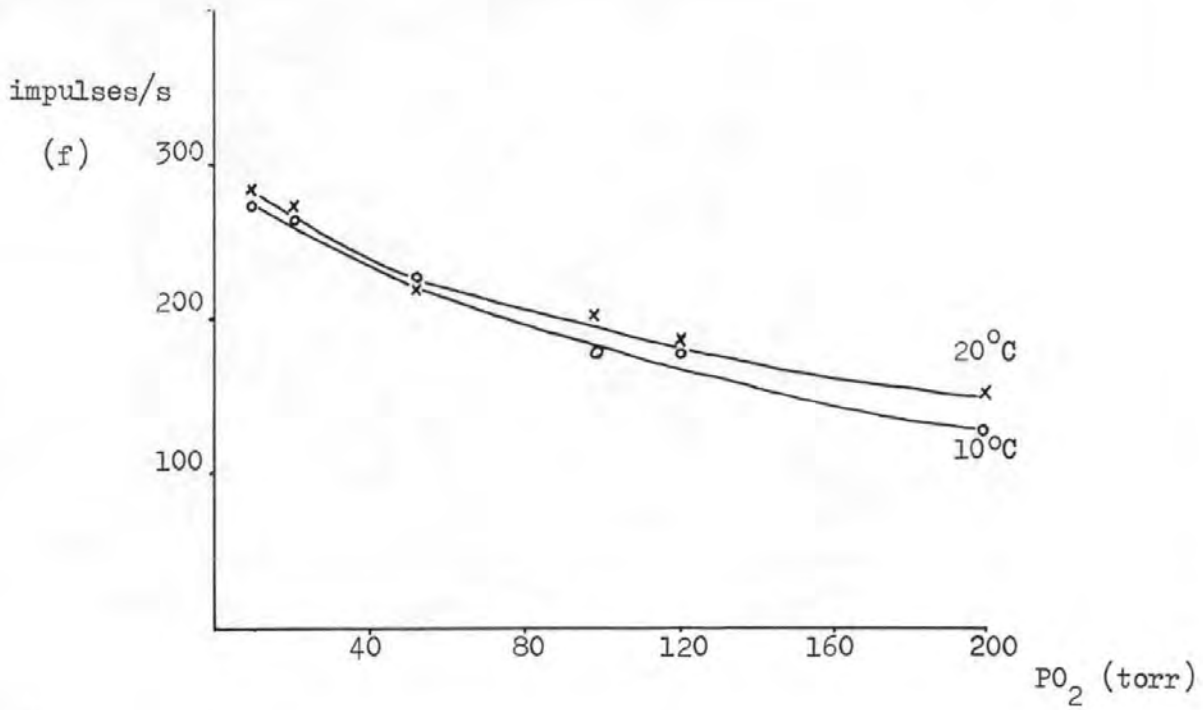
Table 11

NaCl (mM)	$\frac{fe}{fs}$	S.E.
62.5	0.24	0.06
94	0.86	0.07
125	1.0	-
187.5	2.63	-0.17
250	4.56	0.24

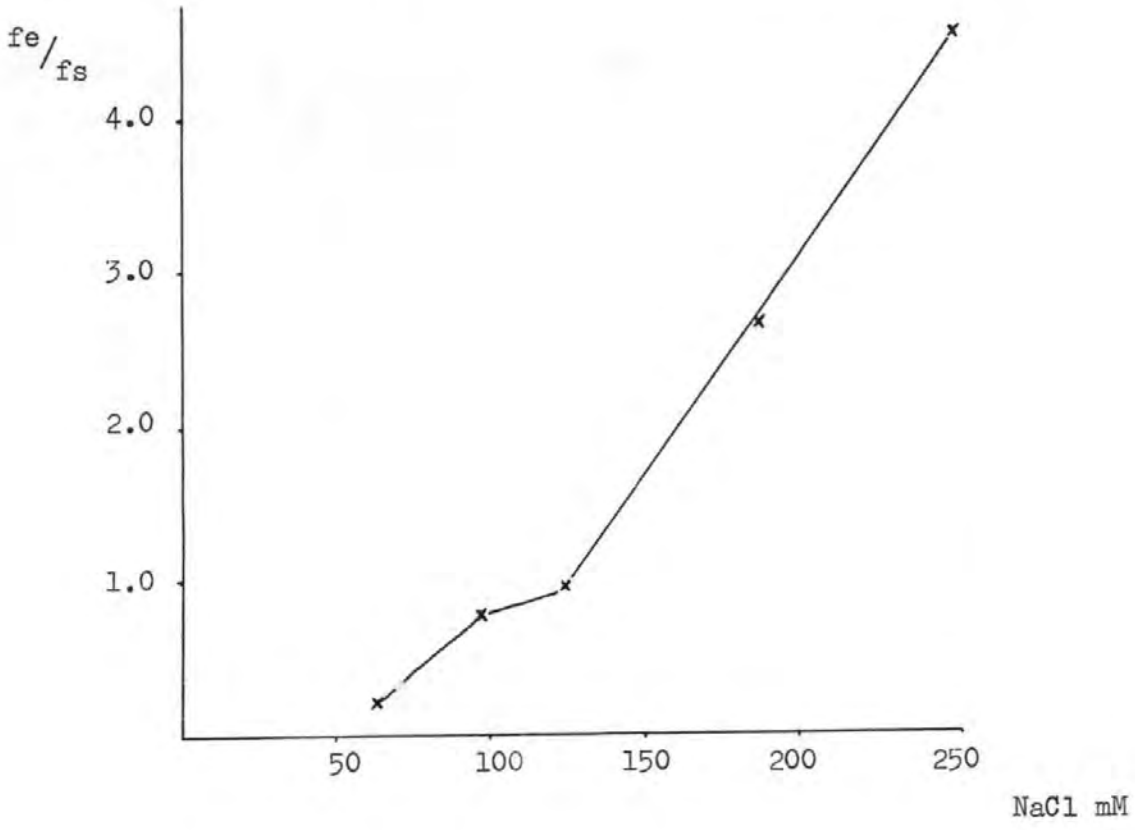
Effect of Na Cl concentration on activity A (Mean of four experiments)

Activity B changes very little above 270 m Osm but increases rapidly as the osmotic pressure is lowered (Fig 24a). When the osmotic pressure was maintained at 270 m Osm while decreasing the Na Cl concentration, the activity remained unaffected. Thus activity B is not

Fig 23

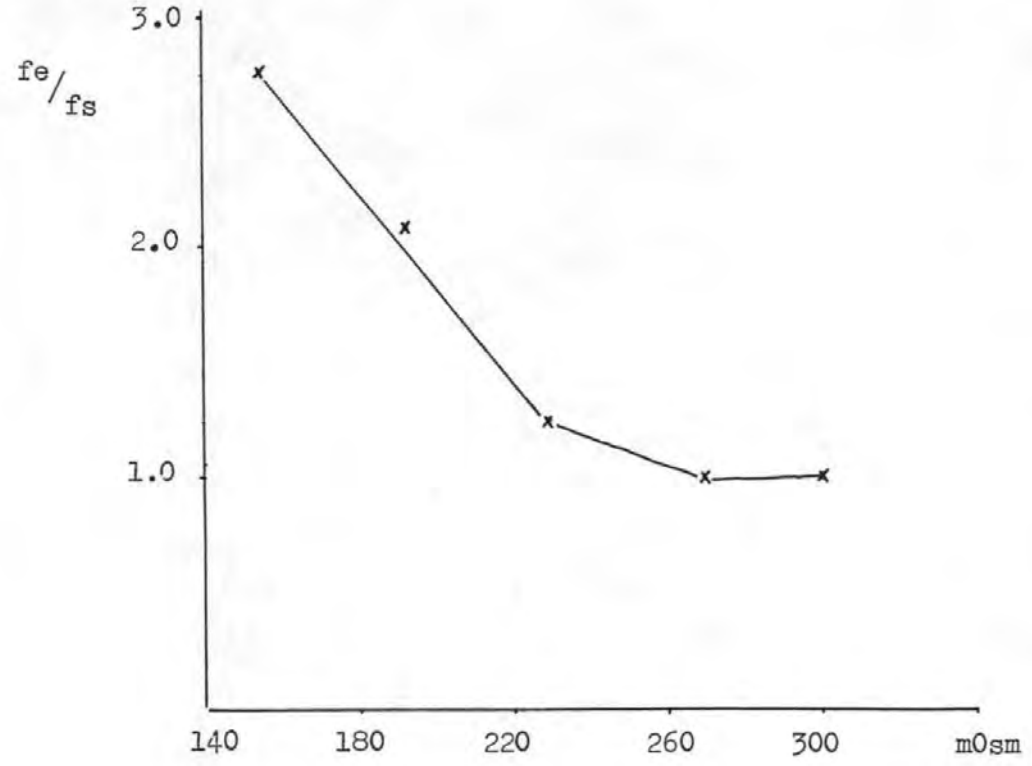


a) Effect of temperature on the response of activity B to PO_2 at pH 7.2

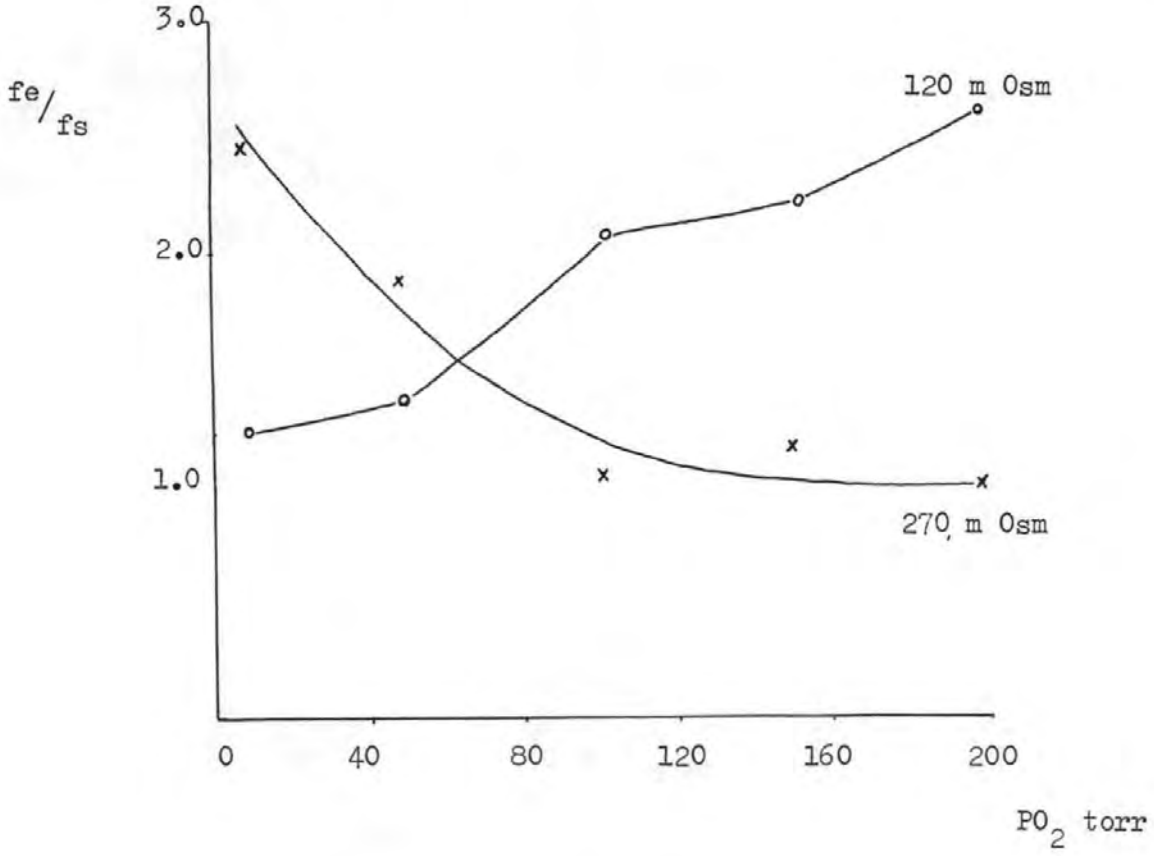


b) Effect of NACl concentration on activity A (15°C, pH 7.8, PO_2 220 torr)

Fig 24



a) Effect of osmotic pressure on activity B (15°C, pH 7.8, PO_2 220 torr)



b) Effect of osmotic pressure and PO_2 on activity B (15°C, pH 7.8)

stimulated by the Na^+ concentration but by the osmotic pressure itself.

Table 12

Osmotic pressure (m Osm)	$\frac{fe}{fs}$	S.E.
300	1.02	0.11
270	1.0	-
225	1.23	0.08
190	2.06	0.18
155	2.74	0.27

Effect of osmotic pressure on activity B (Mean of four experiments)

8) Combined effect of osmotic pressure and PO_2

The activity of B under hypoxic conditions was considerably reduced when the preparation was perfused with hypotonic solution (120 m Osm). A decrease in the absolute value of the activity was observed which was not much higher than under 'normal' conditions (Table 13, Fig 24b). Restoring the osmotic pressure with mannitol caused a return of the sensitivity of activity B to hypoxia. Restoring the PO_2 to normal (200 - 220 torr) in the hypotonic solution also caused an increase in activity B. Thus it appears that the responses to osmotic pressure and hypoxia are inter-dependent and related to the correct maintenance of one or other factor.

Table 13

PO_2 (torr)	Osmotic pressure (m Osm)	
	120	270
5 - 10	1.22 (0.14)	2.45 (0.19)
40 - 50	1.36 (0.11)	1.87 (0.24)
90 - 100	2.06 (0.18)	1.04 (0.19)
140 - 150	2.21 (0.25)	1.18 (0.08)
200 - 220	2.57 (0.21)	1.0 (-)

$\frac{fe}{fs}$ values + standard error at different osmotic pressures and PO_2 levels (Mean of four experiments)

9) Effect of acetazolamide

Addition of acetazolamide (10^{-4}M) to the perfusion solution caused a slight increase in activity B at pH 7.8 and pO_2 220 torr. However the activity was not significantly altered when the pH was lowered to 7.0 nor when the pO_2 was reduced to 10 torr, indicating a densensitisation of the receptors to H^+ ions and pO_2.

10) Effect of 2 - 4 - dinitrophenol

Concentrations of 2 - 4 - dinitrophenol between 10^{-7} and 10^{-9}M caused an increase in activity B. At concentrations between 10^{-4} and 10^{-6}M there was an initial increase in activity followed quickly by almost complete suppression. Activity A was unaffected.

DISCUSSION

The diverse nature of the pseudobranch structure in teleosts poses problems in trying to attribute a common function to this organ. However all teleosts examined possess specific 'pseudobranch type' cells which may indicate at least one common function between species.

Some confusion in the literature regarding the likely function of the pseudobranch has arisen partly from false assumptions about its structure, including the failure to distinguish between 'chloride' and 'pseudobranch type' cells (Parry & Holliday 1960; Holliday and Parry 1962; Parry 1966). The results of this study indicate that differences in structure are related to differences in habitat and the presence or absence of 'chloride type' cells. Fish which possess these cells usually have 'free' or 'semi-free' pseudobranchs and live in salt or brackish water (an exception is the perch (Perca fluviatilis)). Fish which lack 'chloride type' cells have 'buried' or 'covered' pseudobranchs and usually live in freshwater. However there are exceptions to the rule, e.g. the seawater garfish (Belone belone) and pollack (Pollachius pollachius) have 'buried' pseudobranchs and do not possess 'chloride type' cells. There does not appear to be any obvious environmental or phylogenetic reasons for such exceptions.

The results of this study agree with Granels' (1927) classification of four main pseudobranch types, although there is evidence to suggest that all possible transitions probably exist between 'free' and 'buried' pseudobranchs.

The scanning electron microscope is particularly useful for classification of pseudobranch types since it provides a quick and accurate method of assessing the degree of lamellar fusion and the extent of epithelial covering. Even in the apparently 'free' pseudobranch of the bass, a certain amount

of epithelial fusion between lamellae was seen on the buccal (leading) edge of the secondary lamellae. This fusion has progressed much further in 'semi-free' pseudobranchs (e.g. mullet), leaving only a small free area on the opercular (trailing) edge of the lamellae. In 'free' or 'semi-free' pseudobranchs the degree of fusion and epithelium covering is limited to allow the 'chloride type' cells free access to the water. This they achieve by openings in 'free' regions of the lamellar epithelium on the opercular side of the secondary lamellae. In freshwater teleosts such as rainbow trout, the 'chloride type'cells of the pseudobranch are lost, the secondary lamellae become completely fused and the whole organ is covered with an epithelial layer. This modification has been carried a step further in the carp pseudobranch which is buried deep within the surrounding connective tissue. It can be seen therefore that the pseudobranch of teleosts shows progressive structural modification from the respiratory branchial arches, ranging from a gill-like structure projecting into the buccal cavity to a 'glandular' structure buried in a mass of connective tissue. Although modified, the pseudobranch still retains many gill-like features, particularly in its circulatory pattern, secondary lamellae, epithelial covering and the presence of 'chloride type' cells in some pseudobranchs. However one of the most important differences is that the pseudobranch epithelium is always much thicker than that of the corresponding gill. This is due to a layer of specialized epithelial cells ('chloride' or pseudobranch type' cells) which are always interposed between the blood channels of the secondary lamellae and the outer epithelial layer. This is true even in the 'free' pseudobranch but is far more pronounced in the 'buried' pseudobranch where the specialized cells are also covered by connective tissue. Thus the capillary blood of the pseudobranch secondary lamellae is less likely to be involved in gaseous exchange. This is linked with the fact that the

blood to the pseudobranch is already oxygenated at the gills which precludes the necessity for a respiratory function in this organ.

The topography of the 'free' and 'semi-free' pseudobranchs provided an interesting comparison with that of the gills, bearing in mind the pseudobranchs' apparent lack of respiratory function but possible role in osmoregulation due to the presence of 'chloride type' cells. The surface structure of these pseudobranchs was found to bear a close sililarity to that described for the gills of teleosts (Olson & Fromm 1973; Rajbanshi 1977; Hughes 1979; Kendall & Dale 1979) and lampreys (Lewis & Potter 1976). The provision of an increased surface area for gas exchange due to the microridges has been suggested for gills (Olson & Fromm 1973; Lewis & Potter 1976) but this is likely to have no functional significance for the pseudobranch which lacks an apparent respiratory function.

It has been shown previously that microridges are not restricted to respiratory structures, such as the gills, since they have been found on the epidermal cells of the body and fins of teleost fish (Lanzing & Higginbotham 1974; Dobbs 1975; Hunter & Nayudu 1978; Bereiter-Hahn, Osborn, Weber & Voth 1979). The presence of microridges on a variety of teleost epithelial cells probably indicates a common function such as the anchorage of mucus suggested by Hughes & Wright (1970). This hypothesis is supported by Sperry & Wassersung (1976) who suggest that the complex curved or whorled arrangement of microridges facilitates the spread of mucus away from mucous cells.

It is still uncertain if a mucus film always covers the skin and gill surfaces but it is probably necessary to serve a number of functions. Apart from its possible affect on gas exhange, it is likely to reduce surface friction, to provide protection against mechanical abrasion and infection, and may be important for normal regulation of ionic exchange.

Hughes (1979) thought that the most probable situation was that mucus occupied the spaces between ridges thus presenting a flat interface with the water.

The thickness of the mucus film covering the filaments and secondary lamellae of the pseudobranch may vary from area to area. This is indicated by regions of different mucous cell density and differences in the microridge pattern which may affect anchorage of the mucus. Although there is no clear correlation between mucous cell density and microridge pattern, the average height of the microridges is usually greater where there are large numbers of mucous cell openings or 'chloride' cell openings. The distribution of mucous cells and thickness of the mucus film may be related to the speed and direction of the water flow together with the changes in resistance that occur over the filaments and between the secondary lamellae. It is noticeable that in both 'free' and 'semi-free' pseudobranchs the highest concentration of mucous cells occurs on the opercular edge of the filament where there is likely to be high resistance to water flow between the filaments and operculum.

Although anchorage of mucus seems to be the most likely function of the microridges they have also been considered as the structural result of the water flow itself (Hughes 1979), or as providing a region of microturbulence immediately above the secondary lamellae to enhance gaseous exchange (Lewis & Potter 1976). In the mullet pseudobranch the 'free' region of the secondary lamellae is particularly ridged and convoluted. This is likely to cause a region of microturbulence, with continual mixing of water layers immediately above the 'chloride' cell openings which might facilitate ionic exchange rather than gas transfer.

The 'chloride type' cell of the marine pseudobranch is similar to the 'chloride' cell of the corresponding gill, including the presence of an

apical pit. However one interesting feature of the 'chloride type' cells of the pseudobranch is the presence of numerous narrow saccules interposed between the plasma membrane at the base of the cell and the branched tubular system. These saccules have not been reported in the 'chloride' cells of the gill and the possibility that they reflect a morphological adaptation to an additional or new function will be discussed later. In all other respects the structure of the 'chloride type' cell of the pseudobranch is essentially the same as that of the 'chloride' cell in the gill. Both cell types are characterised by a highly branched tubular system arising from basolateral invaginations of the plasma membrane. They also possess abundant mitochondria in the basal and central regions of the cell, and a clear apical zone filled with vesicles. The prominent tubular system and numerous mitochondria are highly characteristic of ion-transporting epithelia from a wide variety of vertebrate and invertebrate animals (Berridge & Oschmann 1972).

The apical plasma membrane of the 'chloride type' cell of the pseudobranch differs slightly from that of the gill since it is usually smoother with fewer microvilli-like projections. Also, the apical pit is often much shallower and less distinct, with the 'chloride type' cell surface flattened and almost level with the surface epithelial cells. This was found particularly in the mullet and smelt but was only occasionally found in the bass.

The lack of a clear apical pit in the 'chloride type' cells of mullet and smelt pseudobranch may be a reflection of these species adaptability to euryhaline environments; a number of studies have shown loss of apical pits in gill 'chloride' cells during adaptation to freshwater (Copeland 1950; Kessel & Beams 1962; Philpott & Copeland 1963; Threadgold & Houston 1964; Shirai & Utida 1970; Fearnhead & Fabian 1971).

The multicellular composite apex of 'chloride type' cells and interdigitating accessory cells described in this study for the seawater pseudobranch and gill was first noted by Dunel & Laurent (1973) in the pseudobranch of various salt water adapted teleosts. A similar organisation was later found in the gills of seawater teleosts (Sardet et al 1979; Hootman & Philpott 1979) and the skin of the freshwater glass catfish (Korte 1979). Korte (1979), who only found a one to one relationship of 'chloride' and accessory cells, proposed that the accessory cell was a resting or non-functional 'chloride' cell whilst Sardet et al (1979) suggested that they were young 'chloride' cells. However Hootman & Philpott (1979) could find no recognisable intergrades between accessory cells and 'chloride' cells, and suggested that the former were not replacement cells for the 'chloride' cell population. They suggested that accessory cells represented chloride cells that had undergone regression because of the common observation of autophagic lysosomes within these cells. Although autophagic lysosomes were occasionally found in the present study there was no other evidence of regression such as mitochondrial degeneration and fragmentation of the tubular network previously described by Shirai & Utida (1970) and Doyle & Epstein (1972). In agreement with Sardet et al (1979) the 'chloride' cells of the gill in freshwater fish were found as isolated cells, unaccompanied by accessory cells, and surrounded only by respiratory epithelial cells.

The difference in shape of the apical cavity as well as the size of 'chloride' cells in the gills of freshwater or seawater adapted fish may be a direct consequence of the development of the multicellular 'chloride' cell complex. If one examines previously published micrographs of 'chloride' cell apices in seawater, it can be seen that extensions of interdigitating accessory cells are present, although unrecognised as such (e.g. Philpott & Copeland 1963, Figs 5 & 6; Petrik, 1968, Fig 2; Shirai & Utida 1970, Fig 9). However several authors have reported 'dense' and 'light' forms

of 'chloride' cells in the gill, with increases of 'dense' form cells during adaptation to seawater (Doyle & Gorecki 1961; Threadgold & Houston 1961, 1964; Newstead & Conte 1969: Shirai & Utida 1970). This could be due to the reorganisation of 'chloride' cells with the appearance of the denser,young 'chloride' cells adjacent to mature cells. Conte and Lin (1967) utilised a labelling technique to show that the turnover rate of labelled D.N.A. in seawater gill epithelium was almost 3 times faster than in freshwater. Using an autoradiographic technique they showed that the site of cellular renewal in the gill filament of Onchorhyncus kisutch is principally in the interlamellar region of the gill filament, corresponding to the position of the 'chloride' cells.

The development of a multi cellular 'chloride' cell complex in the seawater gill and pseudobranch also appears to involve a new type of junction between the cells of the complex. At the apex of the 'chloride' cell, shallow junctions are established between the main cell and the interdigitations of the adjacent developing cells. Sardet et al (1979) found that these junctions allow lanthanum to penetrate while the other epithelial junctions do not. They are typical one strand junctions resembling those of low resistance epithelia e.g. the thin part of Henle's loop, or proximal tubules of mammalian or amphibian kidney (Boulpaep, 1971; Claude & Goodenough 1973; Humbert, Grandchamp, Pisam, Perrelet & Orci, 1976). Thus Sardet et al (1979) suggested that salt water adaptation triggers a cellular reorganisation of theepithelium in such a way that leaky junctions, acting as a low resistance pathway, appear at the apex of the 'chloride' cells. Since the leaky junctions have connections to the tubular network they represent a permanent communication channel from the blood side to the external milieu. Therefore they are ideally situated to allow ions and possibly small molecules to pass between the blood and the water. There are good recent discussions by Kyte (1976) and Ernst & Mills (1977) on how epithelial cells could secrete

or absorb salt depending on the degree of leakiness of their apical junctions.

The key to the cell function is in the understanding of what ions are pumped in and out of the tubular system and how they flow toward the external or internal environment. In salt water, sodium and chloride are excreted against large concentration gradients. Excretion is the result of large in-and-out fluxes, a major part of which is the Na^+/Na^+ and Cl^-/Cl^- exchange diffusion component (Maetz 1971; Motais and Garcia Romeu, 1972). Numerous investigators have measured the isotopic Na^+ and Cl^- fluxes across the gills of intact seawater adapted teleosts and have demonstrated relatively large unidirectional fluxes of these ions which resulted in small net secretions of both Na^+ and Cl^- (see Maetz & Bornancin 1975). Sodium and chloride secretion can be considered separately since transport of Na^+ and Cl^- occurs independently but in parallel (Maetz 1971).

The nature of Na^+ movements across the gills of seawater adapted teleosts is unsettled and represents a major controversy concerning ion movements across this tissue. Currently the models for NaCl secretory mechanisms by the gill 'chloride' cell propose an important role for Na^+, K^+ - activated ATPase, either directly (Maetz 1969, 1971) or indirectly (Kirschner 1977; Silva et al 1977). The enzyme is thought to be involved in the branchial Na^+ extrusion mechanism in seawater teleosts and most of the species studied show high activity levels corresponding to high Na^+ turnover rates (Maetz 1974). During salt adaptation there is an increase in Na^+ K^+ - activated ATPase activity and ouabain binding sites of the gill tissue (Epstein, Katz & Pickford, 1967; Kamiya & Utida, 1968; Karnaky et al, 1976 (b); Sargent et al, 1975). These changes are probably due to increases in the number of 'chloride' cells and development of their tubular system since Na^+ K^+ - activated ATPase is thought to be

associated with the tubular membranes (Mizuhira et al 1970, Utida et al 1971, Shirai 1972, Karnaky et al 1976 (b), Hootman & Philpott 1979). However these modifications alone do not explain satisfactorily why ions are absorbed in freshwater and excreted in salt water, neither do they provide an explanation for the greatly increased salt permeability of the gill epithelium in salt water adapted fish (Maetz 1971). The presence of leaky junctions in the 'chloride' cells of seawater fish and absence in freshwater fish may provide a structural basis for the greater ionic permeability of the seawater gill.

Sardet el al (1979) proposed a model for the extrusion of sodium in seawater 'chloride' cells, whereby Na^+ is pumped into the tubular system and secreted via the leaky junctions using the driving force generated due to the blood being electropositive with respect to the external milieu.

Shirai & Utida (1970) demonstrated the localisation of sodium in the tubular system of the gill of the seawater Japanese eel using a potassium pyroantimonate method based on that of Komnick & Komnick (1963). Using a similar method in this study no clear localisation of sodium could be found in the 'chloride' type cells of the gill or pseudobranch in the mullet and carp. The validity of this method for demonstrating sodium ions has been questioned since it also precipitates potassium and calcium ions as well as forming precipitates with cellular components such as histones, glycogen and biogenic amines (Pearse 1972; Garfield, Henderson & Daniel 1972; Lewis & Knight 1977). Also, potassium pyroantinomate is itself precipitated during dehydration and the distribution of reaction product varies with the type of fixative (Bulger 1969). In the present study much of the reaction product appeared to be associated with glycogen particles whose position was visualised in cells fixed using the conventional glutaraldehyde- osmium tetroxide method. Therefore more convincing evidence of sodium ion localisation is needed at the subcellular

level before definite conclusions can be drawn about this method of secretion from the 'chloride' cell.

Similarly there is no unequivocal evidence for the pathway taken by chloride ions during secretion from the 'chloride' cell of seawater fish. In the present study chloride reaction product was localised at the apical membrane of the pseudobranch 'chloride type' cell, particularly in the region of the leaky junctions. However it was impossible to achieve high resolution localisation using the techniques employed so it was difficult to assess whether the reaction product was actually within the leaky junctions and tubular system, or in the ground substance and attached to the apical membrane. The precipitation of reaction product on the cytoplasmic side of the apical plasma membrane argues against the aggregates being caused by a build up of precipitate against an impermeable barrier. An artefactual aggregation of reaction product would be more likely to occur in the basal cytoplasm since the fixative moves in that direction during penetration of the cell. Philpott (1966) and Fearnhead & Fabian (1971) found similar localization of reaction product in the 'chloride' cells of the gills of salt water adapted fish whilst Petřík (1968) demonstrated reaction product in the plasma membranes and tubular system. Of particular interest is Fig 2 which shows reaction product in the leaky junctions of interdigitating, adjacent 'chloride' cells, although these were not recognised as such at the time. Petrik (1968) also found cells in which reaction product was located only within the apical cavities and not the tubular system, and concluded that this was due to the manifestation of different stages of chloride ion excretion.

In addition to their similarity in structure and localization of 'chloride' reaction product, 'chloride type' cells of the pseudobranch are similar to those of the gill in their staining reactions to a number of

histochemical tests. Both cell types show a light staining reaction to the P.A.S. test indicating the presence of complex carbohydrate. In addition the apical region of the cells is weakly stained by Alcian blue at pH 2.5, which suggests the presence of acid mucopolysaccharides. Philpott (1966), Conte (1969) and Shirai (1972) suggest that mucopolysaccharides and mucoproteins which are polyanionic substances may serve as ion traps or ion exchangers in the vicinity of the transport enzymes. Ion and polyelectrolyte material may combine in the tubular system and progressively concentrate during transportation toward the apical region, converging in vesicles presumably to be discharged into the external medium. However the interpretation of apical vesicles as secretory vesicles has been questioned by Maetz & Pic (1977) who could find no evidence of exocytosis of these vesicles in the 'chloride' cells of mullet gill. Sardet et al (1979) on the basis of freeze-fracture images and histochemical staining suggested that rather than vesicles, there was a vesicular-tubular space between the tubular reticulum or basolateral membrane and the apical membrane. This could be a transient communication channel between the internal and external milieu and could be responsible for the transit of organic molecules or polysaccharidic materials (Lam 1968; Masoni & Garcia Romeu 1972; Masoni & Payan 1974).

The presence of high levels of succinic dehydrogenase activity in the 'chloride type' cell of the bass pseudobranch indicates their highly active metabolic state. Since succinic dehydrogenase is a bound, mitochondrial enzyme this high activity is a reflection of the large number of mitochondria in the 'chloride type' cell. Sargent et al (1975) found an enhanced succinic dehydrogenase activity in the gills of freshwater eels adapted to seawater and demonstrated this to be associated with 'chloride' cells. They concluded that adaptation of freshwater eel to seawater involves the elaboration not only of a Na^+ pump expressed biochemically as Na^+/K^+ - ATPase but also the energy producing apparatus to drive the pump, expressed

biochemically as mitochondrial enzymes including succinic dehydrogenase.

Another enzyme implicated in osmoregulatory mechanisms is alkaline phosphatase. Although no obvious activity could be found using light microscopy a small amount of reaction product was found in the apical region of 'chloride type' cells examined in the electron microscope. However the precipitate was not actually found in the cell but was associated with the intercellular spaces between adjacent cells and with the surface membrane. Pettengill and Copeland (1948) reported light microscope localisation of alkaline phosphatase activity around the apical pit of seawater adapted Fundulus heteroclitus, and suggested that the enzyme was associated with an 'osmotic work-energy mechanism' when the cell is in its excretory phase. There was a marked increase in activity when the fish was moved from seawater to freshwater with considerable phosphatase activity appearing between the nucleus and distal end of the cell. They interpreted this increase in enzyme activity as a gauge of the expenditure of energy on osmotic work necessary for the 'chloride' cell to function in the absorptive phase.

Alkaline phosphatase activity has also been found in the 'chloride' cells and basement membrane of freshwater mud-eel (Ojha & Datta Munshi 1974) but could not be demonstrated in the 'chloride' cells of the skin of the freshwater teleost Channa striata (Banajee & Mittal 1975). This suggests that although alkaline phosphatase activity may reflect the osmotic activity in freshwater and seawater 'chloride' cells, it is not necessarily an essential enzyme system in ion regulation, and may be absent from some cells. The relatively small amount of activity found in the 'chloride type' cells of the pseudobranch may reflect only a minor role for this enzyme in these cells.

The available morphological and histochemical evidence suggests an ion

excretory role for the 'chloride type' cell of the seawater pseudobranch similar to that proposed for the 'chloride' cell in the gill of seawater adapted fish. This may result from a need in marine and euryhaline fish to utilise all available surface area for extra-renal salt regulation. Parry & Holliday (1960) rejected any osmoregulatory role for the pseudobranch because fish that had undergone pseudobranchectomy were apparently able to osmoregulate quite normally and showed no significant differences in survival time to control fish, when exposed to various sea-water dilutions. However they believed that all the cells of the pseudobranch were the same or similar to the 'chloride' cells of the gill, and used two experimental fish, Salmo trutta and Salmo gairdneri which contain only 'pseudobranch type' cells. Furthermore the removal of pseudobranchs in seawater fish may not drastically affect the fish's ability to osmoregulate since the 'chloride cells' of the gill and opercular epithelium can still carry out this function. It is possible that the absence of 'chloride type' cells in the pseudobranch of freshwater fish reflects a lower dependence on extra-renal salt regulation and suggests that the pseudobranch of these fish is not directly involved in osmoregulation.

The possibility that the 'chloride type' cell of the pseudobranch has a new or additional function to that of the gill is suggested by the appearance of a system of narrow saccules interposed between the basal plasma membrane and the tubular system of the cell. The narrow diameter of these saccules compared with that of the tubules, and their position at the base of the cell may indicate a barrier or 'filter' to certain materials passing from the blood into the cell via the tubular invaginations of the plasma membrane. Such a system might be useful to conserve biologically useful macromolecules while still allowing ions to pass through, or might even be involved in the reabsorption of certain electolytes. The need for such a system in the pseudobranch and not the gill is unclear, but it may

be related to a special relationship between the 'chloride type' and 'pseudobranch type' cells in seawater fish. As shown in the present study all blood flowing to the 'pseudobranch type' cells has first to pass between the 'chloride type' cells where it may be modified in both its ionic and organic composition. 'Pseudobranch type' cells appear virtually identical in both seawater and freshwater fish so their function may depend on a similar blood composition in this part of the capillary system. The 'chloride type' cells of the seawater pseudobranch may therefore regulate the blood composition in order to produce a similar environment to that encountered by the 'pseudobranch type' cells of the freshwater fish. However it is obvious that any interpretation of morphological data in functional terms is only speculative and more work is needed to investigate possible enzyme systems associated with the 'chloride type' cell saccules. Investigations on the penetration abilities of tracers such as lanthanum and horseradish peroxidase might also prove useful.

Newstead (1971) in a study of the gills of a tidepool sculpin (Oligocottus maculosus) claimed that the 'chloride type' cells normally found in the gill epithelium could, when osmotically stressed, undergo rearrangement of their cytoplasmic components into the pattern characteristic of 'pseudobranch type' cells. Newstead (1971) concluded that "there is a direct relationship between the two forms ('pseudobranch type' and 'chloride type' cells) and the transition from one form to another can be evoked by disturbance of the normal homeostasis of the blood".

In this study the cytological changes due to drastic osmotic stress, as in the bass, or the gradual stress to trout and smelt, gave no convincing evidence to support Newstead's theory. The cells from the pseudobranch of all three fish studied did undergo ultrastructural changes although the degree and rapidity of change appears to depend on whether the stress is applied quickly or gradually. The cells from the bass pseudobranch showed

a more rapid, and greater degree of change, than the gradually acclimated smelt and rainbow trout. This is to be expected since the bass pseudobranch cells were given no chance to adapt to a changing osmotic environment.

The loss of apical pits in the 'chloride type' cells of the smelt and bass pseudobranch in freshwater and dilute seawater parallels the response of 'chloride' cells in the gill epithelium of Oligocottus maculosus (Newstead 1971) when exposed to fresh water, and that of similar cells in Fundulus heteroclitus (Kessel & Beams 1962; Philpott & Copeland 1963), Anguilla rostrata (Getman 1950; Shirai & Utida 1970), Monodactylus argenteus (Fearnhead & Fabian 1971), Salmo salar L (Threadgold & Houston 1964) and Etroplus maculatus (Virabhadrachari 1961). Although the arrangement of the tubules and mitochondria did become more orderly in some 'chloride type' cells of bass and smelt, they still remained easily distinguishable from 'pseudobranch type' cells.

The 'pseudobranch type' cells of smelt and rainbow trout kept in water of 0 - 34‰ salinity showed little or no ultrastructural change, and no significant reorganisation of cytoplasmic contents was noted. In trout the appearance of the 'pseudobranch type' cells at higher salinities (34 - 60‰) suggests that they are degenerating and that the fish is unable to cope with further loss of body fluids and build up of salts. The characteristic orderly arrangement of mitochondria and cytoplasmic tubules was replaced in some cells (at 60‰) by a more randomly branched system, although most cells still retained their 'pseudobranch type' characteristics.

The appearance of lipid-like, electron dense inclusions in the 'chloride' and 'pseudobranch type' cells of bass after 24 hours exposure to 0.34‰ salinity is probably a result of rapid disturbance of the normal homeostasis of the blood with subsequent effects on the normal metabolism of the cells. The electron dense inclusions are particularly numerous in the 'pseudobranch

type' cells which give an increased staining reaction to the Sudan black B test for lipids, compared with normal cells. It is interesting to note the appearance of similar inclusions in the 'pseudobranch type' cells of trout after injection of the carbonic anhydrase inhibitor, acetazolamide, or injection of dilute hydrochloric acid. Both these procedures are likely to cause disturbance of blood homeostasis with changes in pH, acid-base balance and ionic composition (see later).

Pickering & Morris (1977) found similar lipid inclusions in one cell type of the gills of sexually mature male river lampreys. These cells (previously referred to as male glandular cells (Morris 1957)) showed some ultrastructural characteristics in common with ion transporting cells but were readily distiguished by large lipid structures with electron lucent centres. In the present study the electron dense inclusions often contained an electron lucent centre, although, as Pickering & Morris (1977) pointed out, this may represent a fixation artefact. They found that cells containing lipid droplets were only found in sexually mature males at spawning time, and not in sexually mature females. However they could find no evidence to support the hypothesis that these cells secrete some substance of sexual significance presumably connected with spawning (Morris 1957). They suggested that this cell type is involved in ion transport and that the appearance of a different male cell might be due to differences in the ion regulatory properties of male and female river lampreys at this time. In this connection it was found that the blood serum of the sexually mature male had a significantly higher chloride level and osmotic pressure than the female. The levels of sodium however were similar.

In the osmotically stressed 'pseudobranch type' cells the lipid droplets are found mainly in the apical region of the cell, with some very close to the apical cell membrane. Some droplets appeared to be in the

process of being secreted from the cell, a possibility reinforced by the appearance of lipid droplets in the interstitial and extracellular spaces between 'pseudobranch type' cells. The surrounding of the larger droplets by membranous profiles of smooth endoplasmic reticulum, abundant ribosomes and glycogen particles is similar to that seen in cells of endocrine glands which synthesise steroid hormones (e.g. cells of the adrenal cortex, interstitial cells of testis) (Sabatini & De Robertis 1961; Luse, 1967; Yoshimura, Harumiya, Suzuki, Totsuka, 1968; Long & Jones 1967; Christensen & Gillim, 1969). A process of endoplasmocrine secretion has been described in these cells (Rhodin 1974), whereby the lipid droplets grandually reach the cell membrane which fuses with the outer casing of smooth endoplasmic reticulum and the contents of the lipid droplet are discharged into the extracellular space. The synthesis of hormones is probably accomplished by a co-operation of the smooth endoplasmic reticulum, the mitochondria and the lipid droplets. Many of the enzymes required for this synthesis are located in the mitochondria and the smooth endoplasmic reticulum, whereas the cholesterol, the cholesterol esters, and possibly some of the intermediate hormone precursors are localised in the lipid droplets. The intimate contact between the smooth endoplasmic reticulum, the lipid droplets and the mitochondria facilitates the exchange of enzymes and intermediate products in this synthesis (Rhodin 1974).

It is possible that the appearance of lipid droplets in the 'pseudobranch type' cells of osmotically stressed fish or in cells of sexually mature, male river lampreys is due to the production of a steroid or steroid hormone by these cells in response to a change in salt regulatory mechanisms. Steroid hormones such as the mineral corticoids of the adrenal cortex are known to be involved in the regulation of salt and water metabolism (Potts & Parry 1964).

Possible support for this idea is provided by the staining reaction of normal, carp 'pseudobranch type' cells during prolonged immersion in osmium at 40°C. Osmium tetroxide is deposited selectively within the Golgi apparatus, endoplasmic reticulum and nuclear envelope of the 'pseudobranch type' cells. Friend & Brassil (1970) found selective staining of the Golgi complex and endoplasmic reticulum in the zona fasciulata cells of the rat adrenal cortex and proposed that it was due to the retention and staining of steroids and their intermediary products. They noted similar and consistent staining patterns in other cells concerned with cholesterol and steroid biosynthesis, in the corpora lutea, testis interstitial cells, hepatocytes and intestinal absorptive epithelia.

Osmium staining showed the 'pseudobranch type' cells to have an extensive but scattered Golgi apparatus and smooth endoplasmic reticulum. The densest staining reaction occured in the dark (stage 3) cells. These cells also contain large numbers of glycogen particles packed close to the tubular membranes surrounding the mitochondria. This raises the possibility of these cells acting as a high energy source ready to take part in some metabolically active function.

Parry & Holliday (1960) suggested that the relationship between the pseudobranch and the eye could be explained in terms of an endocrine function for the pseudobranch, related to the control of chromatophores in the skin. They proposed that the pseudobranch produces, or activates, a hormone affecting the chromatophores, and that entry of this hormone into the general circulation is controlled by the choroid gland. The amount of hormone in the circulation is controlled by the state of the capillaries in the choroid gland; when fully dilated there is a maximal blood flow from the pseudobranch into the general circulation and the fish is pale; when the capillaries are fully contracted the circulation through the pseudobranch is restricted and the amount of hormone in the general circulation is thus low, and the fish is dark. Thus in this double system,

the pseudobranch can be regarded as a self replenishing reservoir of hormone and the choroid gland is the 'tap' which can be opened or closed to a varying degree. The control of the 'tap' might well be in the amount of incident light falling on the retina, with or without pituitary intervention. Fish in the dark or blinded fish are then dark because of the associated restriction of the amount of hormone released into the circulation. They supported this hypothesis by presenting evidence to show that injection of homogenised pseudobranch extract into pseudobranchectomised trout caused various degrees of temporary local paling around the site of injection and isolated chromatophores contracted when placed in saline extracts of horse mackerel and cod pseudobranchs. Extracts of fin, spleen and gill tips failed to cause a similar response. This would suggest that the pseudobranch contains a substance capable of causing contraction of fish melanophores.

The coordinating systems for colour changes in fish show great diversity (Fujii 1969). In some fish, blood borne hormones are believed to be predominently responsible for pigment movements, while in others the pigment cells are solely regulated by nerves. Between these two extremes there are many examples where both neural and humoral mechanisms are working, although the latter is generally subservient to the former (Parker 1948). The pituitary gland is strongly implicated in humoral control of chromatophores. Extracts of teleostean pituitaries affect the distribution of pigment in both the melanophores and erythropores of fish (Pickford & Atz, 1957). It has been proposed that two antagonistic melanotrophic hormones are secreted by the fish pituitary; one (M.S.H., melanophore-stimulating hormone, intermedin) causing melanophore dispersion, the other (M.C.H., melanophore-concentrating hormone) inducing melanin concentration in the middle of the pigment cell (Pickford & Atz, 1957; Waring 1963). Other workers consider the experimental data to be explicable in terms of only one hormone (Kent 1961). Apart from the pituitary gland another

endocrine organ which might be involved in chromatophore control is the pineal. Lerner and Case (1960) isolated a potent bleaching agent for frog skin from bovine pineal glands and called it melatonin. Its chemical structure is N-acetyl-5-methoxytryptamine, being closely related to serotonin (5-hydroxytryptamine). Healey and Ross (1966) observed that melatonin caused paling of black adapted Phoxinus, while Fundulus and Carassius carassius were little affected (Fujii 1969).

It is possible that a third alternative is provided by the pseudobranch which may produce or activate a substance similar to the proposed pituitary M.C.H. or pineal melatonin which is necessary for maintenance of the pale colour phase in some teleosts. However, the temporary local paling caused by injection of pseudobranch extracts into pseudobranchectomised fish may cause a misleading interpretation of pseudobranch-mediated chromatophore control since many substances are known to cause contraction of melanophores, although apparently not specifically evolved for this purpose. Thus many alkaline and alkaline earth ions such as K^+, Rb^+, Cs^+, NH_4^{++}, Ba^+ and Sr^+ are generally effective enough to induce melanin aggregation (Spaeth 1913; Kamada & Kimosita, 1944; Fujii, 1959; Watanabe, Naitoh & Tsuchiya, 1965). In some fish Ca and Mg can aggregate melanin while in others they are less potent (Watanabe et al, 1965). Fujii (1959) expressed the view that potassium chloride acts on the melanophore synapses promoting the liberation of monoamine content, although K^+ also has some effect on the melanophore by lowering its membrane polarisation.

Many hormonal substances other than M.S.H. or M.C.H. are known to affect the state of fish chromatophores either in vivo or in vitro, (Fujii, 1969). Robertson (1951) indicated that extracts of mammalian thyroid gland caused marked aggregation of melanophores of rainbow trout.

The melanosome aggregation resulting from the action of adrenalin is well known (Parker, 1948; Fujii, 1961), and is probably due to its action

on melanophore adrenergic receptors (Fujii & Miyashita, 1975). Nor - adrenalin is also effective in causing melanosome aggregation (Fujii, 1961; Fange 1962; Healey & Ross, 1966; Scheline, 1963). Dopamine is extremely active in inducing pigment aggregation (Scott 1965). The paling effects of dopamine and other sympathominetic drugs including ephedine and tyramine were shown by injecting them into dark adapted minnows (Healey & Ross 1966). 5-Hydroxytryptamine (Serotonin) has also been reported to have melanin aggregating properties (Scheline 1963; Scott 1965). Lange (1973) suggested that the covered pseudobranch of the cod is responsible for the formation and possibly the storage of 5-hydroxytryptamine (5 HT) or a closely related substance. This was based on the observation of argentaffin and argyrophil granules in acidophilic cells of carcinoid-like tumours in the cod pseudobranch which Lange (1973) believed these to arise from the acidophilic cells of the pseudobranch. She suggested that these cells were analagous to Kulchitsky or enterochromaffin cells. However in this study no evidence of secretory granules could be found in the 'pseudobranch type' cells of the closely related pollack or any other fish.

The ability of pseudobranch extracts to cause paling of pseudobranch-ectomised fish and inability of gill tissue to cause the same effect suggests that a melanophore concentrating substance is produced by the 'pseudobranch type' cells since these are absent from gill tissue. However another possibility is that contraction of melanophores is caused by catecholamines circulating in the blood of the pseudobranch, although similar levels would be expected in the gill blood which supplies the pseudobranch. Obviously more work is needed to try to isolate the pseudobranch component responsible for contraction of chromatophores.

The darkening of fish after removal of the pseudobranchs probably has a far simpler explanation than endocrine control of chromatophores.

Bilateral pseudobranchectomy causes permanent blindness in fish (Vialli 1926; Denton & Saunders 1972; Ballintijn, Beatty & Saunders 1977) with obvious effects on colour control. This operation causes interruption of the blood supply to the choroid rete mirabile, with a consequent dramatic decrease in oxygen tension in the eye (Ballintijn et al 1977). There is also a gradual loss of visual pigment which suggests that visual pigment regeneration is ultimately dependent on a continual blood supply from the choroid rete and quite possibly on the high ocular oxygen tensions associated with this supply. It is not known whether it is a prolonged lack of oxygen or the general loss of other essential blood borne factors that is responsible for the decrease in the capacity to regenerate visual pigment. Also the initial cause of blindness and resultant body darkening following bilateral pseudobranchectomy (B.P.) is unknown. In this study B.P. fish were very dark and blind, as judged by their behaviour (see Denton & Sanders 1972) within 1 to 2 hours, but Ballintijn et al (1977) found that a substantial amount of visual pigment still regenerated at this time. This suggests that the initial blindness and resulting body darkening is caused by an impairment of retinal function other than a loss of ability to regenerate pigment during the visual cycle.

Unilateral pseudobranchectomy does not cause blindness or body darkening since there is a connection between the left and right efferent pseudobranchial vessels. Thus the choroid rete of the ipsilateral eye can receive blood from the contralateral pseudobranch following unilateral pseudobranchectomy. This inter-connection is responsible for normal oxygen tensions being maintained in both eyes of unilaterally pseudobranchectomised fish (Ballintijn et al 1977).

A functional relationship between the pseudobranch and the eye has been implied from studies in which removal of the eyes or keeping fish in

darkness for long periods of time resulted in a reduction in the size of the pseudobranch (Pflugfelder 1952). Harb & Copeland (1974) found that fish kept in constant light for 7 days or constant darkness for 28 days showed some rearrangement of the mitochondrial cristae of pseudobranch cells into rows of helices. This rearrangement was far more pronounced and organised in fish kept in constant light for 28 days. They interpreted this highly organised arrangement to indicate a specialized metabolic activity different from the usual function of the mitochondria in the pseudobranch.

Wittenberg & Haedrich (1974) believed that the pattern of occurence of the pseudobranch among salt, brackish and freshwater fishes provides a clue to the functional relationship between the pseudobranch and the choroid rete mirabile. With few exceptions, marine teleosts which lack a pseudobranch also lack the choroid rete. All fish in which the choroid rete mirabile is well developed but the pseudobranch is absent inhabit fresh water e.g. Osteoglossiformes (Wittenberg & Haedrich 1974). The Elopiformes, all of which have powerfully developed choroid retia, show a striking gradation in the size of the pseudobranch. Those species which are primarily marine have conspicuous, large pseudobranchs whilst species from brackish or freshwater have very small or minute pseudobranchs. This led Wittenberg and Haedrich (1974) to infer that a function of the pseudobranch which is required for the operation of the choroid rete mirabile may be dispensed with in freshwater. A comparison of the structure of pseudobranchs from freshwater and seawater fish may give a clue to this function, and the possible functional elements involved.

The basic structure and vasculature of the pseudobranchs of freshwater and seawater teleosts are very similar, the most obvious difference being that seawater fish usually have 'free' or 'semi free' secondary lamellae to allow the 'chloride type' cells access to the external environment.

Since freshwater fish generally lack 'chloride type' cells it may be possible to ascribe a function to these cells which is necessary to the choroid rete mirabile of seawater fish.

Wittenberg & Haedrich (1974) suggested that the pseudobranch affects a forced exchange of blood bicarbonate ion for external chloride ion. The pseudobranch of a marine teleost carrying out a bicarbonate/chloride exchange could reduce total blood carbon dioxide to a level which when multiplied in the counter current system of the choroid rete would not be damaging. In freshwater such exchange would be limited by the available chloride, and the pseudobranch could be dispensed with as it has been in some groups.

However this suggestion encounters a number of difficulties since an exchange of external chloride ion for internal bicarbonate ion is only likely in freshwater fish, where there is a net absorption of chloride ions (Maetz 1971). Such a process is described by Maetz & Romeu (1964), Romeu & Maetz (1964) and De Renzis & Maetz (1973) in the gill of the goldfish, and by Kersetter and Kirschner (1972) in the trout gill. This exchange is presumably dependent on cells having access to the surrounding water. In the gills of freshwater fish it is probably the 'chloride' cells which carry out this exchange but it is difficult to envisage such a process in the pseudobranchs of freshwater fish which usually lack 'chloride type' cells and have no contact with the ambient water.

An exchange of external chloride for blood bicarbonate ion is not likely in the 'chloride' cells of seawater fish since there is a net secretion of chloride, with the morelikely exchange of cell bicarbonate ion for blood chloride ion (Maetz 1971; Maetz & Bornancin 1975). The 'chloride type' cells, therefore, <u>are</u> likely to alter the ionic composition of blood flowing through the pseudobranch, but for reasons other than those

suggested by Wittenberg & Haedrich (1974). Thus it does not rule out the possibility that these cells are needed as an integral part of the pseudobranch/choroid rete mirabile relationship in seawater fish. Even so it seems unlikely that the function of the 'chloride type' cells of the pseudobranch is needed solely as a requisite of the choroid rete mirabile since this organ is missing in the seawater Myctophidae which have well developed pseudobranchs. Although Wittenberg and Haedrich (1974) did not note the presence or absence of 'chloride type' cells, they described them as having conspicuous gill-like pseudobranchs which would suggest that they do possess 'chloride type' cells. This would indicate that the 'chloride type' cells have other functions unconcerned with the choroid rete. If in fact the Myctophidae do possess 'chloride type' cells it would be interesting to see if they contain the saccular network found in 'chloride type' cells of other pseudobranchs.

The idea of pseudobranch 'chloride type' cells being necessary for the functioning of the choroid rete mirabile encounters further difficulties when one considers such seawater fish as the pollack and garfish which both possess choroid retes but were found to have buried pseudobranchs without 'chloride type' cells. It seems therefore that the 'chloride type' cell is not the essential functional element of the pseudobranch required for the operation of the choroid rete mirabile although its possible role in ionic regulation of the blood is likely to be of some importance to this organ.

The one element common to all teleost pseudobranchs is the specific 'pseudobranch type' cell so one has to look at the possibility of this cell modifying the blood flowing to the choroid rete mirabile. The position of the 'pseudobranch type' cells in the pseudobranch is ideally situated for such a role since all blood entering the ophthalmic artery has first to pass between these cells. However the presence of a

pseudobranch when the choroid rete is absent (e.g. in Myctophidae) argues against this as the sole function of the 'pseudobranch type' cell. Furthermore, the 'pseudobranch type' cell is not in contact with the surrounding water, so it does not fit in with Wittenberg & Haedrich's (1974) original concept that external chloride is exchanged for internal bicarbonate. They suggested that in 'buried' pseudobranchs, material exchange might occur with the systemic blood of the surrounding tissues rather than the external water. The question then arises as to what material exchange is possible without access to the external water. If Wittenberg & Haedrich's idea of reducing total blood carbon dioxide is to be fulfilled then carbon dioxide and bicarbonate ions would have to be removed by the 'pseudobranch type' cells. A Cl^-/HCO_3^- exchange across the blood barrier does not seem likely since the 'pseudobranch type' cell has no access to a supply of external chloride ions. In the present study no significant amount of chloride was found in the pseudobranch type cells although a small amount of silver chloride reaction product was found at the anti-vascular pole of some cells in the bass. Conceivably this could be due to chloride in the small extracellular spaces between the epithelial and 'pseudobranch type' cells.

Ion transport in many epithelial cells is highly dependent on the enzyme carbonic anhydrase which has a central role in the production of H^+ and HCO_3^- from the dehydration of carbonic acid formed from the hydration of CO_2 (Maetz 1956, 1971). High levels of carbonic anhydrase have been found in the pseudobranch by Leiner (1940), Sobotka & Kann (1941) and Maetz (1956). The histochemical localisation of this enzyme in the pseudobranch has been reported by Vervoort (1958) and Laurent et al (1969). Vervoort found that the enzyme activity was restricted to the granular fraction (mitochondria?) of the acidophil cell cytoplasm and the nuclei of the erthyrocytes. Laurent et al reported localisation of carbonic anhydrase at the vascular pole of the pseudobranch cells. They suggested that the

carbonic anhydrase activity is related to the tubules opening on the vascular side of these cells. The present study supports this view by providing histochemical evidence at the ultrastructural level for localisation of enzyme activity associated with the tubular membranes at the vascular pole of the 'pseudobranch type' cell. Such a location suggests the involvement of carbonic anhydrase in an exchange of materials between the tubules and the blood across the basement membrane.

Another possibility is that carbonic anhydrase is secreted by the 'pseudobranch type' cell. The enzyme has been implicated as having a role in oxygen secretion since it is found in large concentrations in structures involved in oxygen secretion. These include the choroid rete mirabile (Leiner 1939, 1940; Leiner and Leiner 1940; Maetz 1956; Hoffert 1966), the pigment cell layer of the teleost retina (Leiner 1939; Maetz 1956), the swimbladder rete mirabile (Fange, 1950, 1966; Maren 1962) and the gas gland of the swimbladder (Fange 1953). Significantly the pseudobranch of elasmobranchs which have no choroid rete, lacks this enzyme (Leiner 1939). They also lack 'pseudobranch type' cells (Wittenberg & Haedrich 1974) which may explain the absence of this enzyme.

Since the pseudobranch vessels are in series with those of the choroid rete mirabile, any carbonic anhydrase secreted by the pseudobranch could be available for the oxygen concentrating mechanism of the eye. However, Maetz (1956) reported no difference in the concentration of carbonic anhydrase in the afferent and efferent blood going to or from the pseudobranch, and concluded that it does not secrete this enzyme.

Injection of acetazolamide, the specific inhibitor of carbonic anhydrase produces a number of effects including darkening and blinding of the fish (Ballintijn et al 1977), cessation of active secretion of oxygen in the eye (Fairbanks et al 1969; Ballintijn et al 1977), and a decrease in the pH of the vitreous humor of the eye (Maetz 1956). Maetz measured the pH in

the vitreous humor in the retina of the perch (Perca fluviatilis) following injection of acetazolamide and found decreases in pH from 7.6 to 6.9 in an hour, and to 6.6 after 12 hours. Presumably what happens on loss of carbonic anhydrase activity due to the action of the inhibitor, is that tissue and blood PCO_2 increase. This increase, together with the concentrating effect of the choroid rete presumably results in a lowered pH. This drop in pH impairs retinal function and the fish becomes blind. Wittenberg and Haedrich (1974) cited this experiment in support of their hypothesis that the pseudobranch acts in consort with the choroid rete mirabile to create a high oxygen concentration at the retina without simultaneously accumulating an excessive concentration of carbon dioxide. However the drop in pH of the vitreous humor is not necessarily due to inhibition of the pseudobranch carbonic anhydrase since the retinal and choroidal carbonic anhydrases would also be inhibited. In addition the kinetics of gas exchange in the red blood cells (Berg & Steen 1968) would be affected at the gill, pseudobranch and choroid rete. It is not clear whether the factors causing blindness are brought about by oxygen deficiency or pH changes. The influence is reversible initially because recovery occurs in acetazolamide-injected fish (Ballintijn et al 1977). Fairbanks et al (1974) did not attribute the rapid suppression of the ocular oxygen concentrating mechanism to inhibition of pseudobranch carbonic anhydrase. Even though they found the carbonic anhydrase inhibitor CL-11,366 (2-benzenesulfonamido-1, 3, 4 thiodiazole-5-sulfonamide) in the pseudobranch concurrent with early suppression of oxygen concentration, none was detected 10 minutes after injection, and at this time the concentrating mechanism remained suppressed.

No previous attempt has been made to describe ultrastructural changes in the pseudobranch following injection of acetazolamide. Intramuscular injection of 1 - 5 mg/kg ofacetazolamide caused obvious ultrastructural

changes in the 'pseudobranch type' cells of the trout pseudobranch. These changes were present in fish examined after 4 hours, were more pronounced after 24 hours, but had virtually disappeared by 96 hours. This was concurrent with body darkening and blinding of the fish which started within an hour of injecting 5 mg/kg of acetazolamide but disappeared within 72 - 96 hours.

The changes in the 'pseudobranch type' cells probably do not reflect a direct effect of the inhibition of pseudobranch carbonic anhydrase since the inhibitor is likely to have disappeared from the pseudobranch within 10 minutes (Fairbanks & Hoffert 1974). The ultrastructural changes, rounding of mitochondria, appearance of lipid droplets and cavuolation of the cytoplasm are similar to changes following exposure to rapid changes in osmotic environment, or injection of HCl. These effects are most likely due to changes in the interconnecting factors of pH, acid base balance and ionic composition of the blood. Hoffert and Fromm (1973) found that 24 hours after injection of acetazolamide into rainbow trout there was a significant decrease in blood pH (7.47 - 7.12) and a very large increase in pCO_2, (3.8 - 17.6 mm Hg). However there was no significant change in pO_2, oxygen content or the oxygen dissociation curve for whole blood.

As well as inhibiting pseudobranch carbonic anhydrase, acetazolamide would also cause inhibition of erthrocyte and branchial carbonic anhydrase with resultant effects on CO_2 excretion and salt transport at the gills. There is some likelihood that carbonic anhydrase may play, in freshwater adapted salmonids, the central ionoregulatory role which is commonly attributed to the Na^+/K^+ ATPase system under marine conditions (Houston & McCarty 1978). Support for this idea is given by Kersetter and Keeler (1976) who found that ouabain perfusion in situ rainbow trout gill preparations has little effect upon sodium influx. Acetazolamide perfusion, however, significantly reduced the uptake of this ion. This is probably

due to the blocking of carbonic anhydrase catalysed H^+ production with the consequent inhibition of any H^+/Na^+ exchange and failure to convert NH_3 to NH_4^+ prior to NH_4^+/Na^+ exchange. By a similar token, inhibition of carbonic anhydrase catalysed HCO_3^- production might be expected to affect Cl^-/HCO_3^- exchange. However Kersetter and Kirschner (1972) found that acetazolamide has no apparent effect on chloride influx in the rainbow trout gill, and suggested that if a Cl^-/HCO_3^- exchange exists in the trout gill, sufficient HCO_3^- can be supplied by the blood. On the other hand, inhibition of both sodium and chloride uptake by acetazolamide was reported in the goldfish by Maetz (1956). This conflicting data suggests that in the goldfish exchangeable HCO_3^- is produced by carbonic anhydrase mediated hydration of CO_2 entering the gill epithelium from the blood, whilst in the trout the Cl^-/HCO_3^- exchange mechanism is driven by transepithelial diffusion of blood bicarbonate or by a coupled Cl^-/HCO_3^- exchange system located at the nutrient membrane of the 'chloride' cell, neither of which is dependent on carbonic anhydrase.

More recently Haswell & Randall (1976) suggested that branchial carbonic anhydrase may be utilised to convert plasma bicarbonate to CO_2 in the trout gill. The principal pathway for CO_2 excretion has previously been assumed to be via a movement of plasma bicarbonate into the red blood cells where the intracellular carbonic anhydrase rapidly generates CO_2 which then diffuses out following its concentration gradient at the gills. However Haswell & Randall (1976) found that bicarbonate in the plasma of trout cannot be readily converted to CO_2 via the red blood cell due to the presence of a plasma inhibitor. Instead plasma bicarbonate may be excreted by movement into the gill epithelium where carbonic anhydrase could catalyse the production of CO_2 prior to excretion.

None of the functions proposed for branchial carbonic anhydrase seems likely for the 'pseudobranch type' cells since they do not have an external

supply of exchangeable chloride ions, or an apparent pathway for excretion of CO_2. It is possible that a certain amount of CO_2 can diffuse across the covering layer of epithelial cells in 'free' and 'semi-free' pseudobranchs although the average length of the water-blood barrier (12 - 14μ) is considerably greater than in the gills of most teleosts (Hughes & Morgan 1973). Furthermore, diffusion of ions or gases is highly unlikely in covered or buried pseudobranchs because of the far greater diffusion distance involved.

Laurent et al (1968) suggested a function for carbonic anhydrase related to the possible chemoreceptor role of the pseudobranch. They also demonstrated carbonic anhydrase activity in the chemoreceptor areas of rabbit carotid body and frog carotid labyrinth, and suggested that this enzyme may be necessary in systems monitoring blood pH and/or carbon dioxide. Laurent (1969) demonstrated changes in membrane potential with changes in the pH of perfusing solution and suggested that this was consistent with ionic exchanges between the pseudobranch type cells and extracellular spaces where afferent nerve endings were located. He found that reduction of pH lowered the membrane potential and led to a redistribution of K^+ (a possible activating agent of afferent fibres). Laurent & Rouzeau (1972) found that inhibition of carbonic anhydrase, in the presence or absence of exogenous CO_2, caused submaximal stimulation of receptors accompanied by desensitization of the receptors to changes in pH (H^+ ions). They suggested that this was due to intracellular accumulation of H^+ ions which are interchangeable with K^+ and consequently affects the redistribution of this ion between the intra and extracellular milieu. In the present study inhibition of carbon anhydrase activity caused a small but significant increase in nervous activity which was raised slightly by lowering the pH from 7.8 to 7.0. However this was not significant and agrees with Laurent & Rouzeau's (1972) findings of a densensitization to H^+ ions.

Laurent (1974) suggested that an ionic pump is the most likely mechanism to explain the movement of ions across the membrane. In support of this, cytochemical (Laurent et al 1968) and biochemical evidence (Bonnet et al 1973) has been produced to show a very large Na^+/K^+ ATPase activity of a particular type in this membrane. The present study adds further support for this suggestion with the subcellular localisation of ATPase activity at the anti-vascular membrane of the 'pseudobranch type' cell. However due to the ouabain insensitivity of the reaction used (Wachstein-Meisel Pb capture) its interpretation as Na^+/K^+ ATPase is open to dispute. There have been many arguments on the validity of the Wachstein-Meisel method for histochemical demonstration of ATPase activity (Rosenthal, Moses, Beaver and Schuffman, 1966; Moses, Rosenthal, Beaver and Schuffman 1966; Moses and Rosenthal 1967; Novikoff 1967; Hori 1968). The lead ions in the incubation medium cause non-enzymatic hydrolysis of nucleoside phosphates and it results in the production of lead phosphate crystals on plasma membranes (Rosenthal et al 1966). To try and prevent this an incubation medium containing ATP concentrations 2.5 times that of lead was used (after Shirai 1972). Another disadvantage is that fixation of tissues with various aldehydes and the high concentration of lead ions markedly inhibits total ATPase activity, especially Na^+/K^+ ATPase (Moses et al 1966; Marchesi & Palade 1967). Recently ouabain sensitive Na^+/K^+ ATPase activity has been demonstrated in the tubular system of the gill (Hootman & Philpott 1979), using the p-Nitrophenyl phosphate, Strontium captive method of Ernst (1972 a & b). This method appears to provide increased resolution and specificity of reaction product through manifestation of Na^+/K^+ ATPase as K^+ NPPase. Clearly this method would be of use for localization of this enzyme in the pseudobranch.

ATPase activity has been found associated with the cell membrane of the carotid body parenchymal cells (Nada & Ulano 1972) as well as in other receptors (Kuijpers, van der Vleuten & Bonting 1967; Smith, Stell, Brown Freeman & Murray 1968; Iwayama 1969). Iwayama (1969) assumed that the ATPase in the gustatory regions was implicated in the active transport of

cations across cell membranes. Nada & Ulano (1972) suggested that ATPase in the carotid body may participate in the active transport of catecholamines across cell membranes. They cited evidence for uptake of the catecholamines dl- noradrenaline and b-hydroxydopamine by carotid body parenchymal cells and suggested this was similar to uptake of these materials by adrenergic nerve terminals using a membrane transport mechanism (Malmfors 1967; Tranzer and Thoenen 1968). Such a mechanism does not seem likely in 'pseudobranch type' cells which do not contain storage granules typical of catecholamine containing cells.

Krylov and Anichkov (1968) recorded an increase in the electrical activity of the sinus nerve of carotid body through perfusion of ATP with Ringer solution, and suggested the direct participation of ATP in the process of stimulation. Thus any metabolic changes affecting the synthesis of ATP would be likely to affect the receptors. The effects of 2-4 dinitrophenol on the nervous activity of perfused trout pseudobranch are interesting in this respect. The drug has a diphasic affect, stimulating between 10^{-9} and 10^{-7}M, and stimulating then inhibiting between 10^{-6} and 10^{-4}M. The action of 2-4 dinitrophenol by so called uncoupling of oxidative phorphorylation is probably linked to the inhibition of ATP synthesis. The stimulating effects of this drug at low concentrations may be associated with a reduction in the synthesis of ATP necessary for the proposed Na^+/K^+ ATPase ionic pump, and a consequent slowing down of the pump. Complete blocking of ATP synthesis by high concentrations of the drug eventually results in disappearance of nervous activity. Thus it follows that metabolic changes within the 'pseudobranch type' cells may well serve to modify the responses of associated receptors to blood borne stimuli such as pH, pCO_2, pO_2 and ion content.

The high metabolic activity of the 'pseudobranch type' cells is reflected by the high levels of succinic dehydrogenase in these cells.

The staining reaction for succinic dehydrogenase was even more intense in the 'pseudobranch type' cell than the 'chloride type' cell. The reaction was localised in the central and basal regions of the cell, corresponding to the position of the abundant mitochondria. Other evidence of high metabolic activity is provided by Hoffert and Fromm (1970) who found that glucose utilisation by the trout pseudobranch is comparable to that of retinal tissue, which is considered one of the most metabolically active tissues in the body. In contrast to the retina the pseudobranch showed a much lower production of lactic acid and a much higher percentage of glucose oxidised through the tricarboxylic and (T.C.A.) cycle. The oxidative T.C.A. cycle would be expected to operate in the pseudobranch since its blood supply comes directly from the first gill arch, and therefore contains a maximum amount of oxygen. The pseudobranch was also found to have about 100% greater metabolic activity than gill tissue. Comparison of the glucose/lactate ratios for gill and pseudobranch indicates that much more glucose is metabolized via the Embden-Mehyerof (E.M.) pathway in the gill. The appearance of abundant glycogen and lipid droplets in the 'pseudobranch type' cells during osmotic stress could be due to disturbance of normal carbohydrate metabolism, with glucose being converted to glycogen and fat instead of providing energy for the normal metabolic activity of these cells.

Although it is obvious that the 'pseudobranch type' cell is a highly active cell which may be involved in a number of functions it is clear that an unequivocal explanation of these is far from being resolved. The problem is further complicated when one considers the large vacuole-like structures found in some of these cells. These structures have not been reported previously in 'pseudobranch type' cells which raises the possibility that they are artefacts. However a number of observations argue against this. Preservation of other cellular components was generally very good,

with no swelling or contraction of membranes, and apparently normal mitochondria which are usually a good indicator of any cell damage. Also vacuoles were not found in other cell types. Furthermore the vacuoles were enclosed by a definite membrane with a closely associated dense, fibrous cytoplasmic material and bundles of cytoplasmic filaments. In epithelial cells, intracellular filaments are generally thought of as cytoskeletal elements providing the cell with rigidity, as well as tensile strength and resilience. While this may provide one explanation for their presence in the 'pseudobranch type' cell, it is also possible that they have contractile properties similar to the myofilaments of muscle cells. This possibility raises interesting questions in regard to their close association with the vacuoles of 'pseudobranch type' cells. Are these vacuoles capable of expanding and contracting and if so do the filaments have any control over the amount and/or speed of contraction, or do they merely serve as supporting structures for changes in shape of the cell? The contents of the vacuoles are unknown and appear clear under the electron microscope apart from a few small, clear vesicles. It is possible that the vacuoles respond to changes in the osmotic pressure of the blood by swelling or contracting due to the addition or removal of water from the cell. A decrease in osmotic pressure would promote uptake of water by the 'pseudobranch type' cell with consequent swelling of the cell. There are no apparent continuities between the vacuoles and the tubular system or endoplasmic reticulum so any water movement in or out of the vacuole would most likely be due to differences in osmotic pressure between the vacuole and cell cytoplasm. Such a mechanism may provide a role for these cells in osmoreception. Afferent nerve activity increased with decreasing osmotic pressure of perfusing solutions below the normal considered value of 270 mOsm., but was little affected by increasing osmotic pressure above this value. Nerve endings near the 'pseudobranch type' cell may act as pressure receptors which are affected by the swelling of

these cells at low osmotic pressure. No obvious receptor structures were found in association with 'pseudobranch type' cells so it is likely that such receptors would be free nerve endings of fibres running in the interstitial tissue between the 'pseudobranch type' cells. The interstitial tissue is extremely fibrous in nature so the expansion or contraction of this tissue in relation to contraction or swelling of the 'pseudobranch type' cells may provide the necessary pressure stimulus for these receptors. This functional concept of pseudobranch osmoreceptors is similar to that proposed for mammalian osmoreceptors by Verney (1947). He suggested that large vesicles in the supraoptic nucleus of the neurohypophysis act as tiny osmometers, to the surface of which are attached dendrites of the supraoptic cell bodies. The attached endings may act as stretch receptors which respond to changes in the osmotic pressure of the highly vascular environment, and transmit messages to the secretory endings of the supraoptico-hypophysial tracts.

Laurent and Rouzeau (1972) thought that pseudobranch osmoreceptors might act in a different way. They suggested that a decrease in osmotic pressure might act by decreasing the activity of Na^+/K^+ ATPase by a similar method proposed for the kidney (Alexander & Lee 1970). This could affect the distribution of K^+ between the intra and extracellular milieu with a consequent reduction of the membrane potential of the 'pseudobranch type' cells. The stimulating effects of K^+ on the interlamellar receptors would be similar to the effects caused by pH changes and hypoxia (Laurent & Rouzeau 1972). They proposed the presence of two types of interlamellar receptor (B_1 and B_2). They considered that sensitivity to pO_2, pH and perhaps pCO_2 were linked to the same type of receptor (B_1 receptor) since saturation of nervous activity is achieved when one combines several of these stimuli. However a similar conclusion concerning the sensitivity to osmotic pressure (B_2) is not evident since combination with a hypoxic stimulus lowers the

absolute value of the nervous activity such that a combined highly hypotonic and hypoxic solution produces activity not much higher than under normoxic conditions. The latter observation argues against osmotic pressure affecting the Na^+/K^+ ATPase ionic pump in a similar way to pH and pO_2 since a combination of stimuli would be expected to cause maximum saturation of nervous activity. In fact it appears that the pO_2 and osmotic pressure stimuli interfere at some point in the transduction process indicating that the response to one stimulus is dependent on correct maintenance of the other.

Despite the fact that morphological evidence of neural receptors which could intervene in osmoregulatory mechanisms is unknown in fish, the evidence suggests that such structures do exist. Freshwater teleosts must maintain their internal concentration (250 - 300 m Osm) despite a very dilute external enviornment (a few m. Osm) so activation of any alarm signal would be expected to operate when the internal enviornment becomes hypo-osmotic. The situation is reversed in saltwater teleosts, where the internal environment differs little from that of freshwater fish, although they live in a very hyper-osmotic environment (1000 m Osm). It seems possible that the signal for alarm is activated in this case when the internal environment tends to become hyperosmotic. Laurent & Rouzeau (1972) suggested that the trout may possess both systems of alarm since it may be considered as a euryhaline species. They reported that survival of trout, normally indefinite in seawater (dilution by one half), does not exceed several hours after bilateral sectioning of the pseudobranch nerves, thus supporting the idea of a functional relationship between the pseudobranch and osmotic regulation. However in the present study there was no mortality or even apparent distress of rainbow trout placed in diluted seawater after sectioning of the pseudobranchial nerves. There is no obvious explanation for these conflicting results and one can only suggest that it is due to variations in the internal environment of the fish with age or different stages of the breeding cycle.

Any future work should therefore take into account the age and sexual maturity, stage in the breeding cycle, and possibly even the sex of the fish.

Apart from the 'pseudobranch type' cell and 'chloride type' cell the rodlet cell has also been implicated in an osmoregulatory role. These cells have been found in many species of freshwater and seawater fish at many sites throughout the body, although they tend to be most numerous in the epithelium or endothelium of sites suited for secretion into the blood, body cavities or external milieu (Bullock 1963; Leino 1974; Morrison & Odense 1978). The presence of large numbers in the gill, kidney and intestinal epithelia may well imply an osmoregulatory function. Fearnhead & Fabian (1971) suggested that rodlet cells were concerned with osmoregulation from observations that their numbers were substantially reduced in the gills of Monodactylus argenteus adapted to fresh water, compared with those adapted to seawater. In the present study although rodlet cells were normally abundant they could not be identified in the pseudobranch of the bass after 24 hours exposure to seawater diluted 1 : 100 with freshwater. Even after 5 hours exposure only tentative identification of rodlet cells could be made since no cells containing rodlets were found, although a number of cells with contracted fibrous borders were present. These results have to be treated with caution because of the notorious variability in the numbers of cells from fish to fish (Bannister 1966; Leino 1974). However large numbers of rodlet cells (mean 2974 per sq mm) were found in the pseudobranch epithelium of all bass from normal seawater, so it seems likely that a change in external osmotic environment does cause a reduction in the numbers of rodlet cells, although it need not be associated with osmoregulation and may be a stress reaction. Furthermore there has been a certain amount of controversy as to whether these cells are in fact fish tissue cells. They were first described by Thélohan (1892) who considered them to be parasitic in nature. Laguesse (1906) named them Rhabdospora thelohani, believing them to be sporocyst stages of a sporozoan parasite. The view that these

cells are parasites is also held by Laibach (1937); Dawe, Stanton & Schwarz (1964); Hale (1965); Bannister (1966); Iwai (1968); Mourier (1970) and Anderson, Roberts, MacKenzie & McVicar (1976). However, more recent studies suggest that they are not protozoan parasites but are integral constituents of the tissue in which they are found, and that they may have a secretory function (Leino 1972, 1974, 1979; Desser & Lester 1975; Morrison & Odense 1978). The possible glandular nature of the cell is supported by Plehn (1906; Klust (1939); Barrington (1957); Bullock (1963); Bishop & Odense (1966) and Wilson & Westerman (1967). Duthie (1939) believed them to be a type of granular leucocyte , a possibility also proposed by Weinreb & Bilstad (1955); Catton (1957) and Gohar & Latif (1961). Flood, Nigrelli & Gennaro (1975) considered the cell to behave as a foreign body which interacts with the epithelial tissue of the bulbus asteriosus. Al-Hussaini (1949) and Vickers (1962) suggested that rodlet cells may be modified or abnormal goblet cells.

Although the sites and numbers of rodlet cells vary between and within species, often according to season, crowding or ionic concentration of the water (Leino 1974), the apparent lack of host and tissue specificity indicates that the cell is not a parasite. However many observations also appear to be inconsistent with patterns of distribution of normal tissue cells.

Flood et al (1974) described four cell forms which appear to be phases in the life cycle of rodlet cells. The first two phases are similar to the immature rodlet cells described here in that the cells are not encased by a fibrous wall and are characterised by their inclusions either crystalline or amorphous and granular. The next stage described by Flood et al, is the semi-encased stage, also observed by Leino (1974); Desser & Lester (1975) and Barber, Westermann and Jensen (1979), which corresponds to the maturing cell reported here. However, as far as is known the actual development of

the encased stage from the pre-encased stage has not been previously described. This phase provides strong additional evidence that the mature rodlet cell with its fibrous cytoplasmic border does develop within the fish tissue from a specific cell type without a fibrous border.

The appearance of the mature cell may superficially resemble that of a parasite but as Leino (1974) and Desser & Lester (1975) noted, the fibrous cytoplasmic border is clearly not a cyst wall and is part of the cytoplasm, with no membrane separation. Leino (1974) has also shown the presence of desmosomes between rodlet cells and adjacent epithelial cells, a finding confirmed in this study. Desser & Lester (1975) observed tight junctions between the apex of rodlet cells and adjacent epithelial cells. In agreement with Leino (1974) and Desser & Lester (1975), no pathological changes were noted in epithelial cells closely associated with rodlet cells.

The theory that the rodlet cell has a secretory function seems most likely on the basis of its structure and position in most fish tissues. Mature cells are usually found near the surface, in the epidermis or endodermis; the rodlets have a characteristic orientation toward the apex of the cell and the fibrous border may be capable of contractile activity. During the process of maturation the rodlet cell contains a prominent Golgi complex which is typical of certain secretory cells e.g. mucous cells. It is especially large and prominent in cells secreting either protein or complex carbohydrate (Rhodin 1974). The close relationship between the granular endoplasmic reticulum and the Golgi complex in immature rodlet cells provides a clue to the formation of rodlet sacs. The Golgi complex may act as the assembly area for proteins produced on the ribosomes and channelled by the granular endoplasmic reticulum to the Golgi region where they are added to carbohydrate synthesised by the Golgi complex to produce glycoprotein, which is concentrated and packaged for distribution outside the cell.

Morrison & Odense (1978) on the basis of their own histochemical tests and those of previous workers, suggested that the cell product is some form of neutral glycoprotein. The results of this study support that theory since rodlets were stained with the P.A.S. test to show the presence of complex carbohydrate but did not stain with alcian blue indicating that it is not acidic.

At the ultrastructural level the rodlet sacs were densely stained by phosphotungstic acid in chromic acid at pH 0.3 after treatment with periodic acid. This has been reported as a selective stain for glycoprotein Tsuchiya & Ogawa 1973) and for sialic acid (Rambourg 1967; Rambourg, Hernandez & Leblond 1969). It is interesting to note that the central cores of the rodlet sacs were unstained, indicating the absence of glycoprotein. Leino (1979) found that the central core was virtually unstained using the periodic acid-silver methenamine technique whereas the peripheral material was moderately stained. Also, the central core was completely removed by pepsin and trypsin digestion while the rest of the sac was unaffected. Leino (1979) suggested that the rodlet cell granules are composed of two different substances (1)a protease-digestable, silver methenamine negative core, perhaps of quite pure protein (2) a protease resistant, silver methenamine positive periphery, perhaps rich in glycoprotein. The function of the central core remains uncertain but it may be to maintain the shape and rigidity of the rodlet sacs enabling easy expulsion from the cell.

It is still not clear what triggers secretion of the cell. Leino (1974) showed rodlet cells secreting their contents with apparent contraction of the cell and fibrous border. It is possible that mechanical damage incurred in fixing and preparing the tissues may squeeze out the cell contents. However the presence of microvilli-like cytoplasmic projections may indicate the trigger mechanism by which the contents are released due to mechanical

stimulation. Alternatively changes in osmotic pressure may cause secretion of the rodlet sacs or even release of the whole cell as indicated by the virtual absence of rodlet cells in bass kept in diluted seawater.

The presence of immature, mature and maturing rodlet cells together indicates a continuous development of rodlet cells. As Leino (1974) suggested they may have functions similar or complementary to mucous cells whose secretions may be involved in pH control, lubrication and antibiotic effects. It is possible that rodlet cells produce a necessary constituent of the glycocalyx, which contains mainly highly anionic sugars (Threadgold 1976). Sialic acid is a common constituent often linked to glycoprotein or lipid. Because the glycocalyx is polyanionic it has a strong net negative charge and attracts physiologically important cations such as Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}. The secretory product of rodlet cells may help maintain the net negative charge of the glycocalyx and may be released in response to a change in the external ion concentration.

It was surprising that the number of possible rodlet cell openings found using S.E.M. was small considering the large number of these cells seen using the T.E.M. It is possible that most of the rodlet cells are not open to the external environment but only do so to fulfil their special function.

Rodlet cells provide an interesting comparison with mucous cells in the production, packaging and release of secretory products. It seems likely that the presence of large numbers of rodlet cells in the bass gill and pseudobranch epithelium is indicative of a function which may be as necessary to the fish as mucus production by mucous cells.

The role of specific cell types in osmoregulation may be linked to osmoregulatory mechanisms in the pseudobranch vasculature. Vogel, W., Vogel, V., & Schlote (1974) suggested that arterio-venous anastomoses (A.V.A's) of the gill may function in part as intravascular osmoreceptors.

These structures have not been described previously in the pseudobranch but their discovery in the bass pseudobranch and their similarity to those of the gill may help to elucidate their function. The A.V.A's of the bass pseudobranch provide a significant arterio-venous pathway between the efferent filament artery and the central venous sinus (C.V.S.) which may have more than a purely nutritive function. A nutritive arterio-venous pathway is provided by small vessels from the primary afferent artery which supply the tissues of the pseudobranch arch and filaments before being collected in small veins which drain into the C.V.S. and pseudobranchial vein. The pseudobranch differs from the gill in this respect since the nutritive vessels of the gill are supplied by the efferent system. This organisation of the nutritive system seems logical since the efferent blood of the gill is oxygenated and supplies the afferent blood of the pseudobranch. This also argues against the A.V.A. pathway of the pseudobranch having a purely nutritive function since blood in the efferent filament artery is likely to be slightly deoxygenated after passing through the secondary lamellae of the pseudobranch. On the other hand this blood is likely to show a changed osmolality (and ion content) after passage between the 'chloride type' and 'pseudobranch type' cells. The presence of A.V.A's in the bass pseudobranch but apparent absence in the trout pseudobranch may be due to the closer similarity of the former to a true gill, and may be linked to the presence of 'chloride type' cells in this type of pseudobranch. This might provide the link between the A.V.A. pathway and an osmoregulatory role.

Vogel, Vogel & Schlote (1974) suggested that the blood supply to the C.V.S. of the gill is regulated by the A.V.A's probably according to changes in blood composition and/or blood tonicity recognised by A.V.A. cells. Afferent axons may transfer relevant information from this vascular area to the central nervous system while efferent nerve impulses may modulate A.V.A.

reactions. A possible chemoreceptor function of the A.V.A's has also to be kept in mind. Though it still remains to be clarified whether the closely associated nerves are of afferent or efferent nature, it seems reasonable to assume that they establish an important connection between the microvasculature and the central nervous system.

Partitioning of sodium and chloride influxes between a lamellar and sinusal route has been shown in the gills of seawater adapted trout (Girard & Payan 1977). This confirms the current view of the gill circulation whereby all afferent blood from the heart traverses the secondary lamellae and is partitioned between two efferent routes with arterial and venous exits (Morgan & Tovell 1973; Vogel, Vogel & Kremers 1973; Vogel, Vogel & Schlote 1974; Vogel, Vogel & Pfautsch 1976; Girard & Payan 1976; Laurent & Dunel 1976; Holbert, Boland and Olson 1979). This separation is brought about by the anastomoses between the efferent filament artery and the central compartment of the filament. Whereas the arterial route distributes the blood towards the systemic system, the venous system consists of a direct route to the heart.

In a recent review, Hughes (1980) indicated, on the basis of morphometric observations, that the blood passing through the secondary lamellae of tench and trout is richer in red blood cells than might have been expected. He suggested the existence of mechanisms whereby the plasma may be skimmed off and passes into the C.V.S. via some arterio-venous pathway. The existance of small numbers of A.V.A's on the afferent side of the gill circulation has been shown in Tilapia, rainbow trout and eel (Vogel et al, 1973; Vogel et al, 1974; Vogel et al, 1976; Vogel 1978 a, b; Laurent & Dunel 1976), and may provide the morphological basis for such a pathway. The openings of the anastomoses usually appear very narrow and are often guarded by fine hair-like structures which suggest that it might be difficult for red blood cells to pass. The presence of microvilli-like extensions on

the endothelial cells of the pseudobranch A.V.A's may reflect a similar situation. As to the possible functional significance of such differences in circulatory paths for plasma and red cells, Hughes (1980) tentatively suggested that the osmoregulatory functions may be more concerned with the plasma, whereas the respiratory function is more concerned with the red cells. In the gill, this hypothesis is supported by the position of the red cell route (lamellar) close to the water, and the plasma pathways near to the 'chloride' cells. However in the pseudobranch, the 'chloride type' cells are more closely associated with the lamellar route which is further from the water than in the gill and is less likely to be involved in respiratory exchange. Thus there may be no need to skim off the plasma before blood enters the pseudobranch secondary lamellae. An interesting point here, is the difference between the lining of the secondary lamellae blood channels in the gill and the pseudobranch. In the gill the flanges of the pillar cells form an almost continuous layer beneath the basement membrane whereas in the pseudobranch the pillar cell flanges often take the form of a thin fenestrated endothelium which may allow easier diffusion of certain materials from the blood. It is uncertain if these fenestrations belong to a true endothelium or whether the pillar cell flanges have become modified in this form. It is tempting to suggest that these fenestrations form an important pathway for plasma components in the lamellar blood of the pseudobranch which are not found in that of the gill. This may explain the failure to find any A.V.A's on the afferent side of the pseudobranch circulation since separation of red blood cells and plasma would not be desired before passage through the secondary lamellae. It is possible that after passage through the secondary lamellae, plasma is skimmed off by the efferent A.V.A's and passes into the C.V.S. where its osmotic properties are monitored by sensory endings situated therein. Although no conclusive proof of receptors in the C.V.S. has been provided there is

a large nerve fibre population in the connective tissue and it is quite likely that any receptors would be free endings with no obvious specialisation.

The above hypothesis may be reasonable for the bass pseudobranch but is less likely for the trout pseudobranch since no A.V.A's could be found in this organ (although this does not necessarily deny their existence). However there may be no need to separate red blood cells and plasma in the trout pseudobranch which lacks 'chloride type' cells and may not have a direct osmoregulatory function. If in fact separation of these components does occur in the trout pseudobranch, it might be via the alternative method suggested for the gill by Hughes (1980); i.e. plasma passes directly through the walls of the afferent filament arteries and arterioles into the intra-epithelial lymphoid spaces. Although there is no direct evidence for such a pathway, it might be of considerable importance in the trout pseudobranch which has large numbers of nerve fibres associated with extracellular spaces in the secondary lamellae.

Clearly some interesting possibilities are available for separation of blood components in the pseudobranch as well as in the gill, but more detailed morphometric studies on the pseudobranch are needed before any conclusions can be drawn.

There seems to be some confusion in the literature on gill circulation as to what constitutes a venous or a lymphatic system (c.f. Steen & Kruysse 1964; Morgan & Tovell 1973; Laurent & Dunel 1976). The large sinus-like vessel found in the pseudobranch arch appears similar to the branchial vein described for the gills of teleosts by Laurent and Dunel (1976). Also it connects with the central venous sinus (C.V.S.) which the latter considered as part of the arterio-venous system, although Steen & Kruysse (1964) and Morgan & Tovell (1973) described it as part of the lymphatic system. A point in favour of the latter view is the virtual absence of red blood cells within the lumen of the vessel. However the

release of catecholamines by fish upon being captured (Nakano & Tomlinson 1967) and the subsequent dilation of the arterio-arterial route may well explain why little blood was found in the venous sinus. Furthermore Payan & Girard (1977) showed that adrenalin caused a decrease in venous flow probably due to closure of the A.V.A's. This was caused by an α-constrictor effect on adrenoreceptors in this region. These mechanisms may explain why anastomoses were often observed tightly closed and why in some microfil preparations the C.V.S. and pseudobranchial vein were poorly filled.

The control for the opening of the A.V.A's is uncertain although it has been suggested that they are under cholinergic influence (Vogel, Vogel and Kremers 1973). Increased blood flow through the central compartment of the gill is thought to be caused by the vasoconstrictor effect of acetylcholine on sphincters at the base of the efferent filament arteries (Dunel & Laurent 1977; Smith 1977). The anastomoses must be open in this case but whether it is due to a dilatory effect of acetycholine is unknown. At the present time no sympathetic cholinergic vasodilator effects are known in the systemic vasculature of teleosts and all previous observations show only a constrictory response to acetylcholine (Reite 1969; Stray-Pedersen 1970; Nilsson 1972; Holmgren & Nilsson 1974; Smith 1977; Wood 1977). Since acetylcholine is only likely to be released at neuroeffector end organs and is unlikely to circulate in the blood stream (Wood 1975) it is important to find out if any cholinergic nerve terminals are associated with the A.V.A's. Although varicosities and naked asons were often closely associated with cover cells, and endothelial cell processes in the bass pseudobranch, no definite endings or synaptic contacts were found.

In the absence of any cholinergic influence the only control for the opening of A.V.A's may be the removal of the constrictory effect produced by circulating catecholamines. Thus, they may open when the catecholamine

concentration drops to a normal level and remain open until further release of catecholamines into the blood stream, with the consequent affect on α-adrenoreceptors. In the light of current theories on adrenergic and cholinergic control in teleost gills, this seems the most likely mechanism.

Pettersson & Nilsson (1979) suggested that the general effects caused by circulating vaso-active substances in the gill may be reinforced by an autonomic innervation with its direct, rapid and restricted effects. They found that the branchial vascular bed in the cod was controlled by both adrenergic sympathetic (dilatory and sometimes constrictory) and cholinergic parasympathetic (constrictory) fibres, but as yet the site of action of these nerves on the various effectors of the gill vasculature is unknown. In view of the α-constrictory effect of adrenalin on the A.V.A's of trout (Payan & Girard 1977) and eel (Dunel & Laurent 1977) it is possible that these structures are also controlled by adrenergic sympathetic fibres as well as circulating catecholamines. The structure of the specific endothelial cells (Type I and Type II) is interesting in this respect, since similar cells in mammalian arterio-venous anastomoses, as well as those in renal arterioles are now generally considered to be modified smooth muscle cells (Straubesand 1953, 1968; Clara 1956; Meyer 1972).

In general, enlarged endothelial cells are reported to be particularly rich in cell organelles. This, along with their epitheloid shape indicates specialised functions which may be related to regulation of either blood flow or transvascular exchange processes (Rhodin 1967) or even synthesis of extracellular material (Jorgensen and Claeson 1972; Nikolov & Schiebler 1973).

The type I endothelial cells on the arterial side of the A.V.A. often bulge into the arterial lumen and possess microvilli-like extensions which promote an increase in contact between the blood and endothelial cell surface. Thus these cells might analyse the passing blood and may cause

adjustments in the luminal diameter of the anastomosis. However a secretory response of the cells cannot be entirely excluded. Small numbers of specific endothelial granules were found in these cells, although these granules were far more abundant in the normal endothelial cells of the afferent and efferent filament arteries. They were also common in endothelial cells at the openings of the lamellar arterioles but were absent from any cells in the secondary lamellae. Similar granules have been described in endothelial cells of the gill filament arteries (Hughes & Wright 1970; Morgan & Tovell 1973; Vogel, Vogel & Schlote 1974), as well as in endothelia of the rat, man and other vertebrates (Wiebel & Palade 1964; Santolaya & Bertini 1970; Steinsiepe & Wiebel 1970). It has been suggested that the granules contain a pro-coagulative substance which becomes discharged into the vascular lumen in response to high plasma concentrations of adrenalin (Rhodin 1974).

The possibility that A.V.A. cells may be concerned with specific metabolic functions should also be considered. In mammals, metabolism of vasoactive peptides is mainly localized in endothelia of vessels derived, at least partly, from the gill arches (Aiken & Vane 1970; Ryan & Smith 1973).

A,V.A's in the bass pseudobranch were only found in the efferent filament artery although this does not rule out the possibility of small numbers in the afferent filament artery. Similar results have been found in the gills of Salmo gairdneri and Perca fluviatilis (Vogel et al 1976; Laurent & Dunel 1976) although small numbers of afferent A.V.A's have been found in Tilapia mossambica (Vogel et al 1973; Vogel et al 1974), Anguilla anguilla (Laurent & Dunel 1976) and Ictalurus punctatus (Olson, Holbert & Boland 1978). The original concept of non-respiratory interaterial blood shunting in gill filaments (Steen & Kruysse 1964) is seriously questioned by present structural and functional evidence. Even limited afferent-efferent blood shunting is highly improbable, as it would imply a higher blood pressure in the C.V.S. than in the efferent filament artery. As the C.V.S. opens into

veins it is not clear how this could be established. Therefore, blood must be assumed to flow from the arteries, mainly efferent, to the C.V.S.

Apart from allowing a partition of blood diverted from the main filament circulation, Laurent & Dunel (1976) suggested that the C.V.S. may also have a function analagous to the cavernous tissue in the Elasmobranch gill where the corpus cavernosum supplies additional support to the filaments (Wright 1973). It is worth noting that the C.V.S. of the bass pseudobranch was larger than that of the trout, perch or carp while in the latter cases large venous sinuses were found outside the core of the filament particularly around the afferent filament arteries. The filaments of the bass pseudobranch are 'free' so therefore may be in need of greater support than those in covered or buried pseudobranchs. The large extrafilamental sinuses found in covered and buried pseudobranchs, particularly in the carp, imply a capacity for large blood flow or volume. Thus they may provide a means whereby large volumes of blood can be diverted out of the pseudobranch circulation. One can only surmise that such a system is necessary to maintain ionic or osmotic balance, or reduce blood pressure or blood flow to the ophthalmic artery. However no arterio-venous anastomoses have been found in covered or buried pseudobranchs although such structures would be expected to exist if blood was to be diverted rapidly to the venous sinuses. It is possible that only very few A.V.A's are present or they may take a different course to those in the bass pseudobranch e.g. they may not lead directly to the C.V.S. but open into nutritive vessels, or they may be located in the lamellar arteries rather than filament arteries. Serial sectioning of pseudobranch filaments over a considerable distance may be necessary to answer these questions.

The C.V.S. and its extensions may also be important in the collection of material drained from the extracellular spaces surrounding 'chloride type' and/or 'pseudobranch type' cells. A functional relationship

between the 'chloride' cells and the C.V.S. of the gill has been proposed by Laurent & Dunel (1978) who noted connections between the venous system and the extracellular spaces around 'chloride' cells. This might provide an alternative route for intrafilamental ion exchange independent of the lamellar blood circulation. Such a route is very short and may be important during rapid changes in the osmotic environment.

The pattern of blood flow within the filaments and secondary lamellae of the gill can be influenced by many nervous and hormonal factors (Rankin & Maetz 1971; Wood 1974; Payan & Girard 1977), with important consequences on both gaseous exchange and osmoregulatory mechanisms. To date no information is available on patterns of blood flow within the pseudobranch although factors affecting gill circulation may well have similar effects in the pseudobranch. Also variations in the distribution of blood within the gill may affect the ionic composition, gas composition and flow rate of blood to the pseudobranch.

A number of mechanisms have been proposed whereby blood could perfuse different regions of the gill vasculature. As already mentioned the hypothesis of a direct non respiratory blood pathway from the afferent to the efferent filament arteries via the C.V.S. has not been confirmed in any teleost. Another theory is that there is recruitment of blood flow through an increasing number of secondary lamellar as a function of the respiratory needs (Davis 1972; Cameron 1974; Booth 1978; Morgan & Tovell 1973). Recruitment of additional secondary lamellae to act as respiratory units could be passive, resulting from increased pressure on the afferent side of the system; or active, by a relaxation of tone induced by neural and/or humoral factors (Morgan & Tovell 1973). According to Hughes (1972) and Hughes & Morgan (1973), lamellae near the basal portions of the filament are continually ventilated, whereas additional peripheral lamellae are recruited for gas exchange as required by metabolic demands. A further

consideration is provided by the differences in the size of individual secondary lamellae, not only along the length of a given filament but also in filaments from different parts of a gill arch (Hughes 1972). Likewise the frequency of secondary lamellae is variable and consequently the gas exchange area contained in a unit portion of the gill system is not constant. Lamellar recruitment in the pseudobranch is likely to have little or no functional significance as regards gaseous exchange, although it may have effects on osmoregulation by altering the proportion of 'chloride type' and/or 'pseudobranch type' cells in contact with the circulating blood.

As in the gill the distribution of arteries of different diameter in the pseudobranch is well suited to a system of selective recruitment of blood vessels. The primary afferent artery and filament arteries at the centre of the pseudobranch arch are of greater diameter than those supplying other parts of the arch. Also there is a progressive reduction in diameter of the filament arteries distally towards the filament tips, and of the lamellar arteries as they arise consecutively from the filament arteries. All these vessels have muscular walls and are therefore capable of independently regulating blood flow and distribution. The walls of the primary and filament efferent arteries are usually thinner than those of the corresponding afferent arteries and may reflect a reduced ability (or necessity) to control blood flow.

An additional mechanism in the control of blood flow may be provided by the contraction of pillar cells (Newstead 1967; Bettex-Galland & Hughes 1973; Hughes & Weibel 1972). Contraction of these cells would cause vaso-constriction of the central lamellar channels whilst leaving the peripheral circulation route (via the marginal channel) open. The structure of pillar cells in the pseudobranch is essentially the same as that described previously in the gills of teleosts (Rhodin 1964; Hughes & Grimstone 1965; Hughes & Wright 1970; Morgan & Tovell 1973), so would presumably have the

same contractile properties. The structure of the marginal channel is distinct from the other lamellar channels since its outer border is lined by true endothelial cells rather than pillar cells. Laurent & Dunel (1976) could find no evidence for preferential blood flow through the marginal channel of the gills since casting of this channel alone was never observed in microfil preparations. However in the present study, casting of the marginal vessels of the bass pseudobranch were consistently observed, although no such results were obtained in rainbow trout. The reason for this is uncertain but it may be related to differences in species, habitat, or reaction to stress. Bypassing most of the area of the secondary lamellae in the bass pseudobranch would exclude a significant proportion of 'chloride type' cells, so it could possibly function as an osmoregulatory mechanism in seawater fish. Thus it may sometimes be necessary to conserve certain materials (ions, organic molecules etc.) which might otherwise be lost at the 'chloride type' cells of the pseudobranch. The choice of pathway may be dependent on neural and/or humoral factors. Smith (1977) has shown that acetylcholine (ACh) restricted perfusion of the gill secondary lamellae to the inner and outer marginal channels. This implied that one action of ACh might be to reduce the dimensions of the pillar cell channels, perhaps by active shortening of the pillar cells themselves. The pillar cells contain the contractile elements necessary for such shortening but as yet there has been no direct demonstration of their sensitivity to ACh. Since ACh is unlikely to circulate in the blood stream the possibility arises that the pillar cells are directly innervated by cholinergic fibres. In fact innervation of pillar cells has been reported in the eel gill (Gilloteaux 1969) but has not been confirmed in other teleosts. No definite neural structures could be identified in association with the pillar cells of the pseudobranch, although many of the overlapping cytoplasmic extensions in the lamellar channels could not always be definitely attributed to pillar cells or their flanges. However no structures containing clear core

vesicles typical of cholinergic nerve endings (see Richardson 1966) could be found.

Autonomic nervous control of blood flow in the pseudobranch cannot be totally ruled out since sympathetic fibres have been shown to enter the glossopharyngeal nerve supplying the first gill arch of the cod (Pettersson & Nilsson 1979). A branch of this nerve also supplies the pseudobranch in many species, so it may provide an autonomic innervation as well as a sensory and somatic motor supply.

The main interest in the pseudobranch innervation has been directed towards its possible sensory nature and the presence of receptors responsive to hydrostatic pressure, hypoxia, pH, pCO_2 and osmotic pressure. However, the morphological identity of these receptors has still not been shown conclusively and it is often difficult to positively identify fibres as belonging to a sensory, motor or autonomic innervation.

Laurent & Dunel (1966) based their identification of a sensory innervation on light microscope degeneration studies following section of the pseudobranch nerve. They found degeneration of the large myelinated fibres in the pseudobranch nerve and disappearance of the fibres innervating the primary blood vessels. They classed these fibres as belonging to an afferent baroreceptive innervation, the cell bodies of which are located in the ganglion of the glossopharyngeal nerve. Ultrastructural observations in the present study gave similar results with degeneration and disappearance of all large myelinated fibres above 4 μ in diameter, and degeneration of fibres associated with the primary blood vessels.

The baroreceptor terminals are presumably represented by free nerve endings in the adventitia of the blood vessels, although most of the 'endings' found in this study appeared more characteristic of autonomic vasomotor nerves typically associated with the vascular smooth muscle layer of many blood vessels (Rees 1967, Rhodin 1974). Thus most fibres contained large

accumulations of clear vesicles and occasional dense core vesicles with usually only one or two mitochondria. In contrast Rees (1967) found that the presumed baroreceptor nerve terminals of the carotid sinus contained large accumulations of mitochondria as well as myelin figures and small dense bodies resembling glycogen. Although nerves containing a few mitochondria were occasionally found in the adventitia of the pseudobranch vessels there were none showing the same characterisation as those described by Rees (1967). It is possible that the pseudobranch baroreceptor terminals do not show the same specialisation or they may be located in a position not yet examined.

The physiological evidence does support a baroreceptor innervation since rapid changes in perfusion pressure trigger bursts of characteristic impulses (type A activity) with the appearance of high amplitude spikes (up to 250 microvolts). Based on the conduction velocity of these impulses (>10 metres/sec) the receptors are believed to be attached to the large diameter myelinated fibres (see Landon & Hall 1976).

The use of large variations in pressure (between 15 and 80 torr) revealed an initial dynamic burst of activity followed rapidly by a static phase. The activity was apparently affected by changes in pressure rather than the absolute value of the pressure. The appearance of apparently spontaneous but regular bursts of activity occasionally seen at a steady perfusion pressure may be due to brief pulses in pressure, possibly caused by a transient blockage or resistance in the blood vessels or catheter.

On the basis of the physiological and morphological data it seems likely that the pseudobranch vessels do in fact possess both a baroreceptive and an autonomic innervation. However further evidence is needed of the proposed autonomic supply and its possible sympathetic (adrenergic) or parasympathetic (cholinergic) nature. The existence of an adrenergic and cholinergic 'tonus' affecting the branchial vasculature has been proposed

which may be due, partly at least, to an autonomic innervation (Belaud et al 1971; Johansen 1972; Pettersson & Nilsson 1979). A similar system may also be present in the pseudobranch.

A vasomotor innervation may be important in regulating the sensitivity of the baroreceptors (Rees 1967). Contraction of the adventitial muscle cells under autonomic control could conceivably result in a mechanical deformation of the receptor nerves, and in particular those in the immediate vicinity of the advential muscles themselves.

The findings of the degeneration studies in the present study disagree to an extent with those of Laurent & Dunel (1966). The present ultrastructural observations suggest that not only the large myelinated fibres, but all myelinated fibres undergo degenerative changes following denervation. Furthermore many of the unmyelinated fibres of the 3 pseudobranch plexes also underwent ultrastructural changes characteristic of degenerating fibres, and both large and small neurons showed alterations typical of chromatolysis and retrograde degeneration (see Lieberman 1971; Abbot, de Burgh Daly & Howe 1972; Hess & Zapata 1972: Fernando 1973). The failure of Laurent & Dunel (1966) to observe degenerative changes in fibres of the 3 plexuses or neurons may be due partly to the use of light microscope techniques and partly due to regeneration of nerve fibres with the appearance of new unmyelinated and myelinated fibres. It must also be taken into account that the time course of degenerative and regenerative phenomena may be extremely variable due to such factors as temperature, species differences and type of nerve fibre (Fink & Heimer 1967).

Based on the apparent non-degeneration of axons and neurons in the primary and secondary plexes, Laurent & Dunel (1966) suggested that the fibres were of a centripetal nature and were attached to intramural sensory neurons. However the results of the present study question their interpretation of the pseudobranch innervation.

The chromatolysis and degeneration of the intramural neurons calls into question their possible sensory nature. It is well known that interruption of the centrally directed processes of most vertebrate primary sensory neurons causes no chromalytic or degenerative changes in the cell bodies (Moyer, Kimmel & Winborne 1953; Lieberman 1969, 1971). Even many months after section there is apparently no cell death, no atrophy and no chromatolysis. If the pseudobranch intramural neurons are indeed sensory then interruption of their centrally directed processes by section of the pseudobranch nerve would not be expected to cause chromatolysis or cell death. In the present study evidence of chromatolysis (fragmentation and dispersion of the Nissl substance) was present in most neurons within 7 days of section. The characteristic disorganisation of ordered cisternal arrays and replacement by short, vesicular and vacuolar elements has been observed in many studies on chromatolysis of axotomised neurons (Pannesse 1963; Torvik & Skjorten 1971; Fernando 1973; Nathaniel & Nathaniel 1973). Also the observed changes in nuclear shape and increase in the number of electron dense bodies are highly characteristic of many chromatolytic neurons (Barron, Doolin & Oldershaw 1967; Lieberman 1971; Nathaniel & Nathaniel 1973). The internal structure of the electron dense bodies observed in this study, particularly in the small type 2 neurons, varied from a simple amorphous material to a highly organised lamellated stack of membranes, and resembled the lysosomes and lipofucsin granules described in other studies (see Lieberman 1971: Nathaniel & Nathaniel 1973).

In the present study there was no apparent return to normal in many neurons, even 15 weeks after section, and most of the large type I neurons appeared similar to the degenerating neurons described in the hypoglossal nucleus by Fernando (1973). Thus there was an overall increase in electron density of the cell cytoplasmic and dramatic increase in the number of ribosomes, fragmentation and swelling of the endoplasmic reticulum, as well as vacuolation of the mitochondria. On the other hand some cells did show

apparent reorganisation of the cisterns of endoplasmic reticulum into parallel arrays. Thus it is uncertain if these cells represent degenerating or regenerating neurons. It is well known that complete section of a nerve gives only partial regeneration, even under optimal conditions (Gutmann & Sanders 1943). This invariably leads to disintegration of some of the neurons, and it may be difficult to distinguish between degenerating and regenerating neurons. Most mammalian peripheral neurons degenerating after axonal lesions disappear within the first 5 weeks after injury (see Lieberman 1971), but in the cold blooded fish, cell death may occur at a much later time. Obviously the neurons need to be examined over a longer period of time than 15 weeks following injury. It has previously been assumed that cells in an extremely chromatolytic state, or with electron-lucent, organelle-free cytoplasm will eventually degenerate (Pannesse 1963). A few such neurons were found in the pseudobranch 4 weeks after nerve section but were not found at any later stage. It seems likely therefore that these particular cells were destined for degeneration. Comparatively little is known of the histological and cytological features of neuron cell death and even when it occurs after rather long post-operative periods, it may well be that actual degeneration processes, once initiated, proceed very rapidly, so that histological "capture" is rare (Lieberman 1971). Furthermore the use of electron microscopy poses enormous sampling problems, being essentially qualitative, so it is likely that various stages in degeneration or regeneration may be missed.

The effect of 5-hydroxydopamine on the intramural neurons is interesting since it causes degenerative-type changes (vacuolation of mitochondria and swelling of granular endoplasmic reticulum) within 24 hours of injection. 5-hydroxydopamine has previously been used as an electronmicroscopical marker for adrenergic nerves since it accumulates in the adrenergic synapses in the form of large dense vesicles (Tranzer & Thoenen 1967;

Gordon-Weeks 1977). It has been described as a 'false transmitter' (see Gabella 1976) which acts pharmacologically as an adrenergic sympathomimetic compound (Tranzer & Thoenen 1967). As far as is known the effect of 5-hydroxydopamine on the nerve cell body has not previously been documented since most studies have been concerned with its property as an E.M. marker of adrenergic nerve endings. Therefore it is unknown if the ultrastructural changes observed in the pseudobranch intramural neurons are due to a direct affect on the cell body or a secondary affect due to uptake and release of transmitter from their peripherally directed endings. Nonetheless the fact that these changes do occur suggests that at least some of the neurons are adrenergic in nature.

The presence of small numbers of dense core vesicles in some large type I neurons provides further support for their adrenergic nature (see Burnstock & Costa 1975). Furthermore the axo-somatic synapses on type I neurons are made by cholinergic type endings expected of autonomic preganglionic fibres synapsing on postganglionic adrenergic neurons. However the chromatolysis and degeneration of the pseudobranch intramural neurons is not easily explained if in fact they are postganglionic autonomic neurons. This could only be explained in terms of transynaptic degeneration following degeneration of the distal processes of the sectioned preganglionic fibres. This might also explain the rather long time course of degeneration in these neurons.

All the myelinated fibres in the pseudobranch nerve showed characteristic Wallerian degeneration which develops distally to a peripheral nerve injury (Nathaniel & Pease 1963). Such degeneration would not be expected in fibres still attached to the cell body although some degenerative changes would occur proximal to the cut end of the nerve (Cajal 1928). However degeneration of fibres was seen deep within the pseudobranch tissue far from the site of injury, indicating true Wallerian degeneration of distal

fibres. Thus it seems unlikely that these fibres are attached to the pseudobranch intramural neurons. They probably arise from neurons outside the pseudobranch, either as postganglionic sensory fibres supplying the proposed baroreceptor innervation, as preganglionic autonomic fibres supplying connections to intramural sympathetic and/or para-sympathetic neurons, or as somatic motor fibres.

The fact that only about 20% of myelinated fibres had regenerated 15 weeks after section may indicate the presence of at least two fibre populations, one consisting of small fibres (<4 μm) which regenerate, and one of larger fibres which completely degenerate. However, as already mentioned, only partial regeneration of a nerve is expected after complete section (Gutman & Sanders 1943). Furthermore, myelin sheath thickness and axon diameter are usually smaller in regenerated nerves than in normal nerves, although there is a gradual increase in the proportion of axons with large diameters over a period of time (Schroder 1972). However this may take many months and even then might never reach that of normal nerves.

Apart from an autonomic innervation of the primary blood vessels, controlling blood flow, an autonomic control of the 'pseudobranch type' cell also seems likely on the basis of the morphological evidence. The only synapses found on the 'pseudobranch type' cell were made by cholinergic-type endings full of clear vesicles and an occasional dense core vesicle. It is generally accepted that the direction of transmission across a synapse can be correlated with its morphological appearance (Eccles 1964; De Robertis 1966; Peters, Palay and Webster 1970). Thus, accumulations of vesicles generally occur in association with the membrane thickening on the pre-synaptic side. In the present case the nerve terminal represents the pre-junctional (pre-synaptic) structure and the 'pseudobranch type' cell the post-junctional (post-synaptic) element i.e. the reverse of that depicted in the classical model of the arterial chemoreceptor (Castro 1926, 1928). Most of these efferent type endings did not apparently degenerate

after denervation and normal synapses were found up to 15 weeks after section. This suggests that they arise from autonomic postganglionic neurons within the pseudobranch. Their interpretation as cholinergic endings would suggest that they are of parasympathetic origin. The endings may belong to fibres attached to the small type 2 neurons, many of which showed no serious degenerative changes after nerve section, apart from an apparent increase in the size and number of lysosomes. On the other hand, some of the non-degenerating neurons may represent intramural sensory neurons or even possible interneurons. It is obvious that the different types of large and small neurons found in the primary and secondary plexus present a very complicated picture, and is probably representative of a heterogenous population of nerve cells containing both autonomic and sensory neurons as well as interneurons. Some neurons may serve to influence or regulate other neurons by means of excitatory or inhibitory synapses. The failure to degenerate (up to 15 weeks after denervation) of some of the cholinergic-type endings on intramural neurons suggests that they are intrinsic in origin and arise from other neurons in the plexus. Contact between neurons may also be maintained by the gap-junctions observed between the cell bodies of some neurons. Gap junctions function as low resistance electrical synapses which promote rapid cell to cell propagation of impulses and may synchronize the activity of a group of neurons (Bullock 1977).

It is clear that further histochemical evidence is needed to identify the various intramural neurons of the pseudobranch with any degree of certainty. The use of formaldehyde-induced-fluorescence techniques (F.I.F., see Pease 1972) may provide an answer as to whether adrenergic neurons are present within the pseudobranch.

As with the intramural neurons it is difficult to establish the identity of the various fibres of the terminal plexus solely on the basis of morphological evidence. The large number and variety of fibres as well

as differences in their response to denervation indicates a complex innervation which may contain sensory, somatic motor and/or autonomic fibres. Also the origin of an individual fibre is not always certain. The failure of some fibres to degenerate after nerve section suggests that some at least arise from intramural neurons. This applies to most fibres with cholinergic type endings. However, degenerating fibres do not necessarily originate from neurons outside the pseudobranch but may be connected to intramural neurons which undergo degeneration. It was thought inappropriate in the present study to try and quantify the numbers of degenerating and normal nerve fibres in the terminal plexus since enormous variations were found between sections of pseudobranch secondary lamellae. In some sections most fibres appeared to be degenerating while in others most appeared to be normal. This may be due to variations in the innervation within different regions, or differences in the proximity of nerve terminals or distance from the site of injury. The picture may be further complicated by regeneration of some fibres which may occur as early as 7 days after section.

Although the morphological and denervation studies suggest at least two fibre populations within the terminal plexus it should be pointed out that some variations in the appearance of fibres and endings may be explained on the basis of sections taken at different positions along a single nerve fibre. For example, Verna (1971, 1973) illustrated the existence of long nerve endings with different cytological components along their length, making multiple contacts with Type I glomus cells of the rabbit carotid body. Similar evidence is provided by Morgan, Pack & Howe (1975) who showed both afferent and efferent type synapses between the same nerve ending and Type I cells of the rat carotid body. In the present study afferent and efferent synapses were never found on the same ending although there were variations in the morphology of nerve fibres along their length.

The suggestion of Laurent (1974) that the pseudobranch functions as an arterial chemoreceptor similar to that proposed for the carotid body (Biscoe 1971) is based on physiological rather than morphological evidence. Although the electrophysiological evidence of the present study also suggests that chemoreceptor activity originates from the pseudobranch, the identity of the chemoreceptive element(s) is still unresolved. A number of possibilities may be considered.

Laurent (1974) suggested that afferent endings in the interlamellar plexus are functionally linked to the pseudobranch type cell. This functional connection is not physically represented by a synapse but only by a contiguity. Laurent considered it necessary to take into account the existence of an intermediary phase represented by the extracellular milieu which bathes the naked fibres. He also believed that this environment was a function of the metabolic conditions imposed on the 'pseudobranch type' cell, i.e. pO_2, pCO_2, pH and osmotic pressure. Large variations in the membrane potentials of the 'pseudobranch type' cells associated with pO_2 or pH changes provide an argument in favour of ionic redistribution between intra and extracellular milieu (Laurent 1969, see earlier in discussion). In the present study nerve fibres were rarely found within extracellular spaces and were usually contiguous with the surrounding interstitial tissue and 'pseudobranch type' cells. It is uncertain if this is due to species differences or due to differences in preparation procedures. However it allows a slightly different interpretation of the chemoreceptor mechanism(s). As already suggested in this study the endings may be affected by deformation of the fibrous interstitial tissue caused by swelling or contraction of the 'pseudobranch type' cell in response to changes in osmotic pressure. In fact there may also be direct affects on the nerve cell body of intramural neurons within the secondary lamellae. Furthermore it is possible that an afferent innervation of the 'pseudobranch type' cell takes the form of "en

passant" type synapses which do not show any particular specialization. Such "en passant" type synapses have been suggested in the carotid body by Hoglund (1967) and Al-Lami & Murray (1968). The proposed efferent, cholinergic-type endings may regulate the sensitivity of the cell as a transducer for chemoreceptor stimuli and/or control some glandular function of the cell.

In some respects this is a similar but somewhat simpler model than that proposed for the rat carotid body by Morgan et al (1975). The suggested the possibility of a negative feedback loop between the afferent type ending and the Type I glomus cell, whereby the nerve ending not only receives information from the Type I cell but also regulates the degree of activity in the cell. The sensitivity of this system could be further regulated by an efferent type ending on the Type I cell, and the flow of information in the afferent nerve fibre is regulated by an efferent synapse ending on the afferent fibre.

There is no evidence in the present study to suggest a negative feedback loop between the 'pseudobranch type' cell and any nerve ending, although there does appear to be an efferent control by means of a cholinergic-type ending. In addition there may be a second efferent control of nerve fibres via the nerve-nerve synapses seen on some fibres, although it is unknown at present if the endings occur on afferent or efferent fibres.

If the fibres containing cholinergic-type vesicles are thought to represent an efferent innervation, then there are a number of possible candidates for an afferent innervation.

(1) Small naked fibres which possibly end without morphological specialization.

(2) Fibres ending in swellings containing mainly large accumulations of mitochondria.

Apart from normal looking mitochondria some swellings contained accumulations of small whorled or lamellar type bodies which appeared to be modified

mitochondria. Endings rich in mitochondria have been considered to be afferent in a number of studies, including those on intraepithelial nerves of the trachea and extra pulmonary airways of man and rat (Rhodin 1966; Luciano & Reale 1970; Jeffery & Reid 1973).

(3) Large varicosities containing a variety of large vesicles of varying electron density. These are similar to the heterogenous granular vesicle (H.G.V.) containing endings described by Baumgarten, Holstein & Orsman (1970) in the myeneteric plexus of the large intestine of the monkey, guinea-pig and man. It is not known if these H.G.V. cells belong to motor or sensory neurons nor indeed what is the transmitter (although A.T.P. has been suggested). A.T.P. has also been suggested as a transmitter of a non adrenergic, non cholinergic, vasoconstrictory innervation in the gill (Wood 1977). The fibres have yet to be identified histologically, although endings with heterogenous granular vesicles may well be a candidate.

The actions of 5-hydroxydopamine (5-OHDA) and 6-hydroxydopamine (6-OHDA) on the terminal fibres and 'pseudobranch type' cells were rather unexpected and cannot easily be explained. As already mentioned 5-OHDA acts as a E.M. marker of adrenergic terminals (Tranzer & Hoenen 1967) while 6-OHDA causes selective degeneration of sympathetic nerve terminals (Kostrzewa & Jacobowitz 1974). In the present study both compounds caused apparent degeneration of many terminal fibres as well as ultrastructural changes in the 'pseudobranch type' cells and nerve fibres. The changes caused by 5-OHDA within 4 hours of injection were similar to those seen in degeneration studies 4 weeks after denervation. Only the nerve cell membranes in contact with the 'pseudobranch type' cell membrane underwent breakdown and most ultrastructural changes in the nerve fibres were associated with those fibres adjacent to a 'pseudobranch type' cell. The membranes between 'pseudobranch type' cells and other cells in the pseudobranch were unaffected so the effect seems specific to the contiguous membranes between a nerve fibre and 'pseudobranch type' cell. The breakdown of membranes left the cytoplasm

of the 'pseudobranch type' cell in direct contact with that of the nerve fibre. This often came into contact with a large pale cytoplasmic region which appeared in the apical pole of the 'pseudobranch type' cell, close to the region of membrane breakdown. The pale cytoplasmic region also appeared to be surrounded by a broken membrane and may in fact be the remains of the cytoplasmic vacuole described in normal cells. There was no obvious increase in the number of dense core vesicles in the nerve endings or varicosities, which suggests, on the basis of other studies (e.g. Tranzer & Thoenen 1967) that they are not adrenergic fibres. However the breakdown of the nerve and 'pseudobranch type' cell membranes was not expected and suggests that the 5OHDA is affecting the nerve endings in some way which also causes breakdown of the membrane of the 'pseudobranch type' cell. Paradoxically this may reinforce the concept of a close functional relationship between the nerve fibres and 'pseudobranch type' cells. The effects of 5OHDA on the pseudobranch cannot be oviously explained at present although it may well be due to a different response of fish nerves compared with those of mammals. It is also possible that the effects are secondary to interference with other homeostatic mechanisms. The apparent breakdown of the cytoplasmic vacuole suggests that osmotic factors may be involved. On the other hand this could be due to the breakdown of some controlling mechanism provided by fibres of the terminal plexus.

The effects of 6OHDA on the pseudobranch were even more pronounced than those of 5OHDA. Most terminal fibres had disappeared from the pseudobranch within 24 hours of injection and the 'pseudobranch type' cells showed serious ultrastructural alterations including the appearance of numerous lipid-like bodies similar to those which appear in the pseudobranch of osmotically stressed animals or fish given an injection of acetazolamide. Thus there seems to be a serious disturbance in the normal homeostatic balance of the fish which is reflected by the pseudobranch. It is not certain if the disappearance of fibres from the pseudobranch is due to a specific action of

6OHDA on sympathetic fibres or whether it is part of the serious overall changes in the pseudobranch caused by interference with other homeostatic mechanisms within the fish.

Whilst the injection of 5OHDA and 6OHDA provided no convincing evidence to support an adrenergic sympathetic innervation the effects produced by these compounds on the pseudobranch are rather interesting and provide scope for a more detailed study of their action on the pseudobranch, as well as fish innervation and tissues in general.

The chemical control of respiration in fish still remains open to question even though many studies have been devoted to this subject. Most workers think in terms of regulation by chemoreceptors, although the location of these is still uncertain. Some believe the receptors to be located in the venous part of the circulatory system (Taylor, Houston & Horgan 1968), while others suggest an arterial location (Jones, Randall & Jarman 1970; Davis 1970). Bamford (1974) proposed that the brain was the most important site of oxygen detection in the rainbow trout. The gills, pseudobranch and lining of the buccal cavity and pharynx have also been considered as possible sites (Powers & Clark 1942; Konishi 1957; Kulaer 1958; Serbyeniuk, Shishov & Kiprian 1959; Hughes & Shelton 1962; Shelton & Randall 1962; Randall 1966; Randall & Smith 1967; Laurent 1967; Laurent & Rouzeau 1972). Saunders and Sutterlin (1971) suggested the presence of receptors both centrally (brain) and peripherally (gills, pseudobranch or buccal cavity). The central receptors would presumably monitor oxygen tension in the blood or cerebro-spinal fluid and the peripheral either in the blood or water. The gills were considered the most likely site for peripheral oxygen receptors until Sutterlin & Saunders (1969) were unable to detect changes in the level of activity in nerves innervating the gills, in response to changes in ambient PO_2. However their experiments were carried out at $5^{\circ}C$, a temperature at which no chemoreceptor activity could be measured

from the pseudobranch in this study, although activity increased rapidly above this temperature. There could well be a close link between temperature and the functioning of oxygen receptors since under normal conditions water is more likely to be saturated with oxygen at low temperatures than at high temperatures because of differences in diffusional capacity and differences in biological oxygen demand (Odum 1971). Thus oxygen receptor function may be absent at low temperatures when high oxygen levels would normally be expected. The activation and amplification of type B activity in the pseudobranch with rise in temperature may indicate the existence of certain, as yet unidentified, metabolic factors in the chain of transduction. The role of the 'pseudobranch type' cell may be important in such a system.

The fact that the systems responsible for activity A and B are sensitive to a number of agents does not necessarily mean that they correspond to an equivalent number of specialised receptors. It would appear more logical that certain receptors are sensitive to several agents. It should be emphasized, also, that it is not always easy to distinguish between two types of activity from the pseudobranch especially in recordings of spontaneous activity. However the evolution of an increased activity of one or other type in response to a particular stimulus indicates the presence of at least two types of receptor. This agrees basically with the results of Laurent & Rouzeau (1972). In the present study type A activity appeared to be dominant under ambient conditions at 15°C. However it is probable that many B impulses were not detected since their amplitude is small and often indistinguishable from the background noise. Type B activity was always highest in the first 15 - 20 minutes after preparation of the pseudobranch, possibly due to the tissues becoming hypoxic during removal and preparation of the organ. Although Laurent & Rouzeau (1972) found no evidence of rhythmic discharge, in the absence of stimulation a number of preparations in the present study show sequences of rhythmic type A activity

with spikes of similar height at regular time intervals. No such observations were ever made of type B activity. This further suggests that type A activity arises from baroreceptive fibres while type B activity originates from chemoreceptors. There are many reports on the randomness of discharge of chemoreceptor fibres (Eyzaguirre & Levin 1961; Biscoe & Taylor 1963; Eyzaguirre & Koyano 1965; Ishii, Honda & Ishii 1966).

It is still uncertain if the effect produced by Na^+ on type A activity is due to the presence of a special Na^+ receptor or whether it is only the result of the well known action of the Na^+ concentration on nervous tissue and particularly on pressure receptors (Laurent 1974). The concentration of Na^+ may well be responsible for the level of excitability of the receptors. The effects are certainly not attributable to an osmotic factor since a similar response of type A activity was not observed when the osmotic pressure of the perfusion fluid was raised by the addition of mannitol instead of NaCl. Until it becomes possible to anatomically separate the various activities by recording from isolated fibres there can be no direct proof of the existence of separate receptors contributing to type A activity.

In the present study only multifibre recordings were made because of the difficulties encountered in trying to separate single fibres. This was partly due to the dimensions of the nerve (0.1 - 0.15 mm diameter) and partly because of the fragile nature of teleost nerve fibres due to their thin myelin sheaths (Laurent & Rouzeau 1972). The use of much larger fish and hence larger pseudobranch and nerve, may enable easier dissection of isolated fibres.

Similar problems are encountered in trying to interpret type B activity although it seems likely that sensitivity to pO_2, pH and pCO_2 are linked to the same type of receptor since saturation of activity occurs when one combines several of these stimuli. A similar conclusion concerning the sensitivity to osmotic pressure is not so evident (see earlier).

The physiological evidence suggests the presence of at least two and possibly four types of receptor. A baroreceptor innervation seems fairly certain and a chemoreceptor function linked jointly to pO_2, pH and pCO_2 also seems likely. Another receptor may measure changes in osmotic pressure and a fourth type responsive to Na^+ concentration cannot be ruled out. It seems logical to suggest that the baroreceptors are located in the region of the primary blood vessels, probably on the afferent side, and that receptors responsive to pO_2, pH, pCO_2 and osmotic pressure are located in the intralamellar plexus. It is less easy to suggest a location for a Na^+ receptor which could be associated with either position.

The baroreceptors of the pseudobranch may have some effect on the distribution and blood flow in the gills which will in turn affect the arterial pO_2. Many authors (see Randall 1970) have suggested that baroreceptors, involved in the regulation of dorsal aortic pressure, are located in blood vessels dorsal to the gills. Chemoreceptors in the pseudobranch may also be functionally linked to the gills by causing changes in blood flow and distribution to the gill vessels. There is some evidence for this supposition in the work of Davis (1971) who found that bilateral ligation of gill arch 1 caused a marked reduction in arterial pO_2 which was not seen when other arches or combinations of arches, accounting for much more than the gill area of arch 1, were ligated.

The results of this study confirm the pseudobranch as a complex organ which may have a number of inter-related functions for which it has developed specific cell types and a complex innervation. At least one of these functions in the seawater fish appears linked to an osmoregulatory role, possibly as a supplement to the gills, although the modification of the pseudobranchs' 'chloride type' cells may indicate an additional or slightly different function. The possibility of an osmoreceptor mechanism in both seawater and freshwater fish provides further evidence of an osmoregulatory

role. The possibility of the pseudobranch regulating blood composition and blood flow may be significantly related to its position between the first gill arch and the circulation of the eye. Much of its complexity may be related to functions specially evolved for monitoring and regulation of the blood with respect to the gill and/or eye. Indeed the pseudobranch receptor functions in question are linked directly or indirectly to the vascular system. Further work is clearly needed to try and separate the receptor activities and to positively identify the receptor elements responsible. Also more investigation is needed on the possible autonomic innervation of the pseudobranch and its likely control of blood flow and/or functioning of the 'pseudobranch type' cells. Finally, in considering the functions of the various elements of the pseudobranch, the possibility should not be overlooked that this organ may also subserve an endocrine function either by affecting its own performance as a sensory receptor or by acting on some more distant target organ.

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Comparison of 'Pseudobranch' type and 'Chloride' type cells in the pseudobranch of marine, freshwater and euryhaline teleosts

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The degree of similarity between the ' pseudobranch type ' and ' chloride type ' cells in the pseudobranch of bass (*Dicentrachus labrax* (L)), smelt (*Osmerus esperlangus*) and rainbow trout (*Salmo gairdneri*) was studied. The pseudobranch of bass and smelt contains two specialised cell types commonly known as ' chloride type ' and ' pseudobranch type ' cells, whereas rainbow trout only possess the latter type. ' Pseudobranch type ' cells are characterised by the orderly arrangement of parallel tubules around their closely packed mitochondria. ' Chloride type ' cells are identified by their highly branched tubular network, randomly distributed mitochondria, and the presence of an apical pit. Although ultrastructural changes were noted under osmotic stress, the ' chloride type ' and ' pseudobranch type ' cells remained distinguishable from each other and gave no reason to suggest that they were different forms of the same cell type. It was concluded that the two are distinct cell types, performing different, though possibly related, functions.

I. INTRODUCTION

The pseudobranch of fish arises from the hyoid arch and shows progressive structural modification from the respiratory branchial arches, ranging from a gill-like structure projecting into the buccal cavity to a ' glandular ' structure buried in a mass of connective tissue (Granel, 1922). The physiological function and importance of the pseudobranch have been matters of controversy ever since it was first described by Broussonet in 1785. It has been suggested that the organ is involved in enzyme production (Leiner, 1938; Maetz, 1956), endocrine functions (Holliday, 1960), chemoreception (Laurent & Dunel, 1964, 1966) or that it has a salt regulatory function similar to that described for the gills by Keys & Willmer (1932). In support of the latter theory, cells rich in mitochondria are found throughout the pseudobranch epithelium but whether or not the ' pseudobranch type ' cells are distinct from ' chloride type ' cells, which also occur in the gill epithelium, remains uncertain. Newstead (1971) in a study of the gills of a tidepool sculpin (*Oligocottus maculosus*) claimed that the ' chloride type ' cells normally found in the gill epithelium could undergo rearrangement of their cytoplasmic components into the pattern characteristic of ' pseudobranch type ' cells.

The present study attempts to establish the similarity or distinction between ' pseudobranch type ' and ' chloride type ' cells by examining structural changes in the pseudobranch of marine, euryhaline and freshwater species exposed to osmotic stress.

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II. MATERIALS AND METHODS

To ascertain if drastic osmotic stress could cause rearrangement of ' chloride ' cell structure into that characteristic of ' pseudobranch type ' cells a similar experiment to that of Newstead (1971) was carried out on bass *Dicentrachus labrax* (L.). Fish measuring 20–25 cm were exposed to 0·34‰ salinity without previous acclimation to dilute sea water, for periods of 5, 10 and 24 h. Control fish were kept in full sea water of 34‰ salinity. All fish were held at 18–20° C in continuously aerated water.

Experiments were carried out with smelt, *Osmerus esperlangus* L. and rainbow trout, *Salmo gairdneri*, Richardson, to see what effect more gradual acclimation to different salinities would have on the cells of the pseudobranch.

Smelt, approximately 12 cm long, were held in a closed circuit seawater (34‰) tank in the laboratory at 18–20° C. Groups of fish were acclimated to salinities of 24‰, 16‰, 8‰ and fresh water. Each group was acclimated gradually (over 15 h at each stage) to their respective target salinities where they were held for up to 36 h.

Rainbow trout, approximately 20 cm long, were maintained in the laboratory in a closed circulation system of fresh water at 10–12° C. Groups of fish were acclimated (over 15 h at each stage) to salinities of 10‰, 20‰, 34‰, 42‰ and 60‰ salinity, where they were held for 36 h.

All fish were killed by a blow on the head. The pseudobranchs were removed intact and immersion fixed in a solution of 2·5% glutaraldehyde in either 0·1 M phosphate or 0·1 M cacodyhate buffer at pH 7·2 and 4° C for 1 h. The pseudobranch was rinsed in buffer and cut into small pieces before post fixation in 1% osmium tetroxide in buffer for 1 h. The material was dehydrated in a graded alcohol series, followed by propylene oxide, and embedded in Spurr resin (Spurr, 1969). Sections 1 μm thick were cut on a Porter Blum and stained in 1% methylene blue in 1% borax solution. Thin sections were also cut, stained in uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope. Paraffin wax sections of Bouin fixed material were stained with Mallory's triple stain and examined in the light microscope.

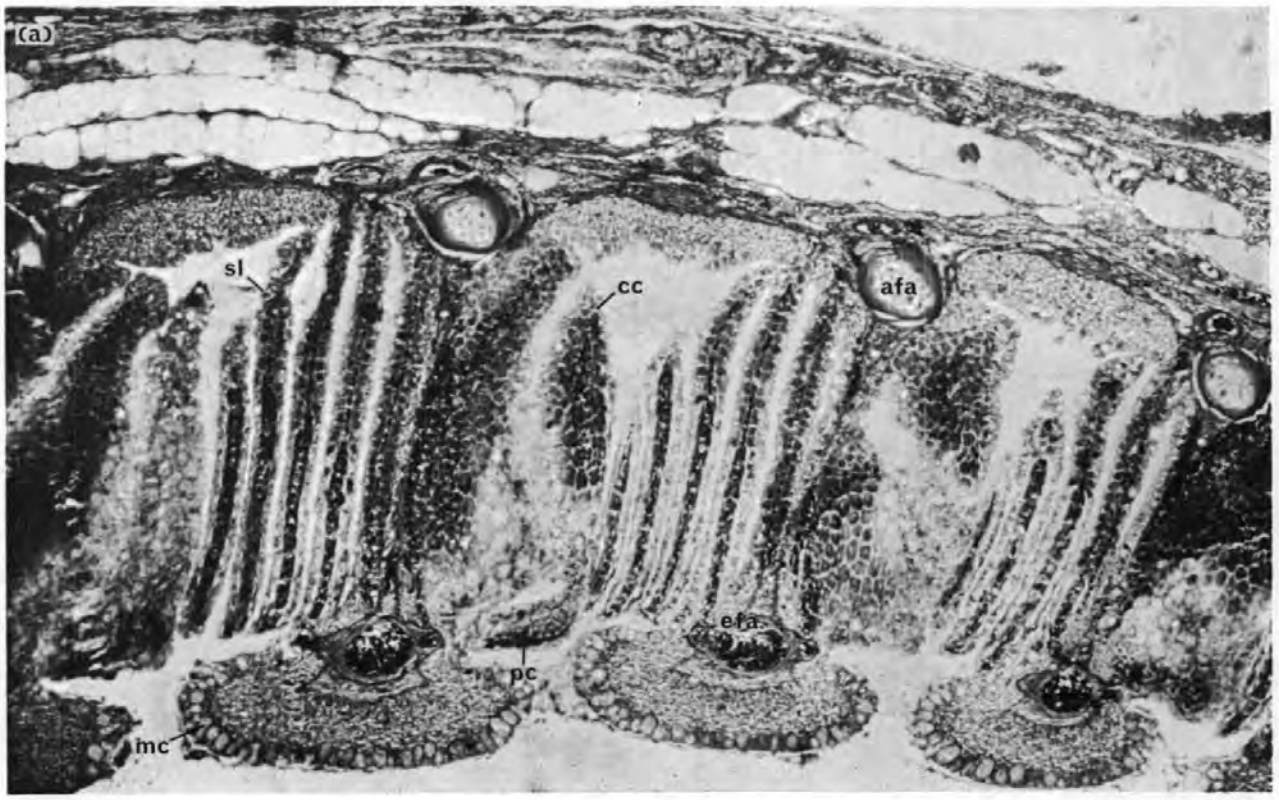
III. RESULTS

STRUCTURE OF THE PSEUDOBRANCH UNDER NORMAL CONDITIONS

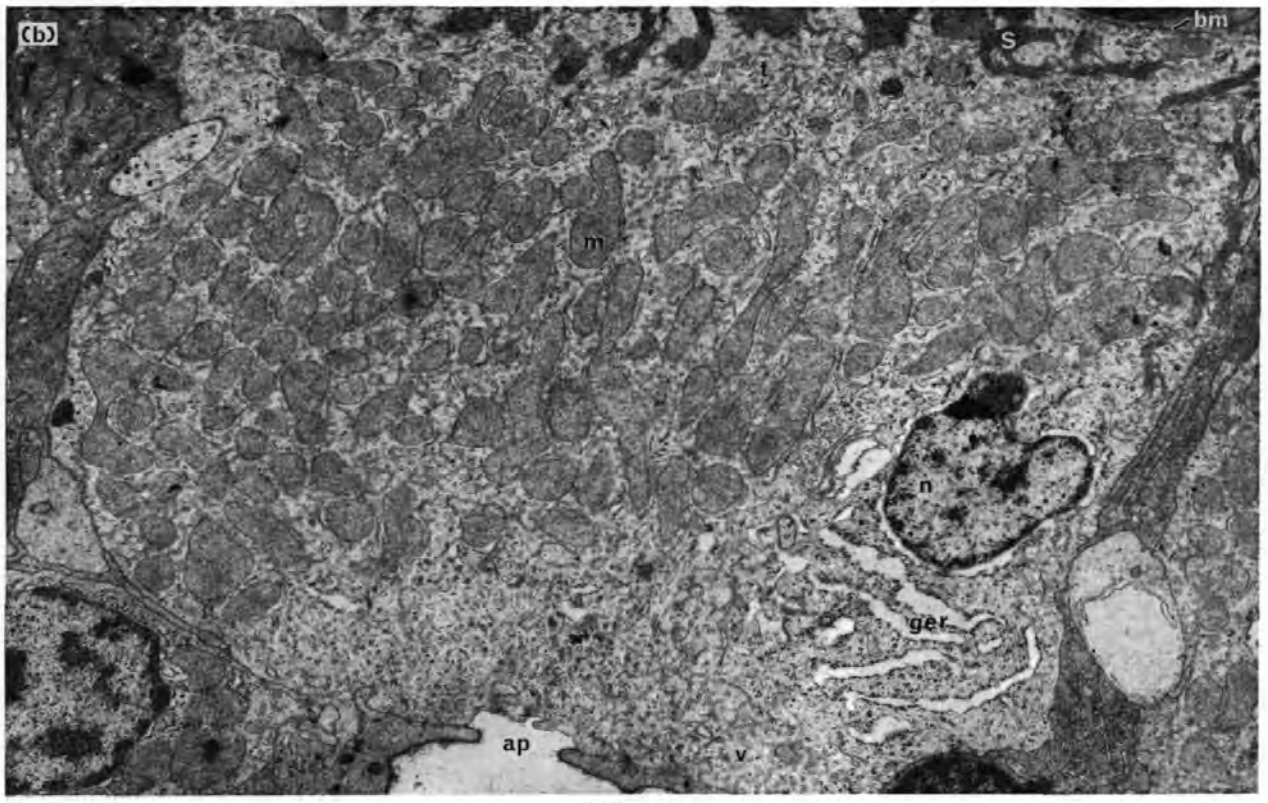
(*a*) *Bass*, Dicentrachus labrax (*L.*)

The appearance of the pseudobranch and the structure of its cells in bass from full sea water was taken as the normal condition of the organ in this species. The structure of the bass pseudobranch resembles that of a normal gill except that it possesses only one row of filaments. It comes under Granel's (1922) category of a free pseudobranch since it is not covered by a fold of epithelium and the secondary lamellae are free of one another, as are the filaments. The pseudobranch is joined to the operculum for most of its length apart from the filament tips which are completely free.

The pseudobranchial epithelium which borders the capillary system of the secondary lamellae is made up of two types of cell, commonly referred to as ' chloride type ' and ' pseudobranch type ' cells. These cells are covered by a layer of flattened epithelial cells which completely isolate the pseudobranch cells from the external environment. The ' chloride type ' cells remain in direct contact with the water by means of apical pits in the overlapping epithelium [Plate I(b)]. The ' chloride ' and ' pseudobranch type ' cells cover approximately half the area of the lamella in the region of the filament afferent artery whilst the ' pseudobranch ' cells cover the remaining area in the region of the efferent artery [Plate I(a)]. The ' chloride type ' cells of the pseudobranch are similar to the ' chloride ' cells of the gill especially as they both possess an apical pit. However, there are some ultrastructural differences, the main one being the presence of numerous narrow saccules connected to the rectilinear tubules formed



(*Plate Ia*)



(*Plate Ib*)

PLATE I. (a) Light micrograph of transverse section of filaments of pseudobranch of bass kept in sea water (34‰ salinity). Stained with Mallory's triple stain. ×160. afa, afferent filament artery; efa, efferent filament artery; pc, pseudobranch cell; cc, chloride cell; sl, secondary lamellae; mc, mucus cell. (b) Electron micrograph of section through 'chloride' cell of pseudobranch of bass kept in sea water (34‰ salinity). ×15 250. m, mitochrondia; ap, apical pit; s, saccules; v, vesicles; t, tubules; ger, granular endoplasmic reticulum; n, nucleus; bm, basement membrane.

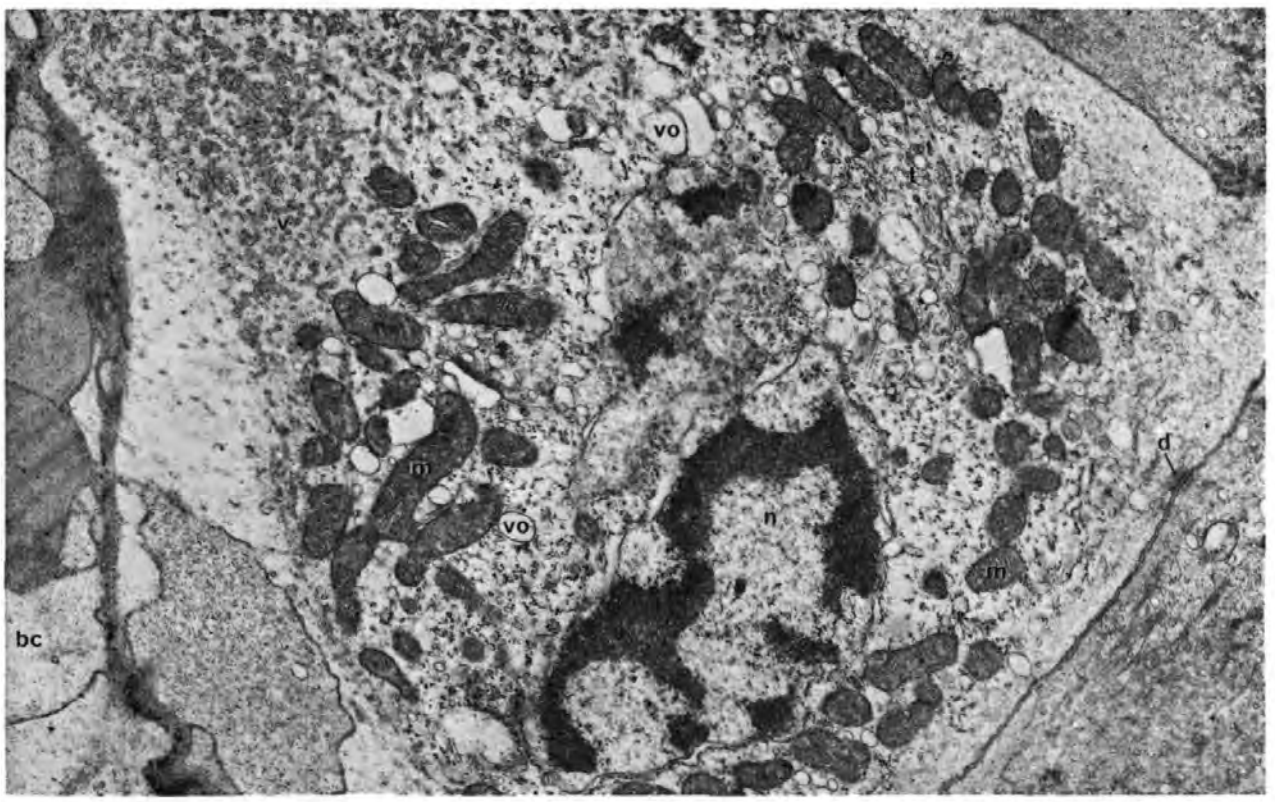
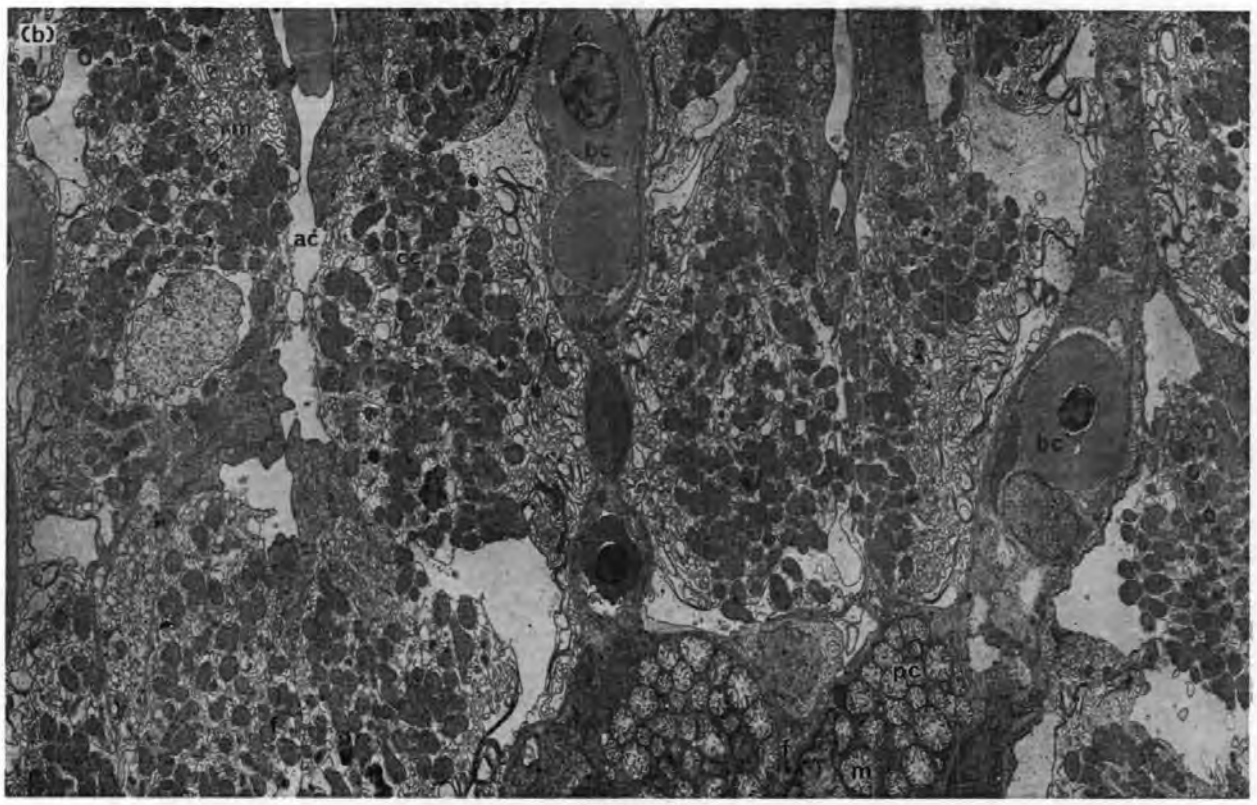
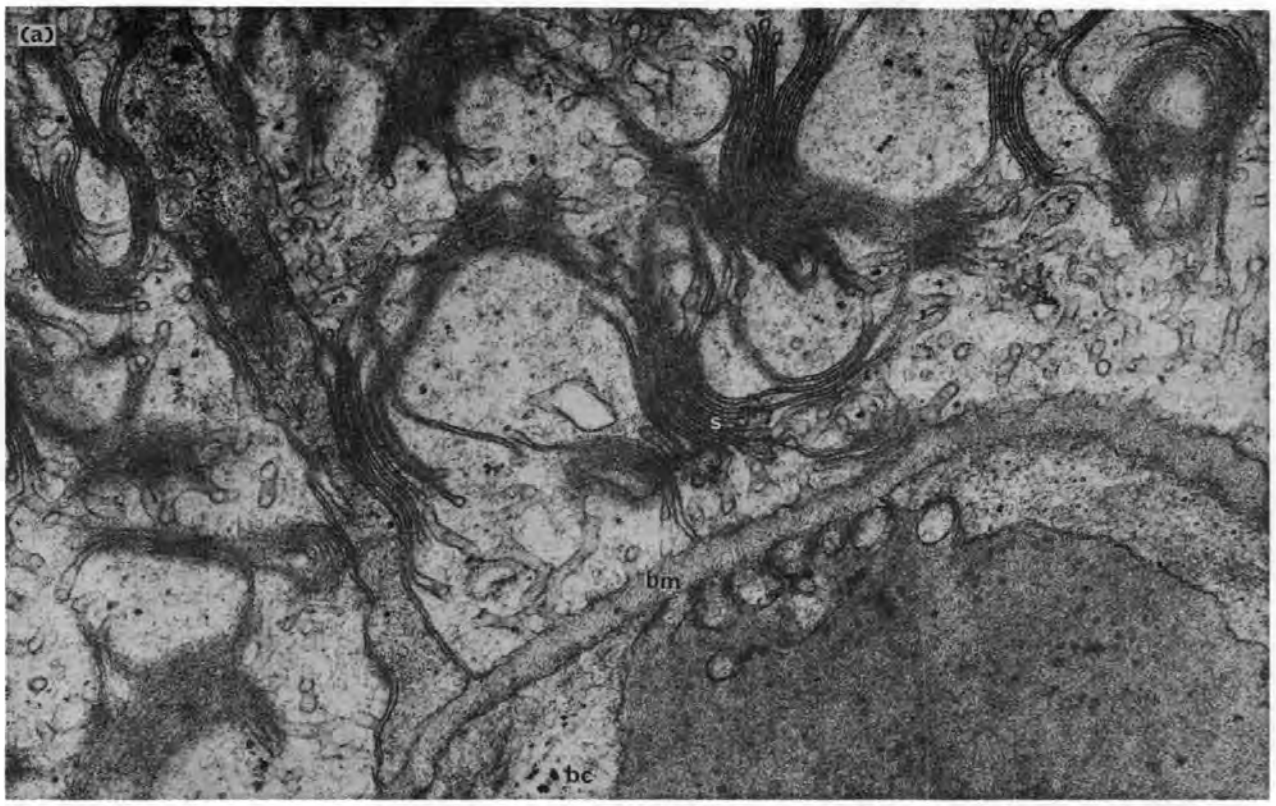


PLATE VI. Electron micrograph of section through 'pseudobranch' cell of pseudobranch of trout kept in 60‰ salinity. ×12 500. n, nucleus; m, mitochondria; t, tubules; v, vesicles; d, desmosome; bc, blood channel; vo, vacuoles.

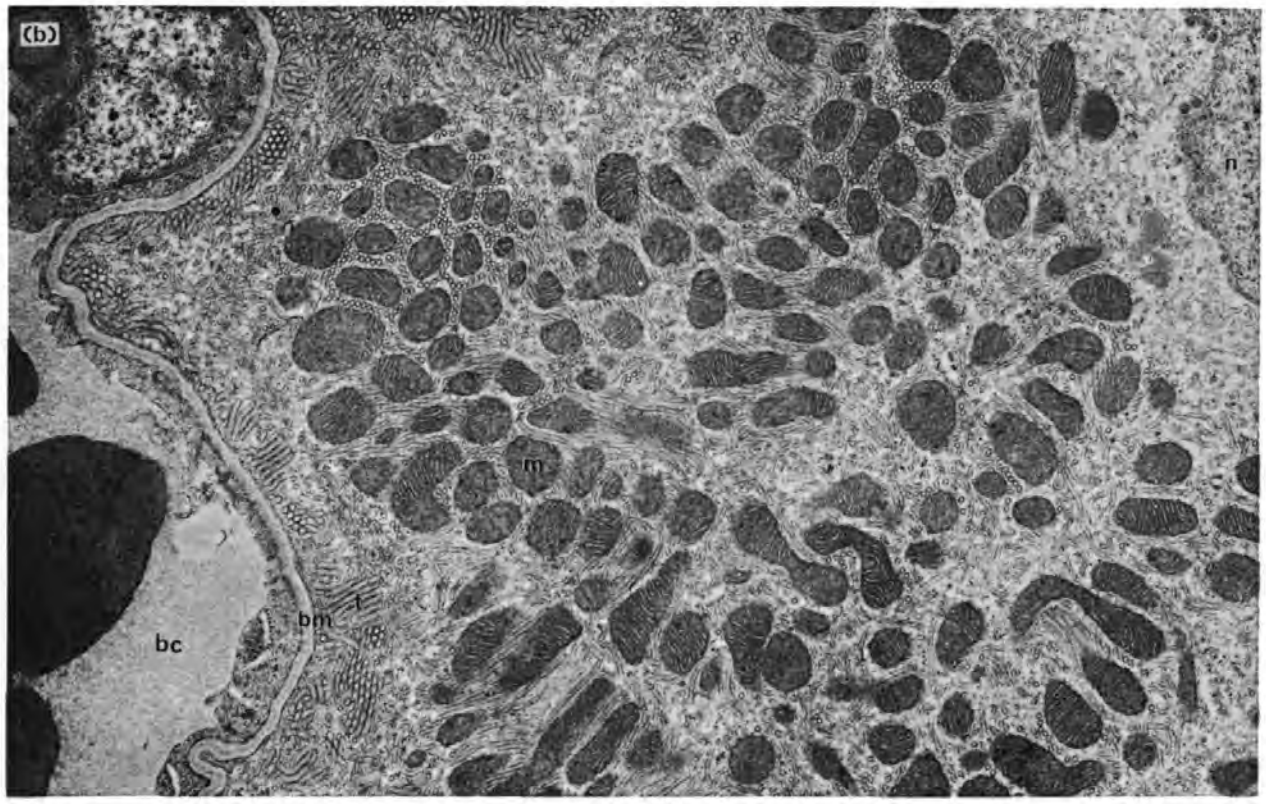


(*Plate Vb*)

PLATE V. (a) Electron micrograph of section through ' pseudobranch ' cell of pseudobranch of bass kept in 0·34‰ salinity. ×27 500. m, mitochondria; g, glycogen; t, tubules; bc, blood channel; di, dense inclusions; bm, basement membrane. (b) Electron micrograph of section through three adjacent secondary lamellae of pseudobranch of smelt kept in fresh water. ×5500. ac, apex of cell; bc, blood channel; m, mitochondria; cc, chloride cell; pc, pseudobranch cell; f, region of fusion between adjacent secondary lamellae.

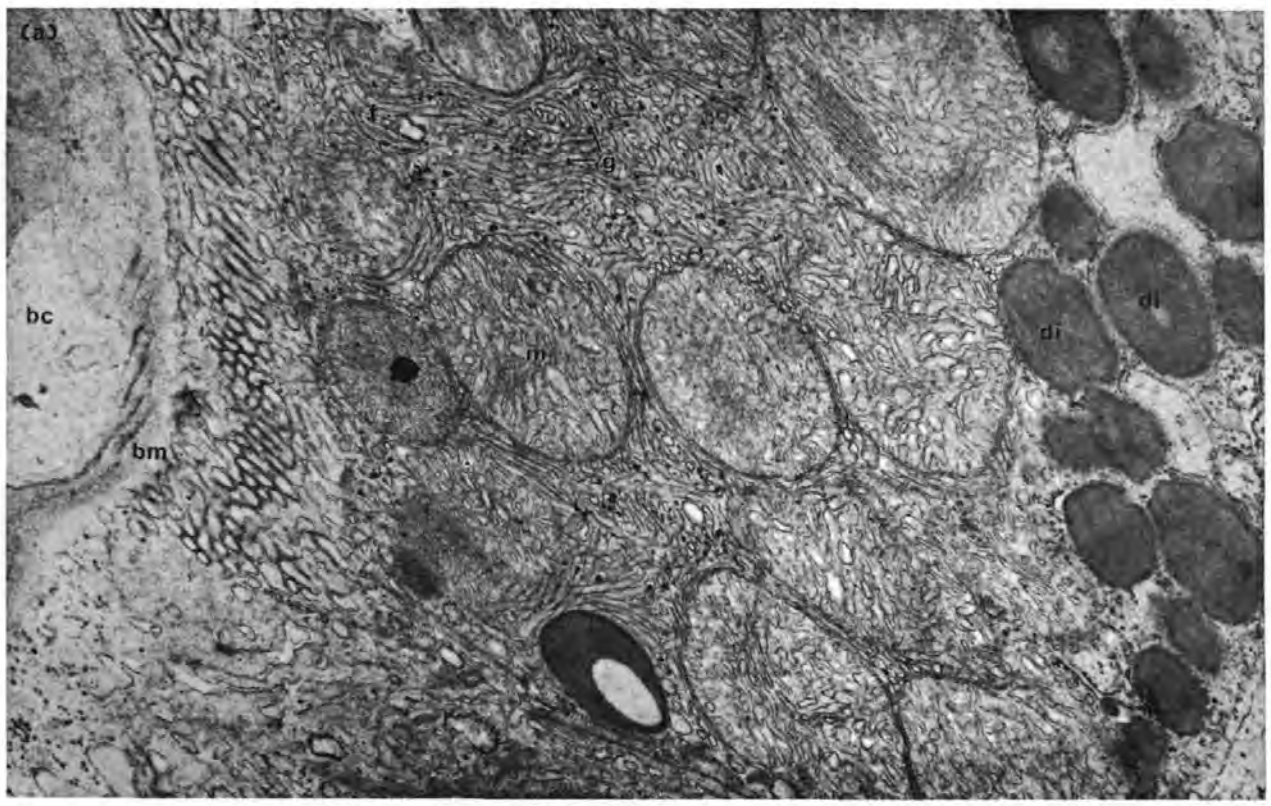


(*Plate IIa*)

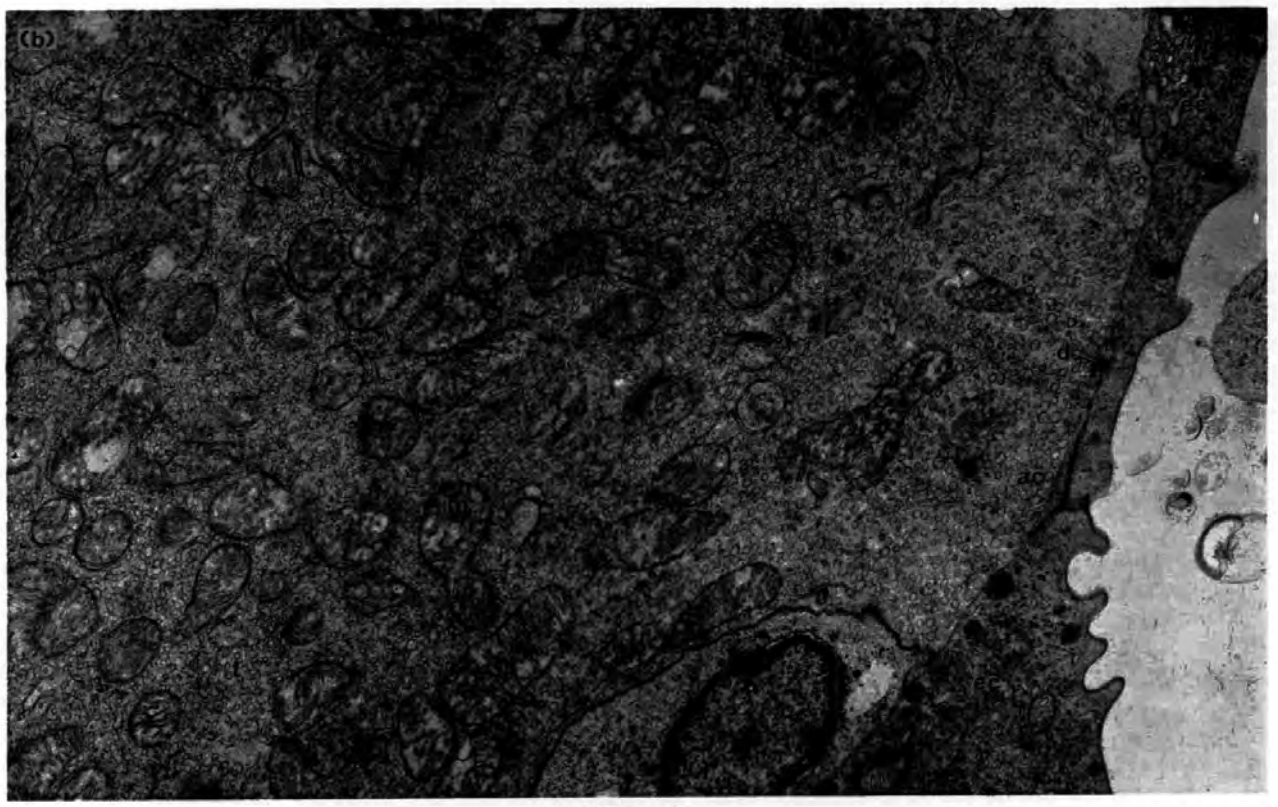


(*Plate IIb*)

PLATE II. (a) Electron micrograph of section through base of ' chloride ' cell of pseudobranch of bass kept in sea water (34‰ salinity). ×44 600. bm, basement membrane; s, saccules; bc, blood channel. (b) Electron micrograph of section through base of ' pseudobranch ' cell of pseudobranch of bass kept in sea water (34‰ salinity). ×18 700. m, mitochondria; t, tubules; bc, blood channel; bm, basement membrane; n, nucleus.



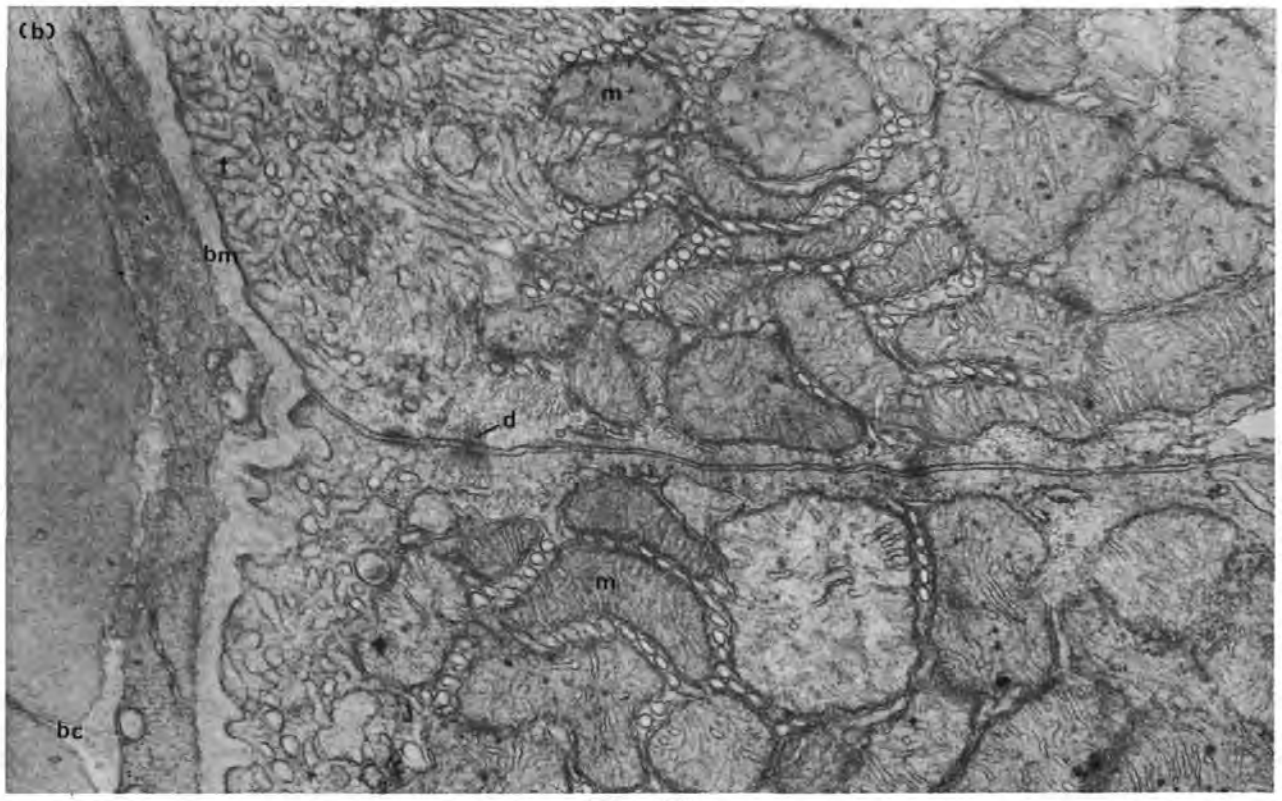
(*Plate Va*)



(*Plate IVb*)

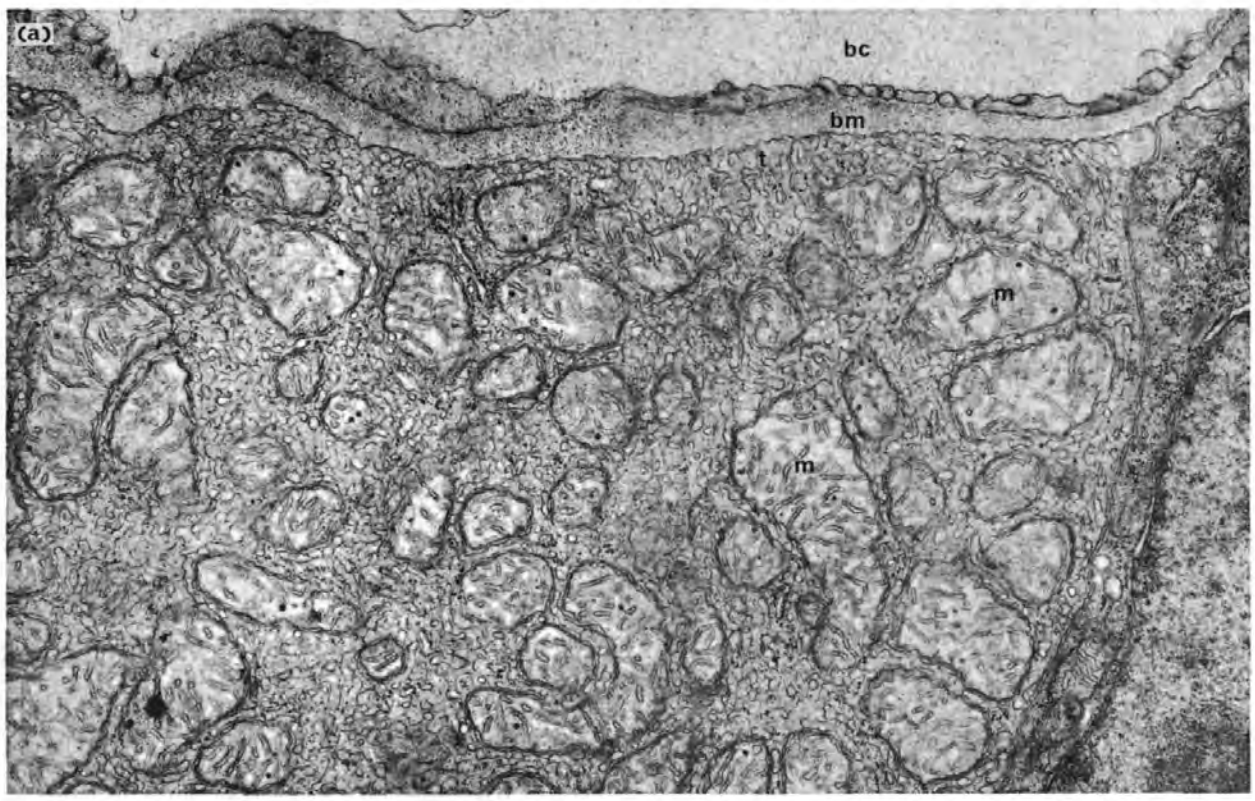
PLATE IV. (a) Electron micrograph of section through base of 'chloride' cell of pseudobranch of bass kept in 0·34‰ salinity. ×25 000. m, mitochondria; t, tubules; bc, blood channel; bm, basement membrane. (b) Electron micrograph of section through 'chloride' cell of pseudobranch of bass kept in 0·34‰ salinity. ×14 700. m, mitochondria; t, tubules; v, vesicles; ec, flattened epithelial cells; d, desmosome; ac, covered apex of cell.

(*Plate IIIa*)



(*Plate IIIb*)

PLATE III. (a) Light micrograph of transverse section through filament and secondary lamellae of smelt kept in sea water (34‰ salinity). Resin section stained methylene blue. ×2200. cc, chloride cell; pc, pseudobranch cell; fc, filament cartilage; cco, central compartment; bc, blood channel. (b) Electron micrograph of section through base of two adjacent 'pseudobranch' cells of trout kept in fresh water. ×40 600. m, mitochondria; bm, basement membrane; t, tubules; bc, blood channel; d, desmosome.



(*Plate IVa*)

from invaginations of the plasma membrane at the vascular border [Plate II(a)]. The presence of these saccules has not been observed in the ' chloride ' cell of the gill.

The central region of the pseudobranch ' chloride type ' cell is rich in mitochondria which are surrounded by a highly branching network of intracellular tubules which connect with the narrow saccules near the vascular border. Occasional profiles of granular endoplasmic reticulum are also seen associated with the nucleus, and clusters of ribosomes are scattered throughout the cell. The mitochondria are absent from the apical region of the cell as are most of the branching tubules which are replaced by large numbers of smooth surfaced vesicles. The apical pit opens between flattened epithelial cells which overhang its edges [Plate I(b)]. As in the gill there is only one apical pit per cell although more than one cell sometimes opens into the same pit. There appear to be fewer cytoplasmic projections and fewer vesicles within the pit of the pseudobranch ' chloride type ' cell than in that of the gill.

The second cell type is specific to the pseudobranch so is commonly referred to as the ' pseudobranch type ' cell [Plate II(b)]. It can be distinguished from the ' chloride type ' cell by several ultrastructural characteristics and the fact that it shows no contact with the external environment, i.e. it does not possess an apical pit. The most characteristic feature of ' pseudobranch type ' cells is the orderly arrangement of parallel tubules around the closely packed mitochondria. The mitochondria are arranged in the centre and vascular pole of the cell but are absent from the distal region. The plasma membrane at the vascular border is greatly enfolded and connects with the intracellular tubules, but there are no narrow saccules characteristic of the ' chloride type ' cells.

(*b*) *Smelt* (Osmerus esperlangus)

The light microscope observations on the smelt pseudobranch show that it does not fall within the category of a ' free ' pseudobranch as indicated by *in situ* examination of the organ. Careful examination of the pseudobranch reveals that although the filaments remain free, as in the bass, the lamellae are fused for much of their length. The smelt pseudobranch from full sea water still contains both ' chloride ' and ' pseudobranch type ' cells which are virtually identical to those found in the bass. Once again the ' chloride type ' cells are associated with the afferent artery, and the ' pseudobranch type ' cells are associated with the efferent artery as shown by the oblique section of a pseudobranch filament in Plate III(a). The lamellae are completely fused in the region of the ' pseudobranch type ' cells, isolating them completely from the external environment. In the ' chloride ' cells region the lamellae are only partially fused between adjacent epithelial cells and there are still ' free ' areas where the chloride cells give rise to apical pits.

(*c*) *Rainbow trout* (Salmo gairdneri)

The pseudobranch of rainbow trout in fresh water loses any resemblance to a normal gill. Although the circulatory pattern is retained, the secondary lamellae and the filaments are fused together so that water can no longer move between them. The organ is also covered by an epithelial layer so that it loses all contact with the external environment. The circulatory system supplies only ' pseudobranch type ' cells similar to those of the bass and the smelt, but there are no ' chloride type ' cells present and thus no communication with the water. The ' pseudobranch type ' cells

of the trout are even more highly organised than those of the bass and smelt, with closer packed mitochondria and tubules [Plate III(b)].

STRUCTURE OF THE PSEUDOBRANCH AFTER EXPOSURE TO WATER OF VARIOUS SALINITIES

(*a*) *Bass*

Both the ' chloride ' and ' pseudobranch type ' cells of bass exposed to 0·34‰ salinity for 5–24 h showed ultrastructural changes. After 5 h the mitochondria of the chloride cells were distended and often vacuolated [Plate IV(a) and (b)]. They usually possessed fewer cristae and were less electron dense than those from full sea water. The numbers of cytoplasmic tubules surrounding the mitochondria increased and they became more extensively branched. In some cells the tubules showed a more orderly arrangement around the mitochondria although nowhere near the extent shown in normal ' pseudobranch type ' cells. The narrow saccules near the vascular border were less extensive and tended to be arranged parallel to the vascular border rather than at right angles as in the normal chloride cell. In some cells the saccules had disappeared completely. There appeared to be a larger number of smooth vesicles at the apex of the cell and the apical pit had disappeared from many cells. The pits were either much smaller and rounded off or were completely covered with overlapping epithelial cells [Plate IV(b)].

There was little further change after 10 h but after 24 h spherical electron dense inclusions had appeared within the cells. The membrane bound inclusions were smaller than the mitochondria with a diameter of 0·4–0·8 μm and contained no apparent substructure. In the case of the ' pseudobranch type ' cells very little ultrastructural change had occurred after 5 and 10 h, apart from a slight increase in the number of glycogen particles. After 24 h there was a large increase in the number of glycogen particles, and many spherical, electron dense inclusions had appeared [Plate V(a)]. The inclusions appeared to be identical to those found in the ' chloride ' cell but were far more numerous in the ' pseudobranch type ' cell. They tended to aggregate at the anti-vascular pole of the cell and were often vacuolated. The tubular network of some cells was more randomly branched than that of cells from full sea water.

(*b*) *Smelt*

No significant changes were seen in either the ' chloride ' or ' pseudobranch type ' cells of smelt held in water of 24‰ and 16‰ salinity. ' Chloride type ' cells from fish adapted to 8‰ salinity contained a more highly branched system of tubules but they were still distinct from the ' pseudobranch type ' cells which remained unchanged. In smelt adapted to fresh water the epithelial sheet covering the lamellae became more extensive and frequently covered the ' chloride type ' cells completely, so that no apical pits were present. Where there was a gap in the layer of the epithelium, instead of a pit, the cytoplasm of the cell often protruded beyond the edges of the epithelial cells [Plate V(b)]. These protrusions of the cytoplasm sometimes had an irregular surface and contained elements of the tubular system, along with an amorphous sometimes granular ground substance. The ' pseudobranch type ' cells of the freshwater adapted smelt corresponded closely to those of the seawater adapted fish.

(*c*) *Rainbow Trout*

In the lower salinities, up to 34‰, the ' pseudobranch type ' cells showed little ultrastructural change although the lamellae did appear slightly distended in some cases and the mitochondria tended to aggregate at the vascular pole of the cell. At 34‰, 42‰ and 60‰ salinity the cells still retained the characteristic pseudobranch appearance although many cells appeared to be degenerating with vacuolation of the mitochondria and cytoplasm. A few cells showed reorganisation of their cytoplasmic contents to a certain extent (Plate VI). Most noticeable was the more highly branched appearance of the tubules (contrasting with the orderly, parallel arrangement characteristic of ' pseudobranch type ' cells) and the disruption of the plasma membrane at the vascular border with loss of communication to the blood channel.

IV. DISCUSSION

The results show that the cells from the pseudobranch of all three fish studied undergo ultrastructural changes upon exposure to osmotic stress. The degree and rapidity of change appears to depend on whether the stress is applied quickly or gradually. The cells from bass pseudobranch showed a more rapid, and greater degree of change than the gradually acclimated smelt and rainbow trout. This is to be expected since the bass pseudobranch cells were given no chance to adapt to a changing osmotic environment.

The cytological changes due to drastic osmotic stress, as in the bass, or the gradual stress to the trout and smelt, gave no convincing evidence to support Newstead's theory (1971) that ' chloride type ' cells could undergo rearrangement of their cytoplasmic components into the pattern characteristic of ' pseudobranch type ' cells. The loss of apical pits in the ' chloride type ' cells of the smelt and bass pseudobranch in fresh water and dilute sea water parallels the response of ' chloride ' cells in the gill epithelium of *Oligocottus maculosus* (Newstead, 1971) when exposed to fresh water, and that of similar cells in *Fundulus heteroclitus* (Kessel & Beams, 1962; Philpott & Copeland, 1963), *Anguilla rostrata* (Getman, 1950; Shirai & Utida, 1970), *Monodactylus argenteus* (Fearnhead & Fabian, 1971), *Salmo salar* L. (Threadgold & Houston, 1964) and *Etroplus maculatus* (Virabhadrachari, 1961). Although the arrangement of the tubules and mitochondria did become more orderly in some ' chloride type ' cells of bass and smelt, they still remained easily distinguishable from ' pseudobranch type ' cells.

In rainbow trout adapted to salinities between 10‰ and 34‰ the distended lamellae and dilated blood channels may be the result of an increased ' work load ' on the pseudobranch due to exposure to increased environmental salinity. It is possible that this response to osmotic stress represents a homeostatic mechanism. The appearance of the ' pseudobranch type ' cells at higher salinities suggests that they are degenerating and that the fish are unable to cope with further loss of body fluids and build up of salts. Also, the characteristic orderly arrangement of mitochondria and cytoplasmic tubules was replaced in some cells (at 60‰) by a more randomly branched system. Although such changes in fine structure were observed, many cells retained their ' pseudobranch type ' characteristics. In addition, the cells that did show rearrangement of their organelles could not convincingly be designated as ' chloride type ' cells.

The ' pseudobranch type ' cells of the smelt were almost identical to those of the rainbow trout and no significant reorganisation of cytoplasmic contents was noted

in fish gradually subjected to reduced environmental salinities. The appearance of spherical electron dense inclusions in the chloride and ' pseudobranch type ' cells of bass after 24 h exposure to 0·34‰ salinity is probably a result of a rapid disturbance of the normal homeostasis of the blood with subsequent effects on the normal metabolism of the cells. The inclusions may represent some waste or breakdown product of the cell, such as lipid from organelle membranes.

The ' chloride ' cell of the gills of teleost fish is commonly regarded as the site of extrarenal salt regulation (Smith, 1930; Keys & Willmer, 1932; Copeland, 1948; Philpott & Copeland, 1963; Threadgold & Houston, 1964). The ' chloride ' cells of marine teleosts are thought to secrete electrolytes whereas those of fresh water and freshwater adapted fish are thought to act in the reverse manner (Krogh, 1937; Pettengill & Copeland, 1948; Maetz & Romeu, 1964; Romeu & Maetz, 1964). Histochemical evidence has been presented to show accumulation of chloride in ' chloride ' cells, particularly in the region of the apical pit (Copeland, 1948; Datta Munshi, 1964; Philpott, 1966; Petřík, 1968; Fearnhead & Fabian, 1971). In addition, the cytological changes reported in ' chloride ' cells in response to both salt anp freshwater adaptation of fish (Getman, 1950; Philpott & Copeland, 1963; Fearnhead & Fabian, 1971) and the corresponding results for smelt and bass pseudobranch (this study) strengthen the hypothesis that these cells are indeed involved in osmoregulation.

Parry & Holliday (1960) objected to the pseudobranch having any osmoregulatory role since fish that had undergone pseudobranchectomy were apparently able to osmoregulate quite normally and showed no significant difference in survival time to control fish, when exposed to various sea-water dilutions. However, they believed that all the cells of the pseudobranch were the same or similar to the ' chloride ' cells of the gill. Whilst it is true that marine and euryhaline fish do contain ' chloride type ' cells as well as ' pseudobranch type ' cells, freshwater fish such as *Salmo gairdneri*, one of their experimental fish, contain only ' pseudobranch type ' cells.

The presence of ' chloride type ' cells on the surface of the pseudobranch lamellae of marine and euryhaline teleosts probably results from a need in these fish to utilise all available surface area for extra-renal salt excretion, but the removal of the pseudobranchs may not drastically affect the fish's ability to osmoregulate since the ' chloride ' cells of the gill can still carry out this function. It is likely that the absence of ' chloride type ' cells in rainbow trout and other freshwater fish reflects a less demanding environment in respect of osmoregulation and that the pseudobranch of these fish is not directly involved in osmoregulation.

The function of the ' pseudobranch type ' cell remains uncertain. No apparent respiratory function exists and from the results of this study, no direct role in osmoregulation is likely. Newstead (1971) believed that ' the more intimate association of mitochondria and tubule membrane in the " pseudobranch type " cells may reflect a shift toward an arrangement more effective in ion transport than in the un-orientated arrangement found in ' chloride ' cells of unstressed animals '. Copeland & Dalton (1959) also express the view that the elaborate organisation of the pseudobranch cell indicates the existence of a morphological adaptation to a special function. They suggest, however, that this specialisation is possibly related to the production of carbonic anhydrase (after Leiner, 1940; Sobotka & Kann, 1941; Maetz, 1956); Parry & Holliday (1960) believe the pseudobranch has an endocrine role related to the control of chromatophores in the skin.

The investigations of Laurent & Dunel (1966) showed that the pseudobranch is richly innervated and they suggest a functional relationship between afferent type nerve endings and the pseudobranch cell. As Laurent & Rouzeau (1972) suggested it is possible that neural receptors intervene in the mechanisms of osmoregulation in fish. Therefore, there may well be an indirect relationship between the pseudobranch and osmoregulation with the pseudobranch cell and its associated nerve ending acting as a functional unit monitoring the osmolarity of the body fluids.

In conclusion, the view expressed by Harb & Copeland (1969) that ' chloride type ' and ' pseudobranch type ' cells are distinct cell types seems the more appropriate explanation of the results obtained here for bass, smelt and rainbow trout.

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Distribution and development of rodlet cells in the gills and pseudobranch of the bass, *Dicentrachus labrax* (L)

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Rodlet cells in various stages of development were found in large numbers in the bass gill and pseudobranch. In the gill, rodlet cells were found in the epithelium at the base of the secondary lamellae and on the filament between adjacent lamellae, whilst in the pseudobranch they were found over the whole area of the secondary lamellae as well as in the filament epithelium.

During early development, rodlet cells are characterised by their amorphous cell inclusions, prominent supranuclear Golgi complex and network of granular endoplasmic reticulum. Later, with formation of a fibrous border the arrangement of the cell organelles undergoes reorganisation; the endoplasmic reticulum becomes distended, numerous vesicles appear and the mitochondria aggregate in the apical region of the cell. One of the most striking features is the development of club-shaped sacs containing electron dense cores, which are orientated towards the open apex of the cell.

Various staining properties of rodlet cells for light and electron microscopy were compared with those of mucous cells found in the same tissues. Possible functions of the cell are discussed.

I. INTRODUCTION

Although the existence of rodlet cells in various fish tissues has been known since 1892, when Thélohan described a peculiar cell he considered to be a parasite, the exact nature and function of these cells has not been elucidated. Laguesse (1906) named them *Rhabdospora thelohani*, believing them to be sporocyst stages of a sporozoan parasite. The view that these cells are parasites is also held by Laibach (1937), Dawe *et al.* (1964), Hale (1965), Bannister (1966), Iwai (1968), Mourier (1970) and Anderson *et al.* (1976). However more recent studies suggest that they are not protozoan parasites but are integral constituents of the tissue in which they are found, and that they may have a secretory function (Leino, 1972, 1974; Desser & Lester, 1975; Morrison & Odense, 1978). The possible glandular nature of the cell is supported by Plehn (1906), Klust (1939), Barrington (1957), Bullock (1963), Bishop & Odense (1966) and Wilson & Westerman (1967). Duthie (1939) believes they are a type of granular leukocyte, a possibility also proposed by Weinreb and Bilstad (1955), Catton (1957) and Gohar & Latif (1961). Flood *et al.* (1975) consider the cell to behave as a foreign body which interacts with the epithelial tissue of the bulbus arteriosus. Al-Hussaini (1949) and Vickers (1962) suggest that rodlet cells may be modified or abnormal goblet cells.

The present study deals with the ultrastructure of immature, developing and mature rodlet cells in bass gill and pseudobranch, their distribution and relation to surrounding cells, and correlation of their structure and staining properties with their possible function.

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II. MATERIALS AND METHODS

Bass measuring 20–25 cm were killed by a blow on the head. The gill arches and both pseudobranchs were removed, cut into pieces, and fixed in 2·5% glutaraldeleyde in 0·1 M phosphate buffer for 1 h at 4° C. The tissues were washed in buffer containing 0·54% glucose and post fixed in 1% osmium tetroxide for 1 h, followed by dehydration in alcohol and embedding in pure Spurr resin (Spurr, 1969). Sections were cut on a Porter Blum ultramicrotome. Sections 1 μm thick were stained with 1% methylene blue in 1% borax and examined in a light microscope, whilst thin sections were stained with uranyl acetate and lead citrate, and examined in a Philips E.M.300 electron microscope.

Thin sections were also stained by 15% phosphotungstic acid (P.T.A.) at pH 2 for 2 h, following oxidation by 1% hydrogen peroxide for 30 min. P.T.A. is reported to be a specific stain for acid mucopolysaccharides and glycoproteins at low pH (Marinozzi, 1967, 1968; Pearse, 1968, 1970). Sections were also treated with 1% P.T.A. in 10% chromic acid (pH 0·3) following oxidation by 1% periodic acid (Tsuchiya & Ogawa, 1973).

Paraffin-wax sections of Bouin or formalin-fixed blocks of gill and pseudobranch were stained by the following methods: Mallory's triple stain, alcian blue at pH 2·5, and periodic acid-Schiff (P.A.S.) (Pearse, 1968).

III. RESULTS

DISTRIBUTION OF RODLET CELLS

In the gill, rodlet cells were found at the base of the secondary lamellae, on the filament between adjacent lamellae and along the edges of the filaments. They were often in close association with chloride and mucous cells, and were usually separated from the blood space by flattened epithelial or suprabasement cells and the basement membrane. No rodlet cells were found in the respiratory epithelium of the lamellae.

Mature rodlet cells in the pseudobranch were found as a single layer of cells just below the surface of the epithelium covering the area of the secondary lamellae (Plate I). As in the gill they were also found on the edges of the filament and between, adjacent lamellae. In the secondary lamellae they were separated from the blood space by a thin layer of flattened epithelial cells, an underlying layer of either chloride or pseudobranch cells, and the basement membrane.

In both gill and psuedobranch, rodlet cells were more numerous in the region of the afferent filament artery compared with the efferent artery. They were present in all the gills and pseudobranchs of 10 fish that were examined. It was difficult to make an accurate assessment of the numbers of rodlet cells present in a gill arch or a pseudobranch since there were large variations between individual filaments and also between individual fish. In a random sample of 10 pseudobranch filaments from each of 5 fish, the density of rodlet cells ranged from 122 to 7575 per sq. mm with a mean of 2974 per sq. mm.

DEVELOPMENT OF RODLET CELLS

Many of the rodlet cells appeared to be in different stages of development. The mature stage (Plate II) and stage preceding it (Plate V) are easily recognisable, being unlike any other cells in the epithelium. Various characteristics of these two stages were used to identify the developing and immature phases of rodlet cell maturation.

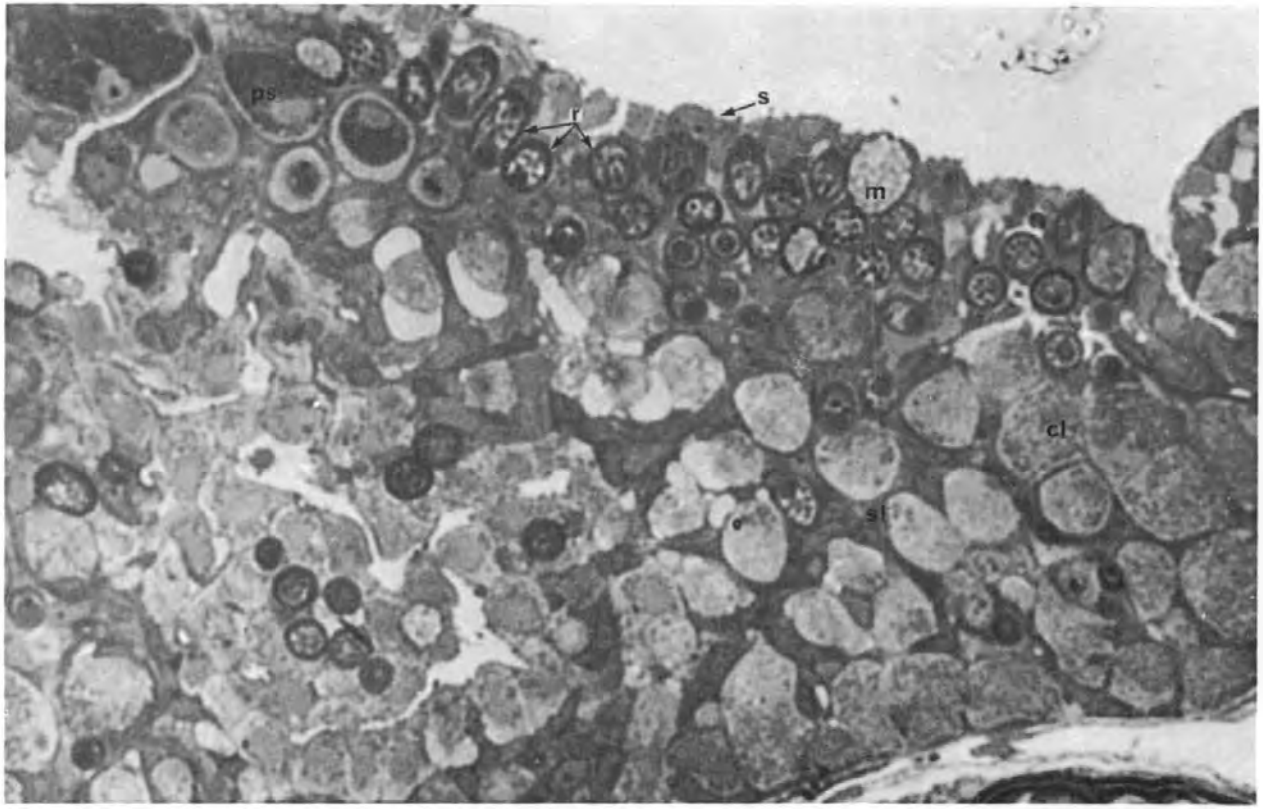

PLATE I. Light micrograph of rodlet cells (r) in the secondary lamellae (sl) of the pseudobranch. cl, chloride cell; m, mucous cell; ps, pseudobranch cell; s, surface of secondary lamellae. Resin section stained with methylene blue, ×852.

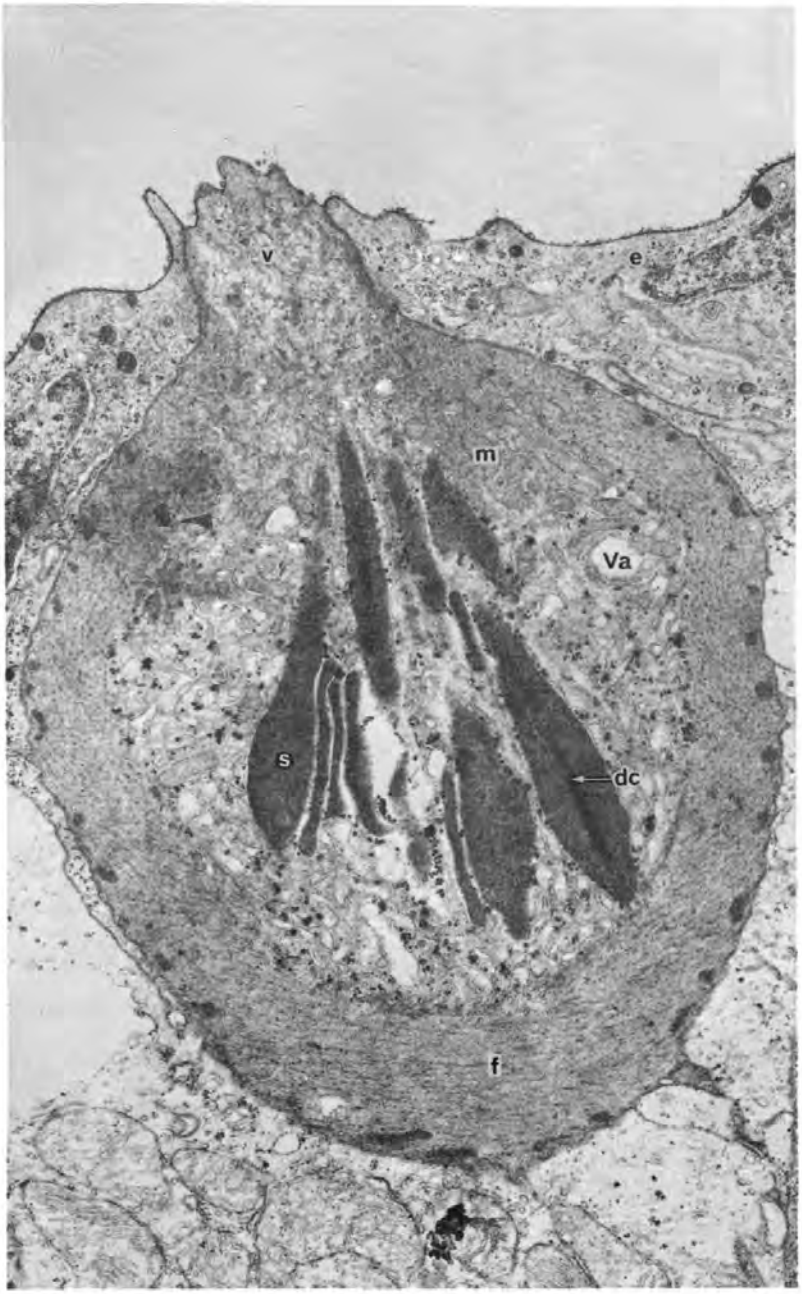

PLATE II. Electron micrograph of mature rodlet cell at the surface of the pseudobranch epithelium. dc, dense central core; e, flattened epithelial cell; f, fibrous cytoplasmic border; m, mitochondria; s, rodlet sac; v, vesicles; va, vacuole, ×20 145.

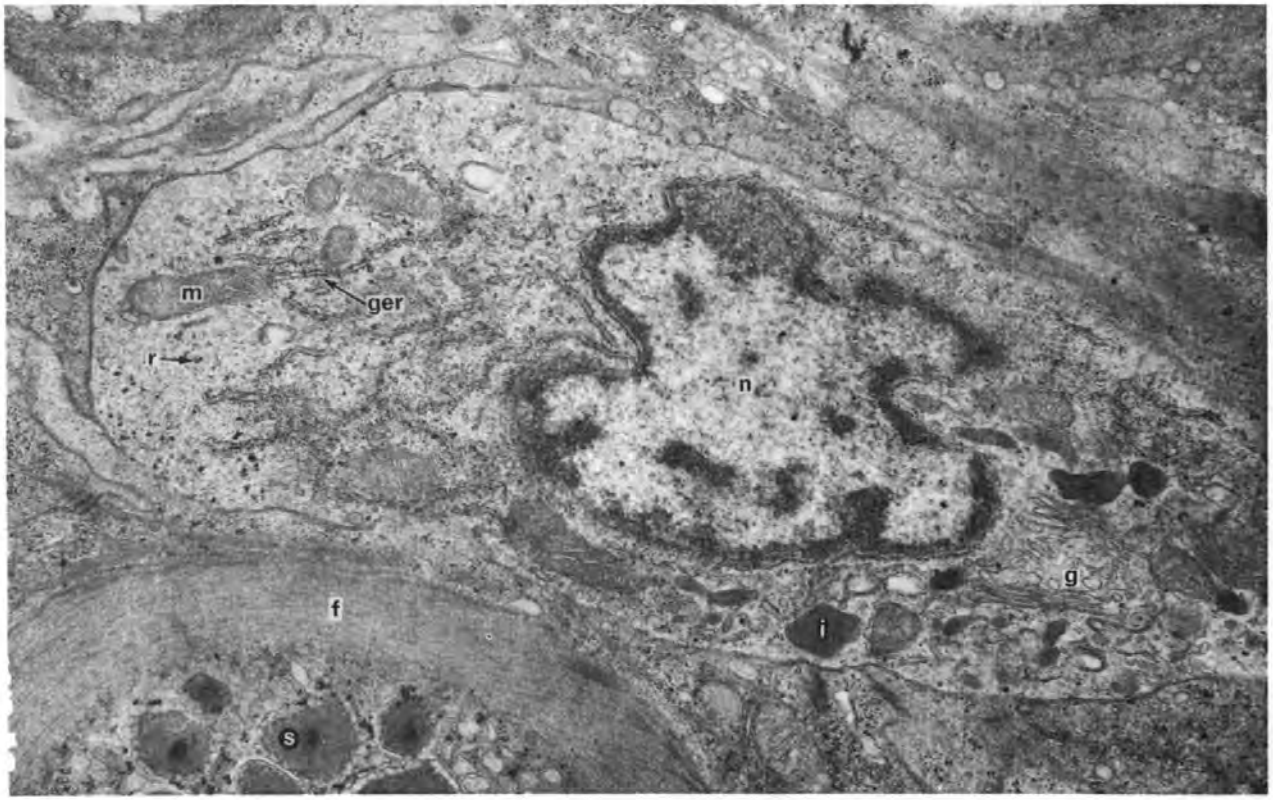

PLATE III. Immature rodlet cell (Type I). f, fibrous cytoplasmic border of mature rodlet cell; g, Golgi complex; ger, granular endoplasmic reticulum; i, membrane bound inclusion; m, mitochondrion; n, nucleus; r, ribosomes; s, rodlet sac. ×20 161.

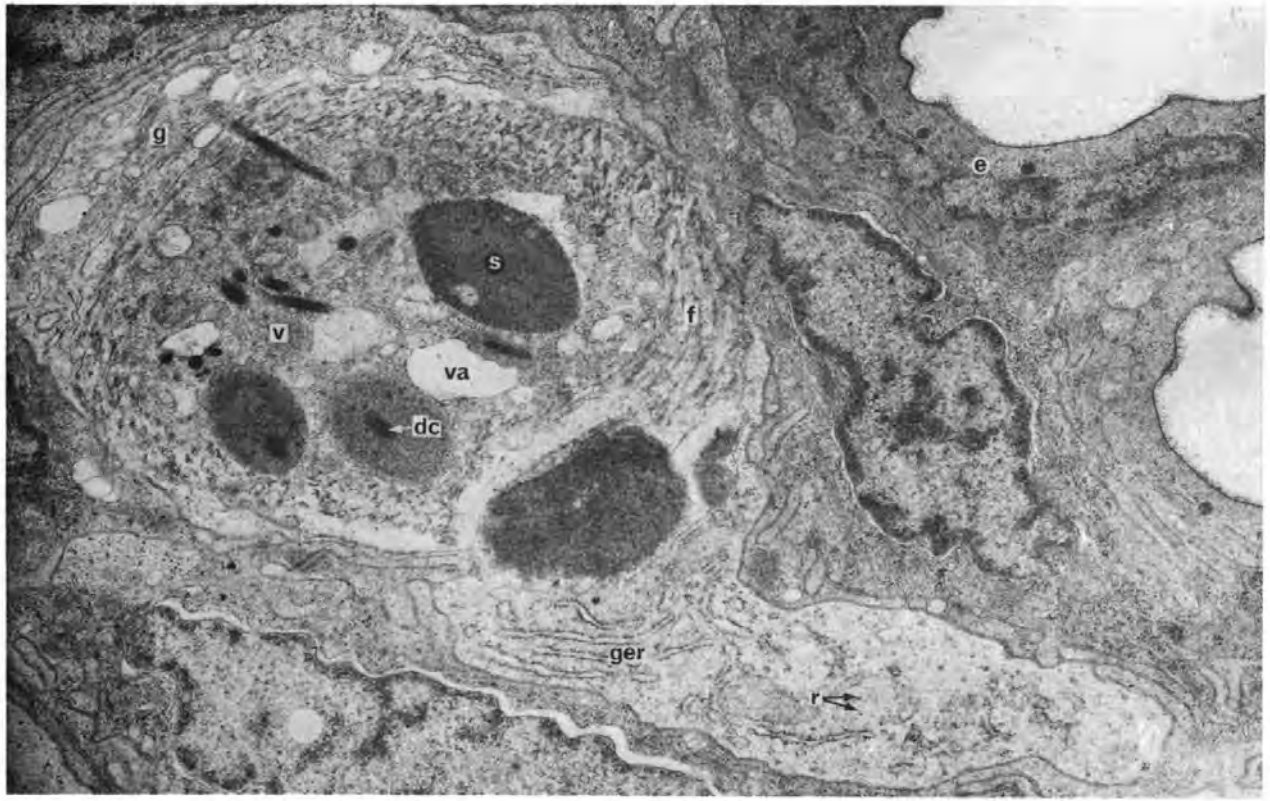

PLATE IV. Developing rodlet cell (Type II). dc, dense central core; e, epithelial cell; f, fibrous cytoplasmic border; g, Golgi complex; ger, granular endoplasmic reticulum; r, ribosomes; v, vesicles; va, vacuole. ×15 020.

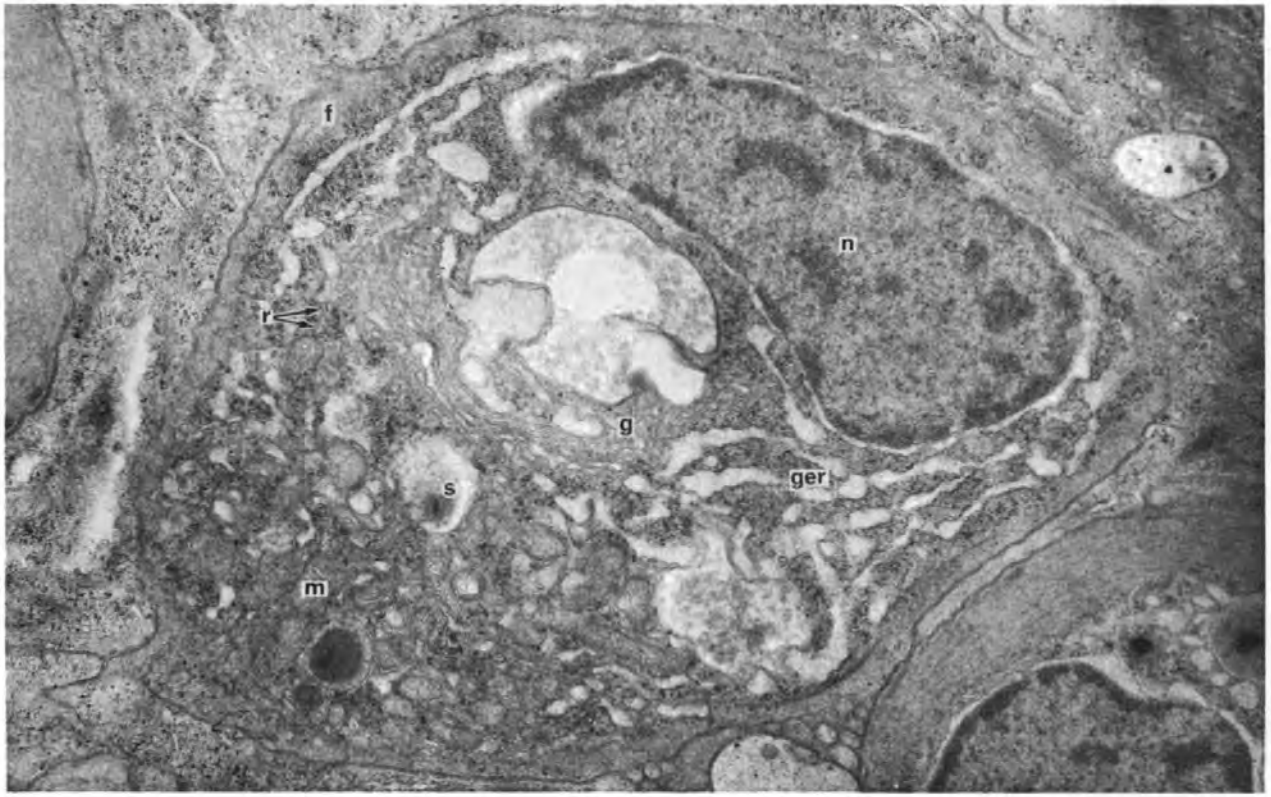

PLATE V. Immature rodlet cell (Type III). f, fibrous cytoplasmic border; g, Golgi complex; ger, granular endoplasmic reticulum; m, mitochondria; n, nucleus; r, ribosomes; s, developing rodlet sac. ×19 075.

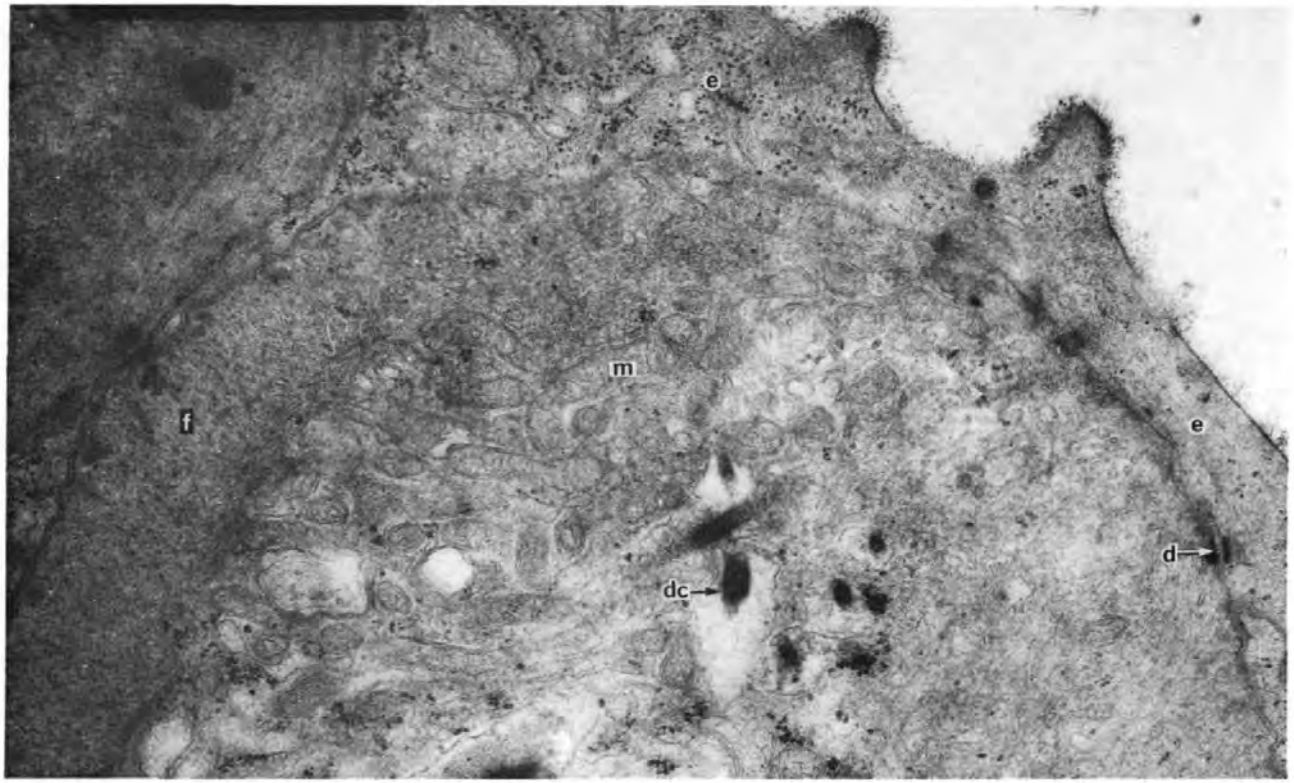

PLATE VI. Apex of mature rodlet cell near the epithelium surface showing demosome contact (d) with an epithelial cell (e). dc, dense central core; f, fibrous cytoplasmic border; m, mitochondria, ×36 720.

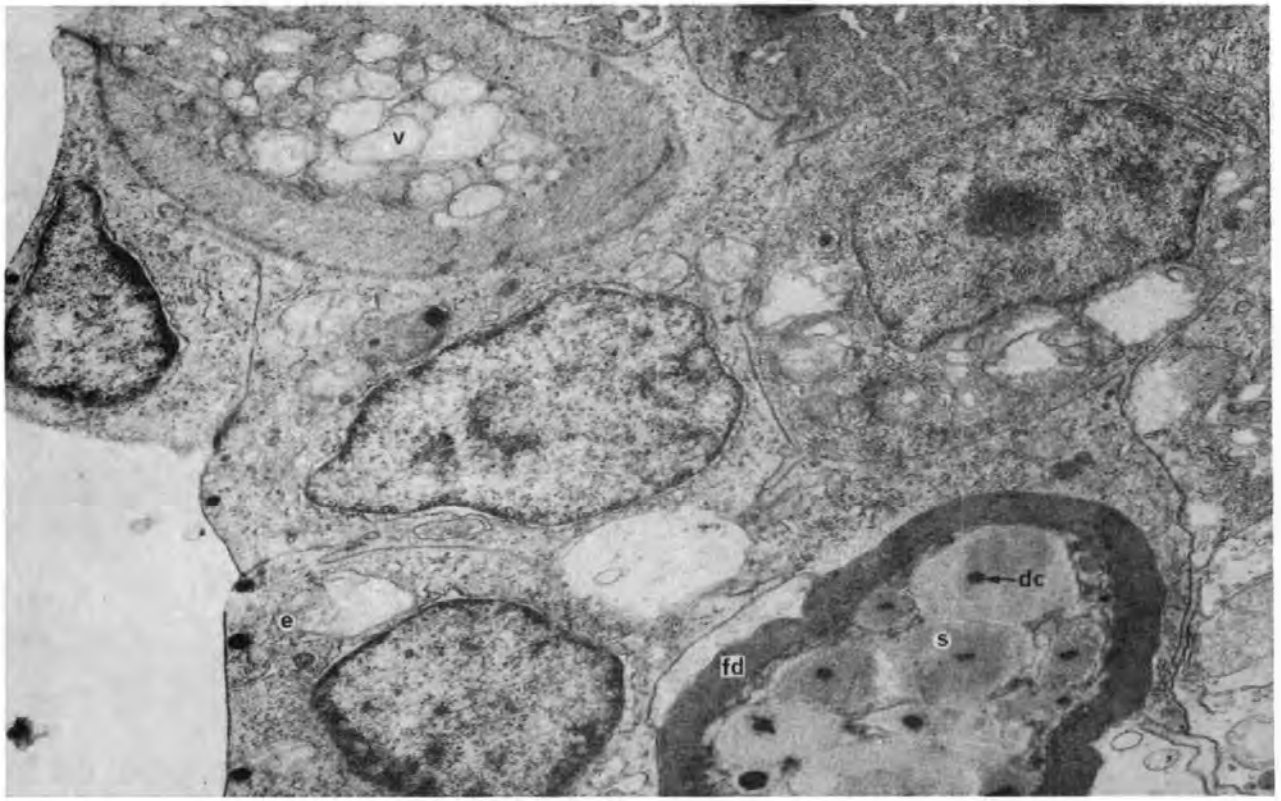

PLATE VII. Some rodlet cells contain large membranous vacuoles (V) or a dark fibrous border (fd). dc, dense central core; e, epithelila cell; s, rodlet sac. 14 435.

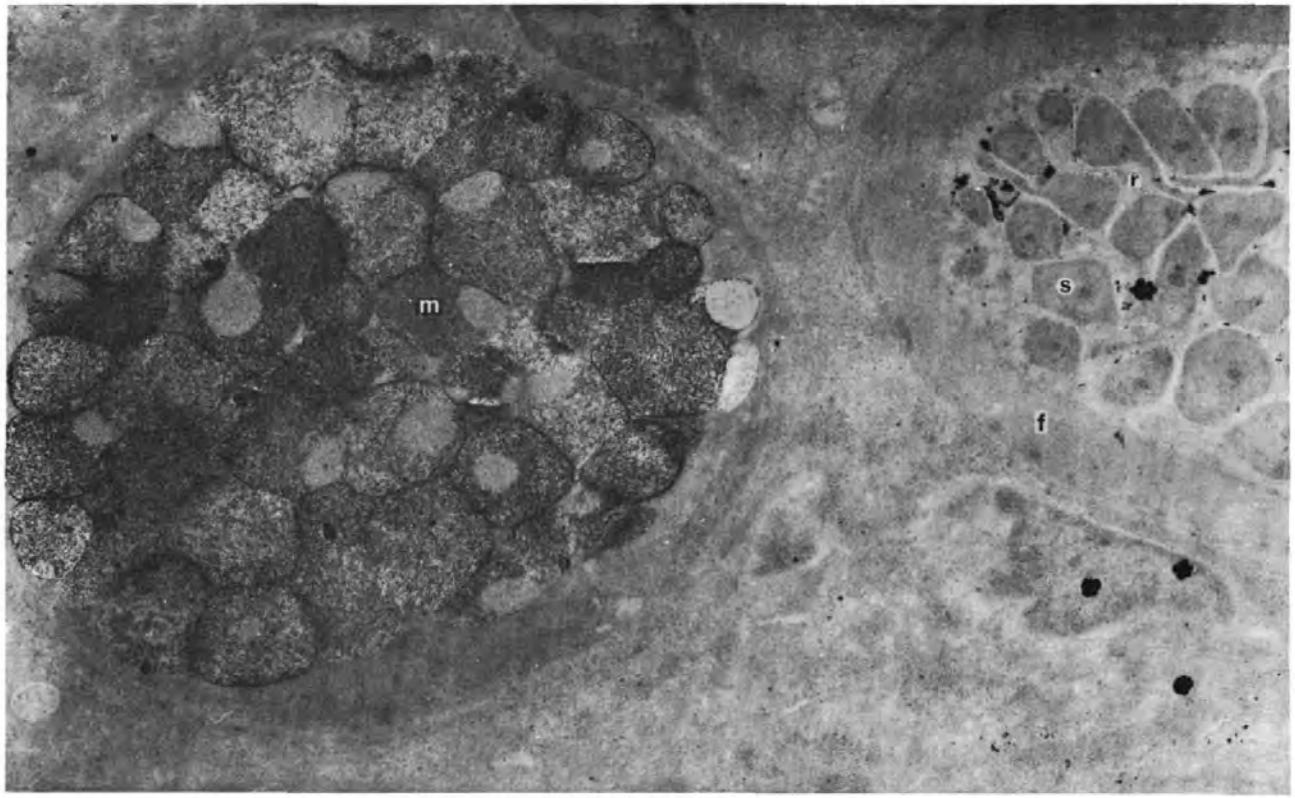

PLATE VIII. Mucous cell and rodlet cell after staining with 15% P.T.A. (pH2). Mucous cell globules (m) and membranes are stained whilst rodlet cell (r) sacs (s) and fibrous border (f) remain unstained. ×12 980.

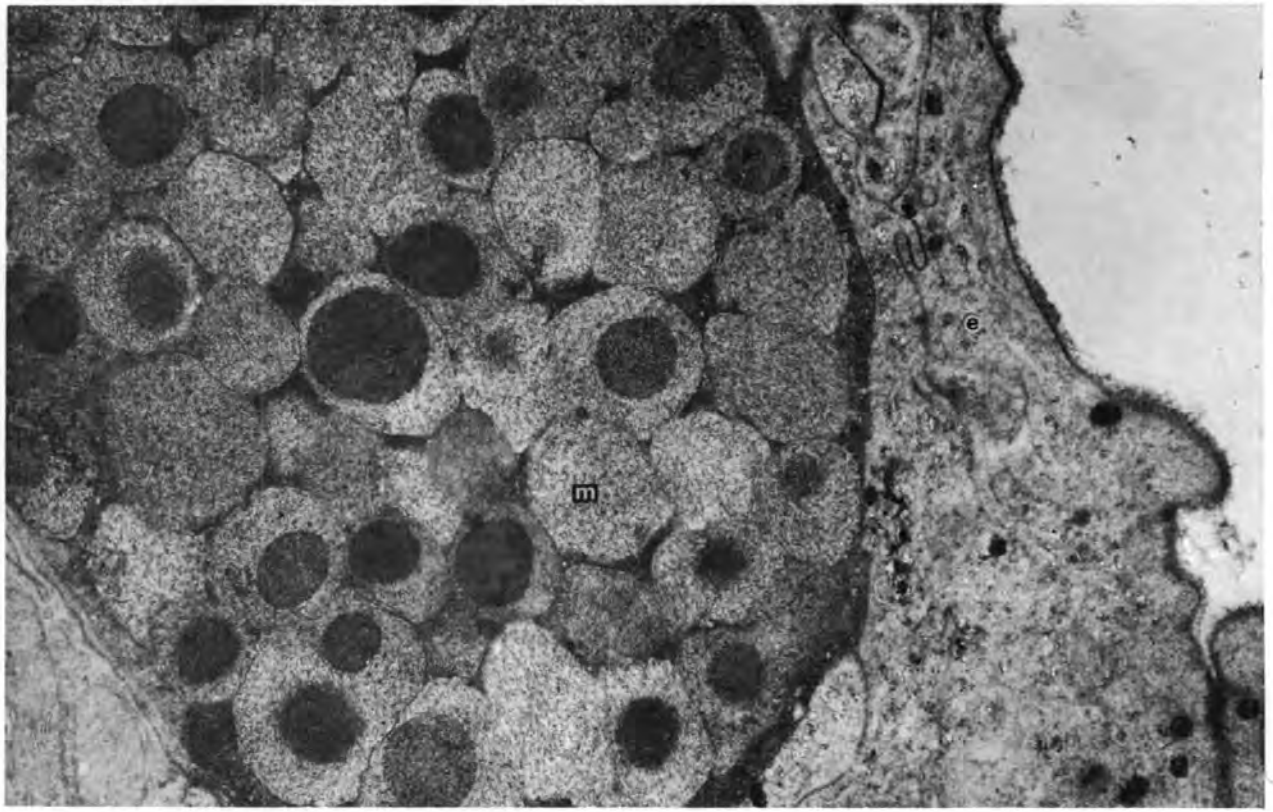

PLATE X. Mucous cell (m) stained with 1% P.T.A. (pH 0·3). The majority of globules are lightly stained with a densely stained central region. e, epithelial cell. ×3 760.

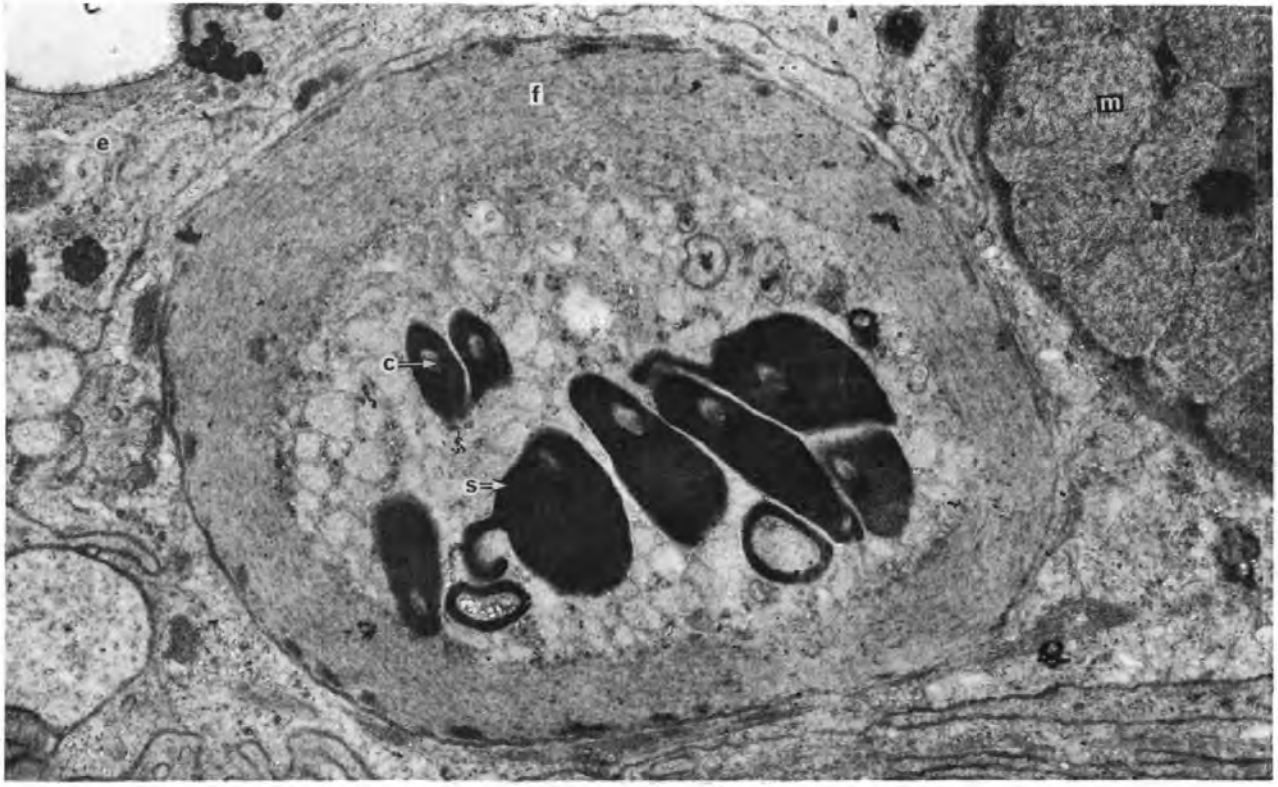

PLATE IX. Rodlet cell stained with 1% P.T.A. (pH 0·3). The sacs (s) are densely stained but the central core (c) remains unstained. e, epithelial cell; f, fibrous border; m, mucous cell. ×18 302.

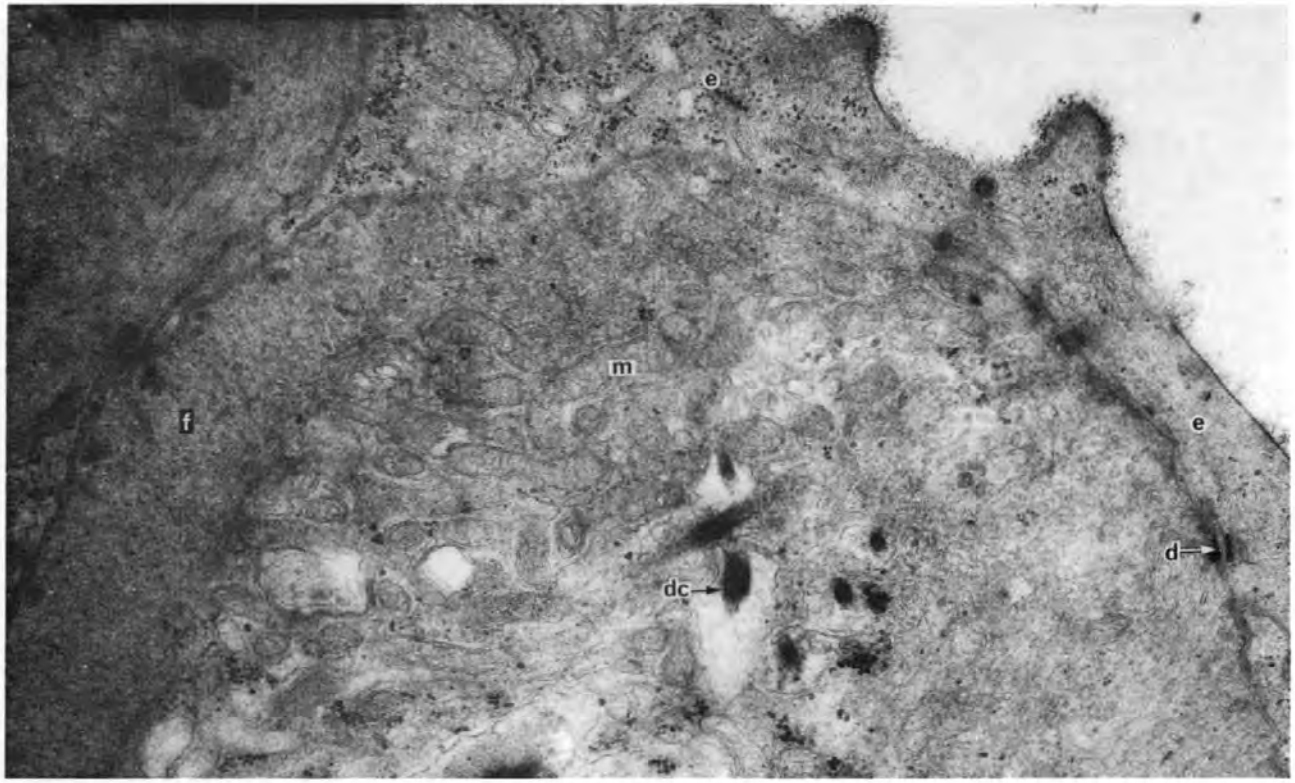

PLATE VI. Apex of mature rodlet cell near the epithelium surface showing demosome contact (d) with an epithelial cell (e). dc, dense central core; f, fibrous cytoplasmic border; m, mitochondria, ×36 720.

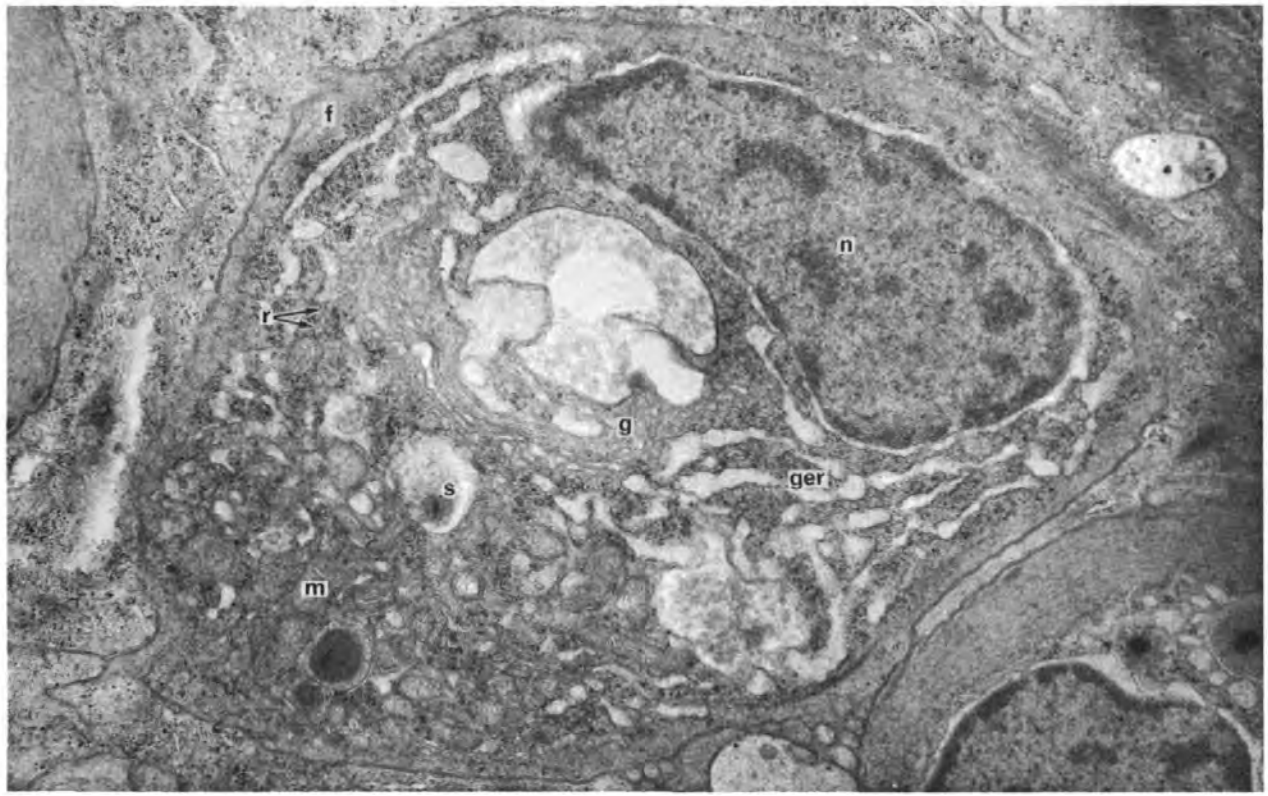

PLATE V. Immature rodlet cell (Type III). f, fibrous cytoplasmic border; g, Golgi complex; ger, granular endoplasmic reticulum; m, mitochondria; n, nucleus; r, ribosomes; s, developing rodlet sac. ×19 075.

The earliest development of rodlet cells can be traced back to a cell (Type I) containing a network of granular endoplasmic reticulum and free ribosomes at one end and a Golgi complex of numerous saccules and vesicles at the other (Plate III). There are a number of electron dense inclusions in the region of the Golgi complex. The nucleus has a light coloured nucleoplasm with darker clumps of heterochromatin, especially around the periphery, and a few mitochondria are scattered throughout the cytoplasm. These cells are often associated with maturing rodlet cells in the sub-surface epithelial tissues.

The next stage appears to be an intermediate cell type (Type II) which contains characteristics of both mature and immature rodlet cells (Plate IV). The cell contains a network of granular endoplasmic reticulum and free ribosomes at one end similar to cell type I. At the opposite end of the cell is a prominent Golgi complex with flattened saccules, large irregularly shaped vacuoles, and numerous smooth vesicles. Cell type II shows the earliest development of a fibrous border, consisting of a layer of widely spread microfibrils beneath the plasma membrane, and surrounding the vesicles and a few rodlet sacs. Some of the sacs contain electron dense cores. A few apparently isolated dense cores are present which may represent the tips of rodlet sacs where they are surrounded by little or no sac material. The cell is close to the surface of the secondary lamellae and is covered only by a thin layer of flattened epithelial cells.

As the cell matures it becomes completely enclosed by a thin fibrous border (0·1 – 0·2 μm wide) beneath the plasma membrane (Cell type III Plate V). The cisternae of the granular endoplasmic reticulum becomes distended and contain a light flocculent material. The granular endoplasmic reticulum is continuous with a large Golgi complex and large irregularly shaped areas containing a similar flocculent material. Numerous free ribosomes are present throughout the cytoplasm and the mitochondria aggregate at the opposite end of the cell to the nucleus which becomes darker and more regular in shape.

In the mature rodlet cell the fibrous border becomes much wider, usually about 0·5 μm but up to 1·5 μm wide (Plate II). It is bounded on the outside by a plasma membrane 100–125 Å thick. A fibrous border contains numerous microfibrils, 300–500 Å apart, arranged in parallel around the circumference of the cell. Beneath the plasma membrane are regularly spaced dense bands which run parallel to the microfibrils. The general appearance of the cytoplasmic border is somewhat similar to that of smooth muscle cell cytoplasm and it is possible that the encasing layer is capable of contractile activity.

The club shaped rodlet sacs take up a large part of the cytoplasm of the mature cell. Each sac is bounded by a membrane and contains a granular matrix which is less concentrated and sometimes almost absent from the peripheral region of the sac. The sacs usually contain a single electron dense core which may extend the whole length of the sac. The cores are circular in cross section and vary in diameter from 1000–1600 Å. The sacs are orientated with the tapered end towards the apex of the cell and bulbous end anterior to the basal nucleus.

A closely packed aggregation of mitochondria is found laterally near the apex of the cell. The fibrous border is absent in the apical region, which contains large numbers of smooth surfaced vesicles and is bounded only by the plasma membrane.

The remainder of the cytoplasm contains many clear vesicles, a few multi-

vesicular lysosome-like bodies and a network of membranous vacuoles containing a lightly staining material. The Golgi complex still occupies a supranuclear position and contains numerous saccules and vesicles. Ribosomes are present throughout the cytoplasm apart from the apical region of the cell.

The apex of mature cells opens between adjacent epithelial cells. The cytoplasm protruding through the opening is bounded by a limiting membrane which is covered with a filamentous glycocalyx. Near the apex desmosomal contact between rodlet cells and surrounding epithelial cells is sometimes found (Plate VI).

Rodlet cells containing only membranous vacuoles or no cytoplasmic contents at all were observed. These may have secreted their contents and are dead or dying cells. A few rodlet cells with very dark borders have been observed (Plate VII) and some cells contained hyperchromatic nuclei. No cells were observed in the process of secreting their contents.

STAINING PROPERTIES

Light microscopy

Rodlet cells gave a positive reaction to the periodic acid-Schiff test but were not stained by alcian blue. Mucous cells were positive to both the P.A.S. test and to alcian blue.

Electron microscopy

The membrane of mucous cell globules was densely stained after sections were oxidised by 1% hydrogen peroxide for 30 min followed by treatment with 15% phosphotungstic acid (P.T.A.) solution (pH 2) for 2 h. Mucous globules were lightly stained whereas the sacs of rodlet cells were unstained (Plate VIII). No other cells in the epithelium were stained.

Rodlet sacs were stained densely after sections were treated with 1·0% periodic acid for 30 min followed by 1% P.T.A. in 10% chromic acid (pH 0·3) for 30 min (Plate IX). The central core of the sacs was unstained. The mucous cell globules were lightly stained with smaller densely stained circular globules scattered throughout the cell (Plate X).

IV. DISCUSSION

Rodlet cells have been found in many species of freshwater and seawater fish at many sites throughout the body, including gill, kidney, intestine, olfactory and pharyngeal epithelia, bulbus arteriosus, heart, liver, gonad, meninges, brain, spinal cord and eye (Bannister, 1967; Bullock, 1963; Leino, 1974; Wilson & Westerman, 1967). Although the sites and numbers of rodlet cells seem to vary between and within species, often according to season, crowding or ionic concentration of the water (Leino, 1974), the apparent lack of host and tissue specificity indicates that the cell is not a parasite. Leino (1974) has even shown the presence of rodlet cells in the gill of swordtails immediately after birth.

Although rodlet cells are found in many tissues throughout the body they tend to be numerous in the epithelium or endothelium of sites suited for secretion into the blood, body cavities or external milieu. The presence of large numbers of rodlet cells in gill, kidney and intestinal epithelia may well imply an osmoregu-

latory function. Fearnhead & Fabian (1971) suggested that rodlet cells were concerned with osmoregulation from observations that their numbers were substantially reduced in the gills, of *Monodactylus argenteus* adapted to fresh water, compared with those adapted to seawater. Preliminary studies at this laboratory have shown that rodlet cells are virtually absent from the pseudobranch of bass after 24 hours exposure to seawater diluted to 1 : 100 with freshwater. These results have to be treated with caution because of the notorious variability in numbers of cells from fish to fish (Bannister, 1966; Leino, 1974). However in this study large numbers of rodlet cells (mean 2974 per sq. mm) were found in the pseudobranch epithelium of all fish from normal seawater, so it seems likely that a change in external osmotic concentration does cause a reduction in the numbers of rodlet cells, although it need not be associated with osmoregulation and may be a stress reaction.

Flood *et al.* (1974) described four cell forms which appear to be phases in the life cycle of rodlet cells. The first two phases are similar to the immature rodlet cells described here in that the cells are not encased by a fibrous wall and are characterised by their inclusions either crystalline or amorphous and granular. The next stage described by Flood *et al.* is the semi-encased stage, also observed by Leino (1974) and Desser & Lester (1975), which corresponds to the maturing cell (Plate V) reported here. However, as far as is known the actual development of the encased stage from the pre-encased stage (Plate IV) has not been previously described. This phase provides strong additional evidence that the mature rodlet cell with its fibrous cytoplasmic border does develop within the fish tissue from a specific cell type containing no border.

It is suggested from these observations that the rodlet cell is a fish tissue cell and not a parasite. The appearance of the mature cell (Plate II) may superficially resemble that of a parasite but as Leino (1974) and Desser & Lester (1975) noted, the fibrous cytoplasmic border is clearly not a cyst wall and is part of the cytoplasm, with no membrane separation. Leino (1974) has also shown the presence of desmosomes between rodlet cells and adjacent epithelial cells, a finding confirmed in this study. Desser and Lester (1975) observed tight junctions between the apex of rodlet cells and adjacent epithelial cells. In agreement with Leino (1974) and Desser and Lester (1975), no pathological changes were noted in epithelial cells closely associated with rodlet cells.

The theory that the rodlet cell has a secretory function seems most likely on the basis of its structure and position in most fish tissues. Mature cells are usually found near the surface, in the epidermis or endodermis; the rodlets have a characteristic orientation toward the apex of the cell and the fibrous border may be capable of contractile activity. During the process of maturation the rodlet cell contains a prominent Golgi complex which is typical of certain secretory cells e.g. mucous cells. It is especially large and prominent in cells secreting either protein or complex carbohydrate (Rhodin, 1974). The close relationship between the granular endoplasmic reticulum and the Golgi complex in immature rodlet cells provides a clue to the formation of rodlet sacs. The Golgi complex may act as the assembly area for proteins produced on the ribosomes and channelled by the granular endoplasmic reticulum to the Golgi region where they are added to carbohydrate synthesised by the Golgi complex to produce glycoprotein, which is concentrated and packaged for distribution outside the cell.

Morrison & Odense (1978) on the basis of their own histochemical tests and those of previous workers, suggested that the cell product is some form of neutral glycoprotein. The results of this study help to confirm that theory since rodlets were stained with the P.A.S. test to show the presence of complex carbohydrate, but did not stain with alcian blue, indicating that it is not acidic.

At the ultrastructural level the rodlet sacs were densely stained by phosphotungstic acid in chromic acid at pH 0·3, after treatment with periodic acid. This has been reported as a selective stain for glycoprotein (Tsuchiya & Ogawa, 1973) and for sialic acid (Rambourg, 1967; Rambourg *et al.*, 1969). It is interesting to note that the central cores of the rodlet sacs were unstained indicating that they are not glycoprotein. Although the composition of the central core remains uncertain its function may be to maintain the shape and rigidity of the rodlet sacs enabling easy expulsion from the cell.

The glycoprotein content of mucous cells was also stained by phosphotungstic acid at pH 0·3. Mucous cell globules, particularly the membranes were stained by 15% P.T.A. at pH 2 whereas rodlet cell contents remained unstained. No other cells in the epithelium were stained by this method which is reported to selectively stain membrane acid mucopolysaccharides (Marinozzi, 1967, 1968; Pease, 1970).

The histochemical results at the light and electron microscope level indicate that rodlet sacs contain glycoprotein but no acid mucopolysaccharides typical of mucous cells.

It is still not clear what triggers secretion of the cell. Leino (1974) showed rodlet cells secreting their contents with apparent contraction of the cell and fibrous border. It is possible that mechanical damage incurred in fixing and preparing the tissues may squeeze out the cell contents. However the presence of microvilli-like cytoplasmic projections may indicate the trigger mechanism by which the contents are released due to mechanical stimulation. Alternatively, changes in osmotic pressure may cause secretion of rodlet sacs or even release of the whole cell as indicated by the virtual absence of rodlet cells in fish from diluted seawater.

The presence of immature, mature and maturing rodlet cells together indicates a continuous development of rodlet cells. As Leino (1974) suggested, they may have functions similar or complementary to mucous cells, whose secretions may be involved in pH control, lubrication and antibiotic effects. It is possible that rodlet cells produce a necessary constituent of the glycocalyx, which contains mainly highly anionic sugars (Threadgold 1976). Sialic acid is a common constituent, often linked to glycoprotein or lipid. Because the glypocalyx is polyanionic it has a strong net negative charge and attracts physiologically important cations such as Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}. The secretory product of rodlet cells may help maintain the net negative charge of the glycocalyx and may be released in response to a change in the external ion concentration.

In conclusion it seems likely that the presence of large numbers of rodlet cells in the bass gill and pseudobranch epithelium is indicative of a function which may be as necessary to the fish as mucous production by mucous cells. Rodlet cells provide an interesting comparison with mucous cells in the production, packaging and release of secretory products.

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A scanning electron microscope study of the pseudobranchs of two marine teleosts

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The pseudobranchs of bass, *Dicentrarchus labrax L.*, and grey mullet, *Liza ramada* (Risso) (*Mugil capito*), were examined in the scanning electron microscope (S.E.M.). Both pseudobranchs appeared gill-like *in situ* but the S.E.M. revealed their gross morphology to be different. The bass pseudobranch was a 'free' pseudobranch, having separate lamellae along the filaments, with areas of fusion on the leading edges of some lamellae, whilst the mullet pseudobranch was 'semi-free' since the secondary lamellae were fused over a large area and only free along their trailing (opercular) edge where the chloride cells were situated. The surface of the epithelial cells presented different patterns of microridges and microvilli depending on their position in the pseudobranch. Three types of cell opening were found at the cell surface, belonging to chloride cells, mucous cells, and possibly rodlet cells. The S.E.M. observations were correlated with those of transmission electron microscope (T.E.M.) studies.

I. INTRODUCTION

A number of studies have been carried out on the topography of fish gills using the scanning electron microscope (S.E.M.) (Olson & Fromm, 1973; Lewis & Potter, 1976; Rajbanshi, 1977; Hughes, 1979). They revealed the presence of microridges and microvilli of various dimensions on the surface of epithelial cells of the secondary lamellae, and confirmed the locations of mucous cell and chloride cell openings. The significance of the lamellar microridges is still uncertain. Hughes & Wright (1970) and Hughes (1979) suggest that their primary function is to anchor the protective mucus coat secreted by the mucous cells, although the possibility that they increase functional surface area has also been considered (Olson & Fromm, 1973).

In the present study the surface structure of the pseudobranchs of the bass, *Dicentrarchus labrax* and the mullet, *Liza ramada* (*Mugil capito*) were examined in the S.E.M. and correlated with transmission electron microscope (T.E.M.) studies.

The teleost pseudobranch, found in the subopercular cavity is a remnant of the mandibular arch and has been modified from a 'free' gill-like structure in most marine teleosts to a covered 'glandular' structure in certain freshwater teleosts (e.g., carp). The topography of the pseudobranch was considered to provide an interesting comparison with that of the gills, since the pseudobranch has no apparent respiratory function (Granel, 1927, after Hyrtl), but may be involved in osmoregulation in marine teleosts which contain chloride 'type' cells (Dunel & Laurent, 1973; Mattey, Moate & Morgan, 1978).

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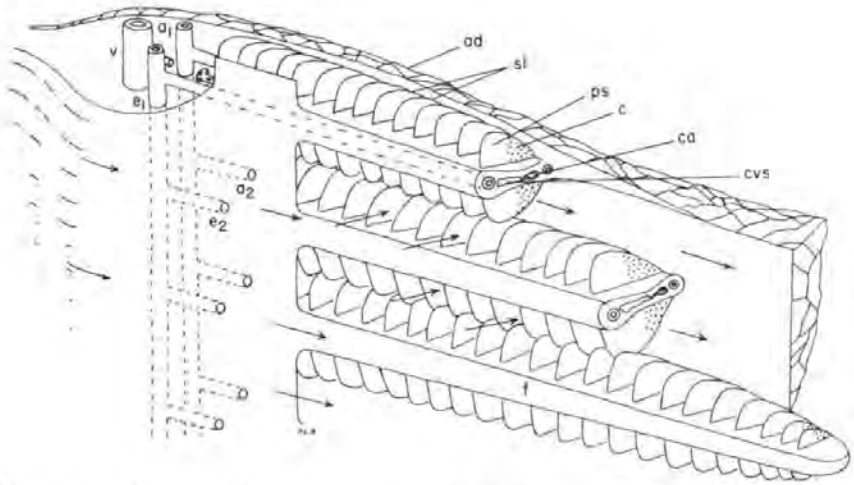

FIG. 1. Section of bass pseudobranch. a_1, primary afferent artery; a_2, afferent filament artery; ad, adipose tissue of operculum; c, chloride cell region; ca, cartilage; cvs, central venous sinus; e_1, primary efferent artery; e_2, efferent filament artery; f, filament (buccal edge); ps, pseudobranch cell region; sl, secondary lamellae; v, pseudobranch vein.

II. MATERIALS AND METHODS

S.E.M. PROCEDURE

The pseudobranchs were removed intact from freshly killed bass (weighing 70–120 g) and mullet (15–30 g), rinsed in tap water and fixed for one week at 4° C in 2·5% glutaraldehyde in 0·1 M phosphate buffer (pH 7·4). They were given three washes of 30 minutes each in phosphate buffer containing 0·54% glucose and dehydrated in a graded alcohol series, followed by immersion in amyl acetate. The tissues were then critically point dried in a Samdri PVT 3, using carbon dioxide as the transitional fluid, and mounted on brass stubs with double sided tape. They were sputter coated with gold in a Polaron E5100 Coating Unit and examined in a Jeol 35C scanning electron microscope.

T.E.M. PROCEDURE

Pseudobranchs from freshly killed fish were fixed for one hour at 4° C in 2·5% glutaraldehyde in 0·1 M phosphate buffer (pH 7·4). The tissues were washed three times in phosphate buffer containing 0·54% glucose and post fixed for one hour at 4° C in 1% buffered osmium tetroxide. They were dehydrated in graded concentrations of ethanol followed by propylene oxide and embedded in Spurr resin (Spurr, 1969). Thin sections were stained in uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined in a Philips 300 transmission electron microscope.

L.M. PROCEDURE

Paraffin wax sections of Bouin fixed material were stained with Mallorys' triple stain or haematoxylin and eosin, and examined in the light microscope.

III. RESULTS

The three dimensional structure of the bass and mullet pseudobranchs was revealed using the S.E.M. There was no covering layer of mucus which other workers have found on gills fixed in glutaraldehyde at similar concentrations (Olson & Fromm, 1973; Lewis & Potter, 1976; Hughes, 1979). Although both pseudobranchs gave the impression of gill-like organs *in situ* the S.E.M. showed their gross morphology to be different. Line drawings were made comparing their structure (Figs 1 & 2).

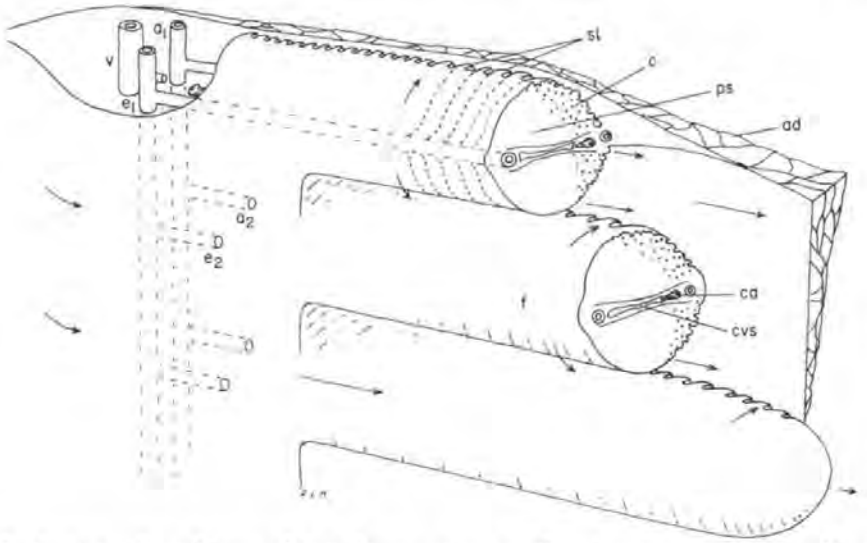

FIG. 2. Section of mullet pseudobranch. a_1, primary afferent artery; a_2, afferent filament artery; ad, adipose tissue of operculum; c, chloride cell region; ca, cartilage; cvs, central venous sinus; e_1, primary efferent artery; e2, efferent filament artery; f, filament (buccal edge); ps, pseudobranch cell region; sl, secondary lamellae; v, pseudobranch vein.

The bass pseudobranch has the more typical gill-like structure with triangular shaped secondary lamellae arranged alternatively on either side of the supporting filaments (Figs 3 & 4). The pseudobranch has only one row of parallel filaments which are attached to the opercular epithelium at the base of the organ (the gill arch) but are 'free' for much of their length. In some areas fusion of the secondary lamellae is seen (Figs 3 & 4). Fusion always occurs on the leading (buccal) edge of the lamellae overlying the region of the 'pseudobranch type' cells previously shown in a T.E.M. study (Mattey *et al.*, 1978). The degree of fusion varies from filament to filament and in some cases occurs only on one side (Fig. 3). The secondary lamellae are always free along their trailing (opercular) edge in the region of the 'chloride type' cells. 'Chloride' cell openings are visible in the basal and central areas of the lamellar epithelium in this region (Fig. 5). They are seen as a collection of irregularly shaped pits in the epithelium surface. They are found at poorly defined epithelial cell junctions and are usually 2–4 μm wide. Microvilli like protoplasmic projections can ocassionally be seen within the pits (Fig. 6). 'Chloride' cell pits were not seen on the outer margins of the lamellae. Mucous cell openings were observed mainly on the filaments but rarely on the secondary lamellae. They were more numerous on the opercular edge of the filament than the buccal edge (c.f. Figs 3 & 4). Droplets of mucus were seen within the pores (Fig. 7).

Another type of opening (1–2 μm in diameter) was sometimes found in the interlamellar filament epithelium and at the base of the lamellae. This type is characterised by its conical, circular shape (Fig. 8). The epithelium is raised in a continuous circular ridge and protoplasmic projections can be seen within the opening. This type of opening may correspond to that of the rodlet cell previously described in the bass pseudobranch by Mattey,

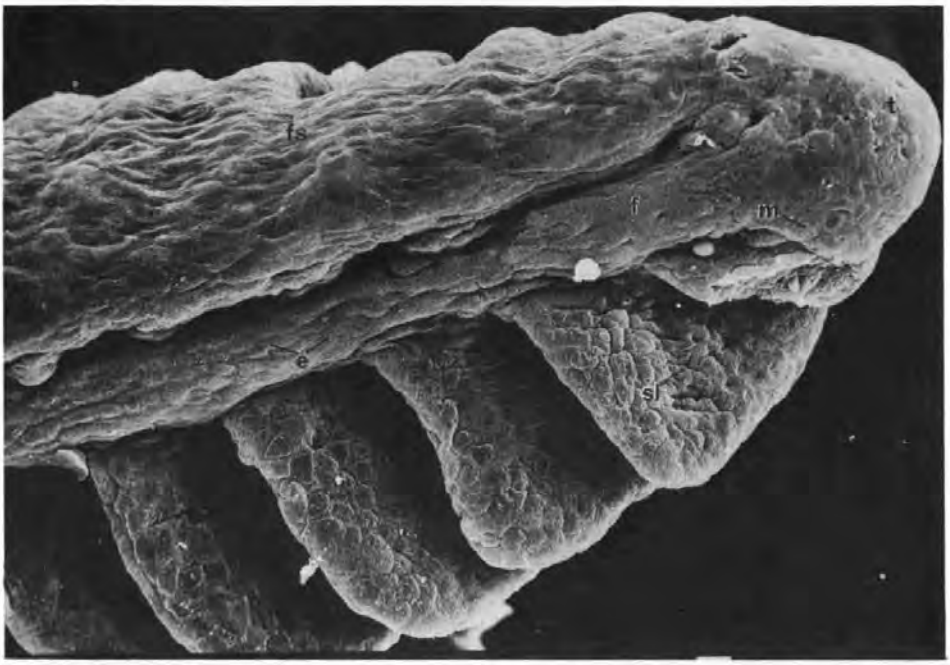

FIG. 3. Bass pseudobranch filament (buccal edge). e, epithelial cells; f, filament; fs, area of epithelial fusion covering secondary lamellae; m, mucous cell pores; sl, secondary lamella; t, filament tip. (× 433).

FIG. 4. Bass pseudobranch filament (opercular edge). e, epithelial cells; f, filament; fs, area of epithelial fusion covering secondary lamellae; m, mucous cell pores; sl, secondary lamellae; t, filament tip. (× 353·5).

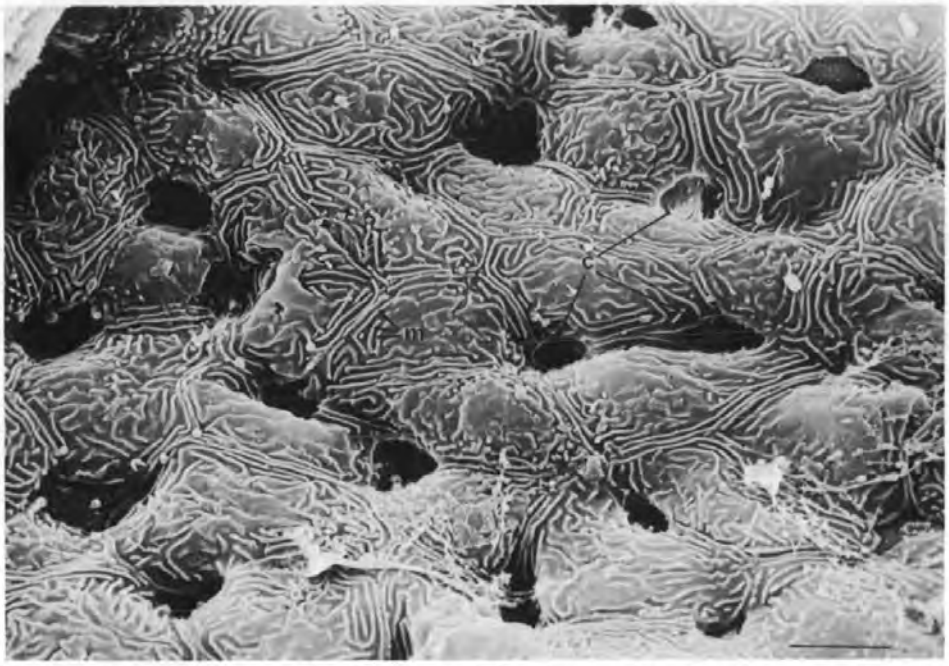

FIG. 5. Chloride cell pits (c) of bass pseudobranch secondary lamellae. m, microridges. (× 2660).

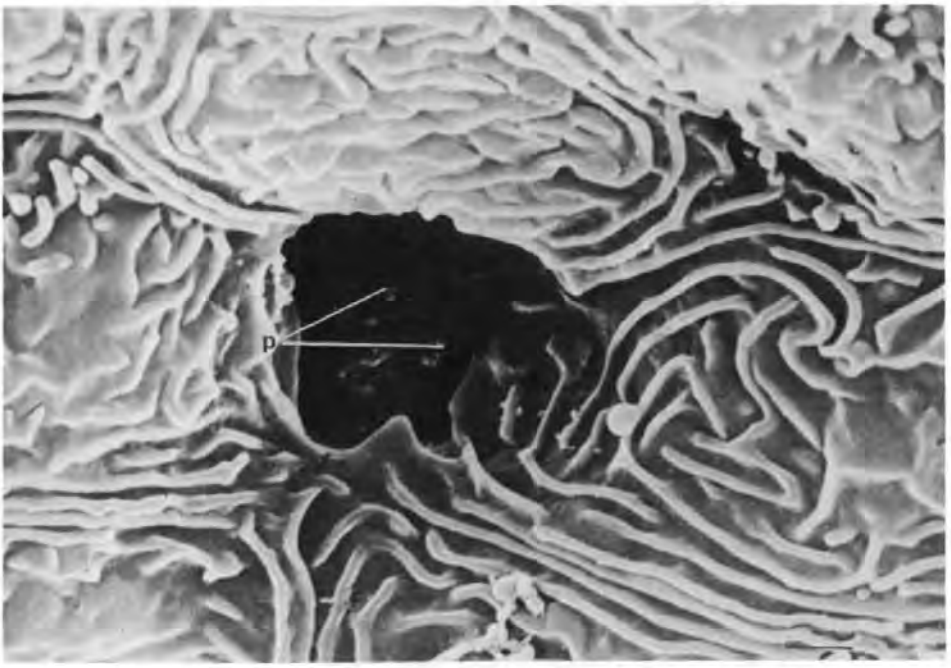

FIG. 6. Microvilli-like projections (p) within chloride cell pit (Bass pseudobranch). (× 9513).

Morgan & Wright (1979, in press). A T.E.M. micrograph of rodlet and mucus cells is shown in Fig. 9.

The gross morphology of the mullet pseudobranch is different to that of the bass (c.f. Figs 3 & 10). It still retains the single row of parallel filaments but the secondary lamellae are no longer apparent. Light microscopy has revealed them to be fused and covered with a thin layer of epithelium except for the opercular edge where the 'chloride type' cells are situated (Fig. 11). The secondary lamellae in this region still retain a 'free' aspect but the epithelial cells are much folded and possess highly convoluted microridges, and numerous microvilli which give the surface a serrated appearance (Fig. 12). Many 'chloride' cell pits are present in flattened regions of the surface epithelium where the microridges are sparse or absent. The openings are approximately circular with a diameter of 1·5–3·0 μm. Numerous mucous pores were found on the edges of the filament but no other type of opening was seen.

The surface structure of the filaments and secondary lamellae of both pseudobranchs is similar to that previously described for the gills of fish (Olson & Fromm, 1973; Lewis & Potter, 1976; Rajbanshi, 1977; Hughes, 1979). The epithelial cells are usually pentagonal or hexagonal in shape. Individual cells are easily seen in the mullet pseudobranch, since they are usually bordered by long straight microridges at the cell junctions where they form a double ridge with an adjacent cell (Fig. 13). This double ridge is often 1·5–2 times higher than the other microridges. In the bass, the ridges of adjacent cells are often some distance apart and the cell boundaries are not clearly defined. Mucous pores are often found at the junctions of

FIG. 7. Mucus pores (mu) on filament of bass pseudobranch. d, mucous cell droplets; m, microridges. (×4865).

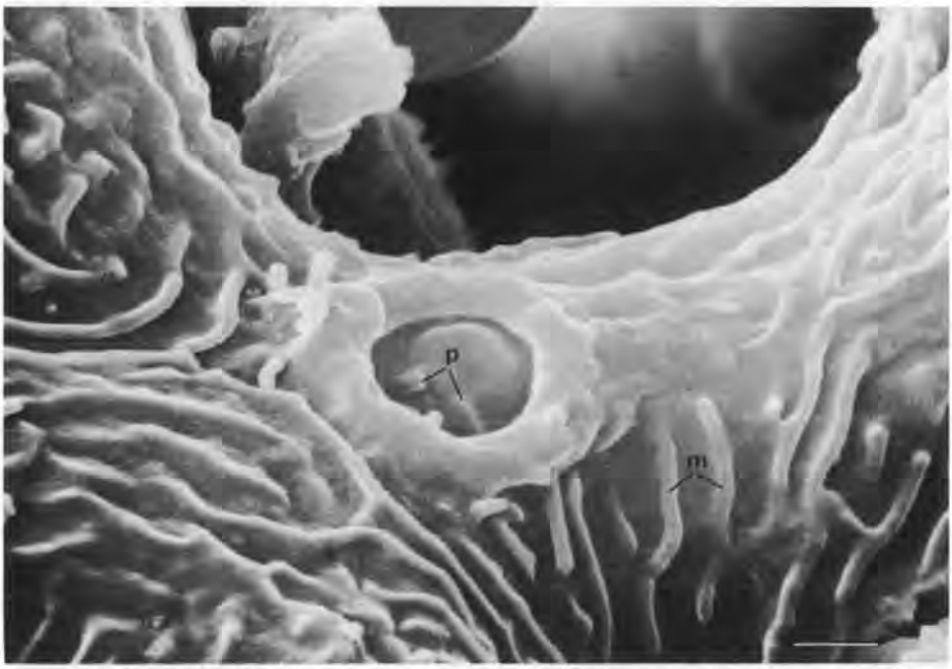

FIG. 8. Possible rodlet cell opening in the interlamellar filament epithelium. m, microridges; p, cytoplasmic projections. (× 11 060).

filament epithelial cells and were larger in the bass (3–6 μm) than in the mullet (1·5–3 μm). In the mullet, dome-like protrusions, probably representing closed mucous cells, were found on the buccal surface of the filament (Fig. 10).

The length of the microridges varied considerably from short microvilli-like projections to long, convoluted ridges up to 8 μm long (Fig. 13). However there does appear to be a general pattern depending on the position of the epithelial cells in the filament or secondary lamellae. In the bass, the epithelial cells on the edges of the filament possess long microridges arranged in concentric whorls. Cells at the marginal base of the secondary lamellae close to the filament have short microridges or microvilli, as do those along the edges of the lamellae. The epithelial cells covering the ' pseudobranch type ' cells have flattened, highly convoluted microridges (Fig. 14) whereas those covering the chloride cells are narrower and further apart (Fig. 5). The average height of the microridges, as measured from transmission electron micrographs, is usually greater in the region of the chloride cells or areas of high mucous cell density (Table I).

Certain patterns of microridge arrangement are also present in the mullet pseudobranch. On the leading and trailing edges of the filament the epithelial cells have long straight microridges, sometimes arranged in a concentric whorl. However cells with microvilli-like ridges are also present, especially near the tip of the filament. The epithelial cells covering the fused secondary lamellae are flatter and microridges are sparse or absent. Where the secondary lamellae become free the serrated appearance and deep folding of the epithelial cells is highly characteristic (Fig. 12).

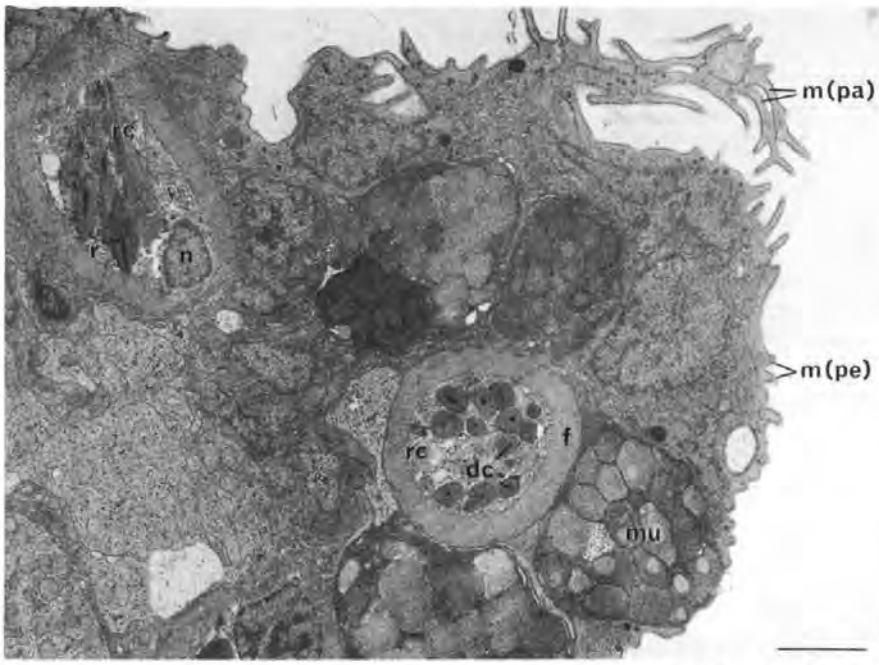

FIG. 9. Transmission electron micrograph of rodlet and mucous cells. dc, dense core; f, fibrous border; m, microridges sectioned parallel (pa) or perpendicular (pe) to the surface of the epithelial cells; m, mucous cell containing mucous globules; n, nucleus of rodlet cell; r, rodlets; rc, rodlet cell. (× 5803).

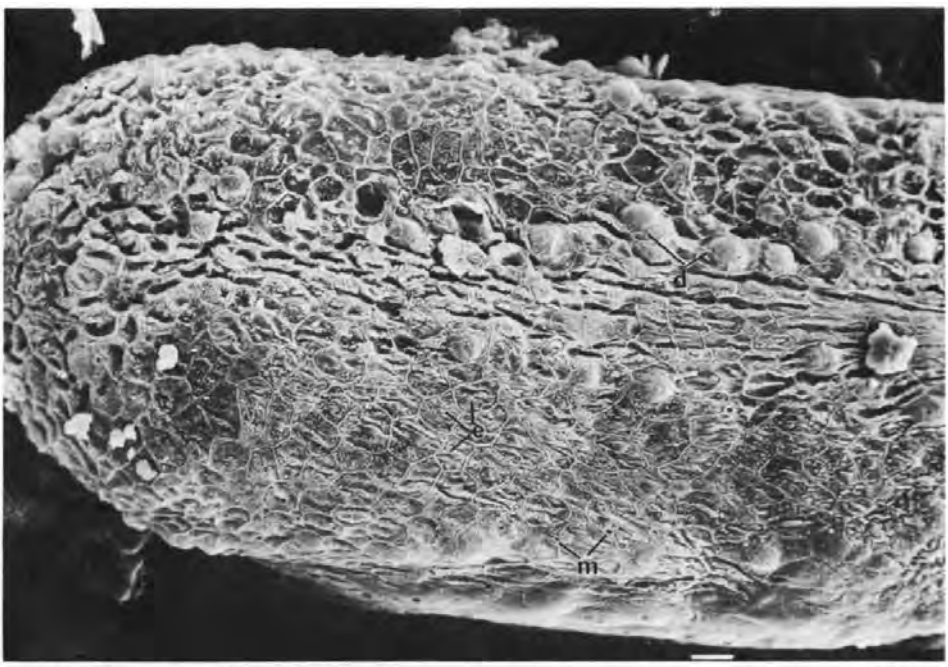

FIG. 10. Mullet pseudobranch filament (buccal edge). d, dome-like protrusions; e, epithelial cells; m, mucous pore. (× 437·5).

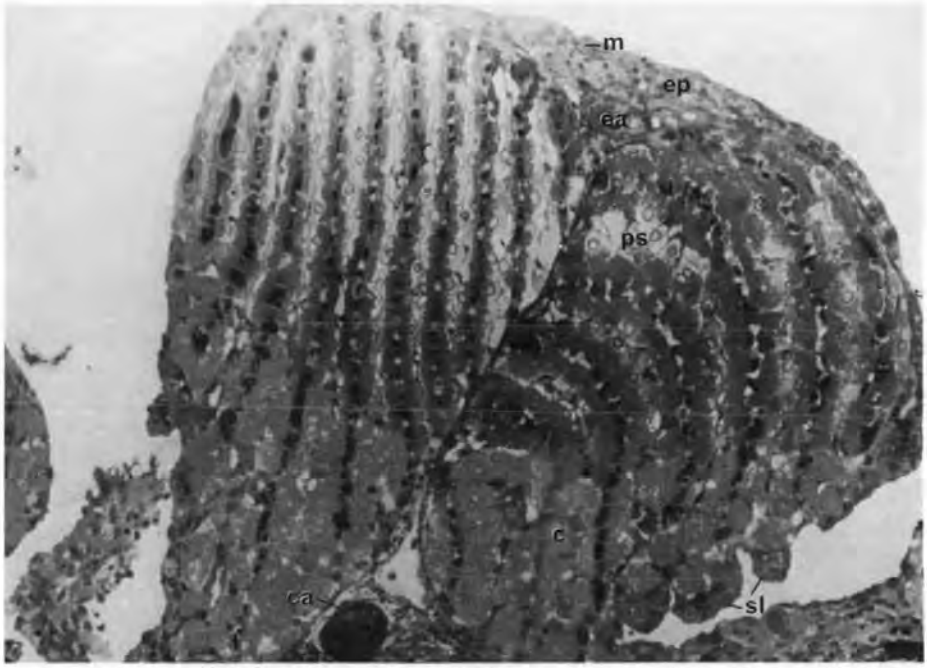

FIG. 11. Light micrograph of mullet pseudobranch filament (T.S.) showing position of chloride cells (c) and pseudobranch cells (ps). ca, cartilage; ea, efferent filament artery; ep, epithelial cells; m, mucous cell; sl, secondary lamellae (free region). (Methylene blue × 258).

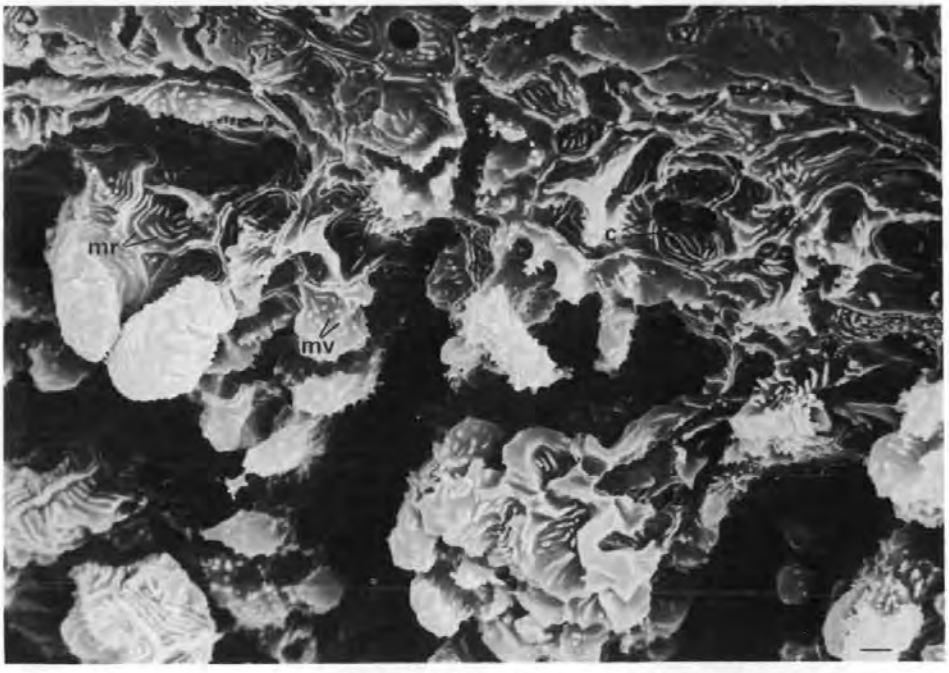

FIG. 12. 'Free' area of mullet pseudobranch secondary lamellae showing chloride cell pits (c); mr, microridges; mv, microvilli. (× 1715).

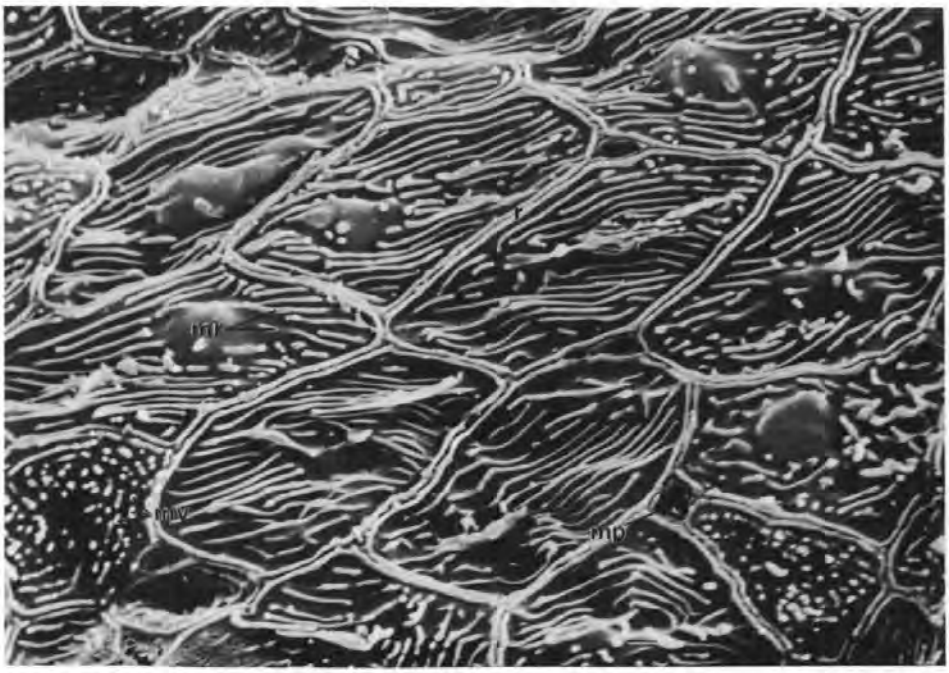

FIG. 13. Epithelial cells of mullet pseudobranch filament (buccal edge) showing double ridge (r) at the cell junctions; mr, microridges; mu, mucous pores; mv, microvilli. (× 2870).

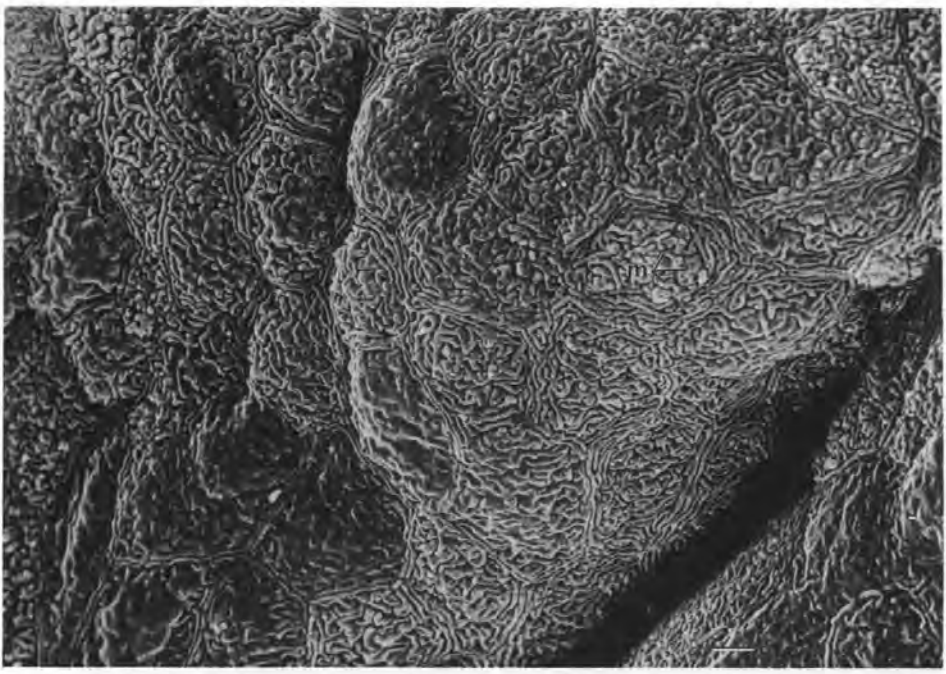

FIG. 14. Epithelial cells covering the pseudobranch cells of the bass pseudobranch; m, microridges. (× 2560).

TABLE I. Correlation of microridge height with distribution (Bass pseudobranch)

Area	Height of microridges (μm)
Filament (buccal edge)	0·19—0·88 (*0·29)
Filament (opercular edge)	0·19—0·81 (0·32)
Secondary lamellae (pseudobranch cell region)	0·12—0·42 (0·23)
Secondary lamellae (chloride cell region)	0·14—0·65 (0·28)
Secondary lamellae (margin)	0·10—0·54 (0·21)

* Mean of 25 readings.

IV. DISCUSSION

Previous studies have found that fixation of gills in high concentrations of glutaraldehyde (25 or 50%) was necessary to give clear results under the scanning electron microscope (Olson & Fromm, 1973; Lewis & Potter, 1976; Hughes, 1979). Olson & Fromm (1973) and Lewis & Potter (1976) found that after fixation in 4% and 5% glutaraldehyde respectively the tissues were covered with a thick layer of mucus, making it impossible to examine the surface structures. Lewis & Potter (1976) suggested that mucus production may be stimulated by fixation in glutaraldehyde concentrations of 2–5%. However, Hunter & Nayudu (1978) found that fish epidermal cells fixed in 5% glutaraldehyde had mostly closed mucous cell orifices which appeared as small domes at the junction of two or three cells. In the present study dome-like structures were also seen on the filaments of mullet pseudobranch, and could indicate the presence of underlying mucous cells.

The pseudobranchs of mullet and bass, although densely populated with mucous cells remained free of mucus after fixation in 2·5% glutaraldehyde. However the tissues were fixed for one week as opposed to the 2–4 h employed in previous studies. Olson & Fromm (1973) suggested that 50% glutaraldehyde might dissolve the mucus from the gills so it is possible that long fixation at a lower concentration has the same effect. However, the slow dehydration, immersion in amyl acetate and critical point drying procedure may be more important factors for providing a mucus-free surface. There did not appear to be any gross tissue shrinkage using this method and the results compare favourably with those of previous S.E.M. studies on gills.

The present study provides a good example of the structural modification undergone by the teleost pseudobranch. The gill-like bass pseudobranch is a typical ' free ' pseudobranch although a certain amount of fusion is observed along the buccal edge of the secondary lamellae. This fusion has progressed much further in the mullet pseudobranch leaving only a small free area on the opercular edge of the lamellae. The bass and mullet pseudobranchs probably have some osmoregulatory function since they both possess ' chloride type ' cells. These need to make contact with the external environment via apical pits so the degree of fusion and epithelium covering is limited to allow them free access to the water.

In freshwater teleosts such as the rainbow trout, *Salmo gairdneri*, the 'chloride type' cells of the pseudobranch are lost, the secondary lamellae become completely fused and the whole organ is covered with an epithelial layer (Mattey *et al.*, 1978). This modification has been carried a step further in the carp (Cyprinus) pseudobranch which is buried deep within the surrounding connective tissue. The pseudobranchs of these species are representative of the four main types of pseudobranch classified by Granel (1927) on the basis of their epithelial covering. The scanning electron microscope is particularly useful for classification of pseudobranch types since it provides a quick and accurate method of assessing the degree of lamellar fusion and the extent of epithelial covering. It also shows the close similarity between the pseudobranch surface structure and that described for the gills of teleosts (Olson & Fromm, 1973; Rajbanshi, 1977; Hughes, 1979) and lampreys (Lewis & Potter, 1976). The provision of an increased surface area for gas exchange, due to the microridges, has been suggested for gills (Olson & Fromm, 1973; Lewis & Potter, 1976), but this is likely to have no functional significance for the pseudobranch which has no apparent respiratory function (Granel, 1927, after Hyrtl).

It has been shown previously that microridges are not restricted to respiratory structures, such as the gills, since they have been found on the epidermal cells of the body and fins of teleost fish (Lanzing & Higginbotham, 1974; Dobbs, 1975; Hunter & Nayudu, 1978). The presence of microridges on a variety of teleost epithelial cells probably indicates a common function, such as the anchorage of mucus suggested by Hughes & Wright (1970). It is still uncertain if a mucus film always covers the skin and gill surfaces but it is probably necessary to serve a number of functions. Apart from its possible affect on gas exchange, it is likely to reduce surface friction, to provide protection against mechanical abrasion and infection, and may be important for normal regulation of ionic exchange. Hughes (1979) thought that the most probable situation was that mucus occupied the spaces between ridges thus presenting a flat interface with the water.

The thickness of the mucus film covering the filaments and secondary lamellae of the pseudobranch may vary from area to area. This is indicated by regions of different mucous cell density and differences in the microridge pattern which may affect anchorage of the mucus. Although there is no clear correlation between mucous cell density and microridge pattern, the average height of the microridges is usually greater where there are large numbers of mucous cell openings or chloride cell openings. The distribution of mucous cells and thickness of the mucus film may be related to the speed and direction of the water flow together with the changes in resistance that occur over the filaments and between the secondary lamellae. It is noticeable in both pseudobranchs that the highest concentration of mucous cells occurs on the opercular edge of the filament where there is likely to be high resistance to water flow between the filaments and operculum.

Although anchorage of mucus seems to be the most likely function of the microridges they have also been considered as the structural result of the water flow itself (Hughes, 1979), or as providing a region of microturbulence immediately above the secondary lamellae to enhance gaseous exchange (Lewis & Potter, 1976). In the mullet pseudobranch the free region of the secondary

lamellae is particularly ridged and convoluted. This is likely to cause a region of microturbulence, with continual mixing of water layers immediately above the chloride cell openings which would facilitate ionic exchange rather than gas transfer.

The number of openings thought to belong to rodlet cells was surprisingly small considering the large number of these cells seen using the T.E.M. in a previous study on the bass pseudobranch (Mattey *et al.*, 1979). It is possible that most of the rodlet cells are not open to the external environment but only do so to fulfil some special function, as yet unknown.

We wish to thank Mr B. Lakey for his helpful assistance in the use of the Jeol 35C scanning electron microscope.

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